Franz J. Hock
Editor

Drug Discovery and Evaluation: Pharmacological Assays

Fourth Edition

SpringerReference

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Fourth Edition

With 26 Figures and 28 Tables

Springer Reference

Editor
Franz J. Hock
CorDynamics
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In Memory of Hans Gerhard Vogel 1927–2011.

Preface to the Fourth Edition

The fourth edition of the book *Drug Discovery and Evaluation: Pharmacological Assays* is presented here.

The concept of the book has changed since the third edition, and we have now split the chapters for better reading and web search. The volume data has again risen considerably compared to the third edition. In particular, a large number of assays and new topics have been added.

Several chapters are new, and most of the chapters have been revised and have been thoroughly updated as well. I am indebted to my colleagues rewriting and updating the chapters and I also thank the authors who provided new topics for this book.

The approach to drug discovery has changed continuously during recent years. Decades ago, most of the drugs were found by serendipity in clinical trials. New drugs, however, were found in animal experiments by a classical approach. This classical approach has advantages and disadvantages. The main advantage was the relatively high predictability of success. The major disadvantage was that we got little or no information about the molecular mechanisms involved in the observed effects. New mechanisms always required new models.

The costs of developing new drugs are exploding, while the output is decreasing. A change in paradigm, the target-based or mechanism-based drug discovery approach, was welcomed with great enthusiasm. Combinatorial chemistry could generate thousands of new compounds. They were tested in high-throughput systems. This made it highly effective for the identification of target-selective compounds. Although this technique showed very great advantages from a scientific and practical viewpoint, it did not translate into higher success rates. The target-based approach has therefore been replaced again by the physiology-based approach – the classical drug discovery – or the function-based approach, which seeks to induce a therapeutic effect by normalizing a disease-specific abnormality.

I am aware that the rapid progress in biology will once again change the methodological approaches in the coming years. In addition, electronic media will continuously help scientists to access and share information. However, on the other hand, it is becoming more and more evident that many young pharmacologists have only limited training in classical pharmacological methods. When searching for these classical methods, researchers will find only insufficient information on the methodological details in electronic

databases. To this end, I hope the current book may bridge this gap by comprehensively covering those classical pharmacological methods, sometimes utilized for over 100 years, with modern pharmacological methods.

At this point, I would like again to express my sincere thanks to all colleagues who contributed to the new edition of this book. Their names and affiliations are given in alphabetical order.

A special thanks goes to Hans Gerhard Vogel, who was the Editor-in-Chief of the first three editions. He was furthermore the initiator of the *Drug Discovery and Evaluation* titles at Springer consisting of *Pharmacological Assays, Safety and Pharmacokinetic Assays*, and *Methods in Clinical Pharmacology*. Gerhard Vogel passed away in 2011. Personally, I am personally very much indebted to him. He introduced me to pharmacology and was always my mentor thereafter. It is an honor to continue his work.

Autumn 2015 Franz J. Hock

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About the Editor

Franz Jakob Hock Since retiring from Aventis in 2002, Dr. Hock has leveraged his experience as a freelance consultant specializing in Safety Pharmacology. Dr. Hock was a research scientist at Hoechst, Hoechst Marion Roussel, and Aventis from 1976 to 2002. He initially worked on methods in general pharmacology and nephrology, before becoming Head of a Laboratory devoted to pharmacological methods for drugs influencing memory and learning. He was ultimately Head of Laboratory for General/Safety Pharmacology at the Frankfurt site of Aventis Pharma Deutschland GmbH.

Dr. Hock received his MSc in Neurobiology from the Technical University Darmstadt and his D.Sc. in Zoology from the University Kassel, Department of Biology, Institute of Neuroethology and Biocybernetics.

He received the degree of Fachpharmakologe DGPT ("certified expert pharmacology") in 1981. In 1983, he spent a sabbatical year at the University of California, Irvine, at the Center for the Neurobiology of Learning and Memory (Director Prof. Dr. James L. McGaugh).

He lectured for several years to students of Biology at the University of Kassel and the Technical University of Darmstadt. He has published over 100 original papers on methods in pharmacology and on new compounds. Furthermore, he held 28 patent applications to protect or broaden the application of lead structures.

He is currently a member of the Task Force General/Safety Pharmacology German/Swiss Pharmaceutical Companies. A member of several national and international scientific societies, Dr. Hock is a founding member of "Safety Pharmacology Society," "Neurowissenschaftliche Gesellschaft e.V.," and "European Behavioural Pharmacology Society." For several years, he served as a member of the Program Committee of the Safety Pharmacology Society. He is a member of several domestic and international scientific societies.

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Part I

Cardiovascular Activity

Studies in Isolated Organs

Michael Gralinski, Liomar A. A. Neves, and Olga Tiniakova

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α-Sympatholytic Activity in Isolated Vascular Smooth Muscle

Purpose and Rationale

Angus and Wright (2000) described in detail various techniques to study the pharmacodynamics of isolated large and small blood vessels.

Furchgott and Zawadzki's (1980) discovery of important role of endothelium ensured that all studies of vascular pharmacology must consider the role/status of the endothelium and its endogenous factors, such as endothelial-derived hyperpolarizing factor, nitric oxide, endothelin, and prostaglandins (Angus and Wright 2000). To make sure that a vessel contains only smooth muscle layer, the endothelium should be removed (by rubbing intimal surface with a wire, plastic tube, or bubbling the air).

Han et al. (1990) studied different subtypes of α1-adrenoceptors in isolated rat aortas, renal arteries, mesenteric arteries, and portal veins.

Noradrenaline and other sympathomimetic drugs increase vascular smooth muscle tone by stimulation of α-adrenergic receptors. Contractions can be antagonized by α-adrenergic receptor-blocking agents such as phentolamine. Drugs can be tested for their capacity of reducing vascular smooth muscle contractions induced by the adrenergic receptor-activating agent noradrenaline. Moreover, effects of peptides, such as bradykinin, can be tested with strips of aorta or pulmonary artery.

Procedure

As donor animals, Pirbright White guinea pigs of either sex weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 200–300 g are used. The vessels to be tested are the thoracic aorta or the arteria pulmonalis. The animals are sacrificed by stunning and exsanguination. The pulmonary artery or the thoracic aorta is quickly removed, and if required, the intimal surface of the vessel is gently rubbed with a wire or polyethylene tube to remove the endothelium. The vessel is then cut into either helical strips of 1–2-mm width and 15–20-mm length or into rings 3–4 mm in length. The strips (or rings) are mounted in an organ bath with a preload of 1 g Krebs–Henseleit buffer solution containing 11.5 M glucose maintained at 37 °C and oxygenated with 95 % O_2 and 5 % CO_2. The functional loss of endothelial cells is confirmed by the loss of relaxation response to acetylcholine (1 μM). Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg). Isometric force is measured with a force transducer (UC-2, Gould-Statham, Oxnard, USA).

Experimental Course

Following an equilibration period of 60 min, contractions are induced by repeated administrations of (–)noradrenaline HCl in concentrations of 2×10^{-6} M for testing the contractions of the pulmonary artery and in concentrations of 2×10^{-8} M for testing the contractions of the aorta. After obtaining a stable plateau of identically sized contractions, cumulative doses of the test compound are added into the organ bath. Consecutive concentrations are given when the response of the previous dose has reached a plateau.

Controls at the end of the experiment: If a compound does not show vasorelaxing activity at any dose, the sensitivity of the preparation is tested by adding phentolamine (1×10^{-7} M).

If a compound shows vasorelaxing activity, the reversibility of the relaxation is tested by increasing the noradrenaline concentration.

Evaluation

The contractile force is determined before and after drug administration.

Percent inhibition of spasmogen-induced contraction by test drug is calculated as compared to the maximal contraction with a spasmogen alone (=100 %).

IC_{50} values are determined from the individual dose–response curves. IC_{50} is defined as the dose of drug leading to a 50 % relaxation of noradrenaline-induced contraction.

Modifications of the Method

The isolated vena cava of rabbits can be used for assaying α-adrenolytic activity. The rabbit is sacrificed by CO_2 anesthesia. The vena cava inferior is removed and cut into strips. The percent inhibition of epinephrine- or norepinephrine-induced contractions is determined.

The effects of bradykinin and bradykinin antagonists can be tested in isolated guinea pig artery and isolated rabbit aorta which contains predominantly the BK_1-receptor type (Regoli and Barabé 1980; Hock et al. 1991).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues, and antagonists in rat isolated pulmonary artery. Endothelium intact pulmonary artery rings were contracted with 3×10^{-8} M phenylephrine, and a cumulative dose–response curve of relaxation was constructed.

Fagura et al. (1997) studied the presence of α1-adrenoceptor subtypes in the rabbit ear artery and rat thoracic aorta using two selective competitive antagonists, 5-methylurapidil (α1A-selective) and BMY 7378 (α1D-selective), and an irreversible antagonist chloroethylclonidine.

Angus and colleagues (1986b) compared norepinephrine response in carotid, mesenteric, renal, and femoral large arteries of the pig, greyhound, and mongrel dog in the presence of propranolol. They concluded that endothelium-dependent relaxation to norepinephrine and substance P varies greatly across five large arteries of the dog (Angus et al. 1986a).

Teng and colleagues (2013) studied adenosine receptor subtypes in mouse mesenteric arteries using knockout and wild-type animals.

Yamamoto and Koike (2001a, b) investigated the distribution of α1-adrenoceptor subtype in the mouse mesenteric artery, abdominal aorta, and thoracic aorta using the following antagonists – prazosin, WB4101, BMY7378, and 5-methylurapidil.

Jähnichen and colleagues (2004) used selective α1A-adrenoceptor antagonist B8805-033 to study noradrenaline-induced contraction of rat tail artery.

Mulvany and Halpern (1977) described a technique to measure contractility of small arterial resistance vessels in the mesenteric bed of normotensive and spontaneously hypertensive rats.

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α-Sympatholytic Activity in the Isolated Guinea Pig Seminal Vesicle

Purpose and Rationale

The seminal vesicles of guinea pigs and rats are tubular organs whose longitudinal and annular muscles are innervated by the sympathetic system. The inhibition of contractions induced by norepinephrine or the α_1-selective agonist phenylephrine indicates α-sympatholytic activity. Sharif and Gokhale (1986) recommended the use the isolated rat seminal vesicle as a rather sensitive and specific model.

Procedure

Male guinea pigs weighing 300–600 g are sacrificed by a blow to the neck. The rats weighing about 300 g were anesthetized with 87 mg/kg ketamine and 13 mg/kg Xylocaine intraperitoneally. Both seminal vesicles are identified, freed from the adhering connective tissue, and resected proximally at their point of entrance into the vas deferens. After the semen was squeezed out, the isolated seminal vesicles were put into oxygenated Krebs solution (95 % O_2 and 5%CO_2) and washed. The seminal vesicles were cut at both ends. The remaining middle portions, about 1 cm in length, were mounted over an organ bath which was connected to a Grass FT03 force transducer (Grass Instruments, Inc., Quincy, MA, USA) for detection of isometric contractions. The tension of contraction was recorder by Gould RS 3400 polygraphy (Ballainvilliers, France). The Krebs solution was maintained at 37 °C and bubbled with 5 % carbon dioxide in oxygen. Before adding the tested drugs, the system was equilibrated for 1.5–2 h under an optimal tension of 1 g (Hsieh et al. 2014).

Following an equilibration period, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 1–5 μg/ml or phenylephrine HCl in concentrations of 10–50 μg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three minutes later, the previous concentration of norepinephrine or phenylephrine is added. As standard, phentolamine is used in concentrations of 3–30 $\times$ 10^{-7} M.

Evaluation

Contractions of the seminal vesicle induced by the α-adrenergic agonists after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in-depth analysis, full dose–response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

Modification of the Method

Leitch (1954) recommended the use of isolated seminal vesicles of rats for the assay of sympatholytic drugs.

Hsieh and colleagues (2014) evaluated the role of sympathetic and parasympathetic nerve system on the smooth muscle of rat seminal vesicles.

Analysis of expression and contractile function of α1-adrenoceptors in seminal vesicles of normotensive and hypertensive rats was provided by Yono and colleagues (2012).

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α-Sympatholytic Activity in the Isolated Vas Deferens of the Rat

Purpose and Rationale

The vas deferens has been used as a model for many diverse studies of different aspects of autonomic neurotransmission; it was first introduced by Hukovic in 1961 (Hukovic 1961). For a detailed review on the vas deferens as a model used to establish sympathetic cotransmission, see the publications by Burnstock and Verkhratsky (2010) and also Westfall and Westfall (2001).

The vas deferens of the guinea pig or preferably the rat is used for quantitative evaluation of adrenergic antagonists. The response of this organ to α-adrenergic agonists consists of a strong rapid contraction followed by quick relaxation on washing the agonists out of the tissue.

Procedure

Male Wistar rats weighing about 300 g are used. The animals are sacrificed by a sharp blow to the neck, and the vasa deferentia are dissected free from the extraneous tissues and suspended in a organ bath containing Tyrode solution being oxygenated with a 95 % O_2 and 5 % CO_2 mixture at 32 °C. Isotonic registration is performed at a preload of 0.5 g. Changes in length are recorded isotonically using a lever transducer (e.g., 368 type B, Hugo Sachs Elektronik, Freiburg, FRG).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 0.5, 1.0, 2.0, or 4.0 μg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three minutes later, the previous concentration of norepinephrine is added. As standard, phentolamine is used in concentrations of 3–30 $\times$ 10^{-7} M.

Evaluation

Contractions of the vas deferens induced by the α-adrenergic agonist after addition of the test

compound are compared with the initial values and expressed as percentage thereof. For in-depth analysis, full dose–response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

Modifications of the Method

Electrical stimulation of the isolated ductus deferens results in the release of norepinephrine. Stimulation-induced contractions of this organ are inhibited by clonidine which impairs adrenergic neurotransmission by activating inhibitory α-receptors. The ductus deferens is suspended in an organ bath bubbled with carbogen and maintained at 37 °C. Tension is adjusted to 25 mN. Following a 45-min equilibration period, supramaximal amplitude stimulation by a HSE type 2 stimulator (Hugo Sachs Elektronik, Freiburg) is applied. After stabilization of the response, clonidine is added to the organ bath in accumulated doses. Test compounds are added 5 min prior to clonidine administration. The percent potentiation of clonidine-induced inhibition of contractions is determined.

The techniques, describing vas deferens preparation in detail, are available in the following reviews – Westfall and Westfall (2001) and Burnstock and Verkhratsky (2010).

The Hukovic preparation of vasa deferentia includes dissecting the vas deferens along with the hypogastric nerve (Hukovic 1961).

Taylor et al. (1983) used the rat vas deferens for pharmacological characterization of purinergic receptors.

Nerve–muscle preparations of the vas deferens have been reviewed by Holman (1975).

Hughes et al. (1974) used the electrically stimulated mouse vas deferens for assessment of the agonistic and antagonistic activities of narcotic analgesic drugs.

Ross et al. (2001) used the mouse vas deferens to study structure–activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors.

Oka et al. (1980) recommended the vas deferens from rabbits as a specific bioassay for opioid κ-receptor agonists.

Mutafova-Yambolieva and Radmirov (1993) studied the effects of endothelin-1 on electrically or drug-induced contractile responses mediated by purinergic or adrenergic receptors in the isolated prostatic portion of rat vas deferens.

Ward et al. (1990) used isolated vasa deferentia preparations from rat and mouse to study the pharmacological profile of the analgesic pravadoline.

Cordellini and Sannomiya (1984) pretreated guinea pigs with reserpine. In the isolated vasa deferentia, concentration–effects curves to phenylephrine were established in the presence of cocaine. The antagonistic effect of phenoxybenzamine was used for receptor occupancy studies.

Donoso et al. (1992) studied neurotransmission in epididymal and prostatic segments of isolated superfused rat vas deferens preparations.

Vaupel and Su (1987) used the vas deferens preparation of guinea pigs to study sigma and phencyclidine receptors.

Eltze (1988) used the field-stimulated (95 % of maximum voltage, 0.1 Hz, 0.5 ms) portion of rabbit vas deferens to study muscarinic M1 and M2 receptors.

Dumont et al. (1997) used the isolated guinea pig heart and the isolated rat vas deferens for in vitro bioassays of calcitonin gene-related peptide (CGRP) agonists and antagonists.

Poyner et al. (1999) found concentration-dependent inhibitions of the electrically stimulated twitch responses of guinea pig vas deferens by calcitonin gene-related peptide, amylin, and adrenomedullin.

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α1-adrenoceptor similar to that found in the vas deferens.

Burt et al. (1995) proposed that noradrenaline contraction in the epididymal portion of the rat vas deferens is mediated by α1A-adrenoceptors.

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues, and antagonists in rat isolated vas deferens. The prostatic half was suspended under 0.5-g resting tension and equilibrated in Krebs

solution at 37 °C. Contractile responses of the prostatic vas were induced by electrical field stimulation at 0.2 Hz, 1.0 ms, and 60 V through parallel platinum electrodes on either side of the tissue.

Analysis of expression and contractile function of α1-adrenoceptors in the vas deferens of normotensive and hypertensive rats was provided by Yono and colleagues (2012).

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α-Sympatholytic Activity in the Isolated Rat Spleen

Purpose and Rationale

α-Stimulant agents (e.g., epinephrine, norepinephrine) or electrical stimulation induces contractions in sympathetically innervated organs such as spleen smooth muscle. These effects can be antagonized by drugs with α-blocking activities such as phentolamine.

Procedure

Male Sprague-Dawley rats weighing 180–220 g are used. The animal is sacrificed in CO_2 anesthesia. The spleen is removed and cut longitudinally into two halves. Each part is placed in an organ bath containing nutritive solution. The bath solution is bubbled with 95 % O_2 and 5 % CO_2 mixture and maintained at 37 °C. Following a 30-min incubation period under a tension of 0.5 g, contractions are elicited by administration of epinephrine (10^{-6} g/ml) or norepinephrine 10^{-6} g/ml). After obtaining 3 approximately identical spasms, the test compound is administered followed by the addition of the spasmogen 5 min later. The contractile response is allowed to plateau and recorded.

Standard compound:

- Phentolamine

Evaluation

The contractile force is recorded at its maximal level before and after drug administration. The percent inhibition of epinephrine- or norepinephrine-induced contraction is determined.

Modifications of the Method

Burt et al. (1995a, b) demonstrated that phenylephrine-induced contractions of the rat spleen are mediated via α1B-adrenoceptors. The contraction consists of an initial phasic component due to release of intracellular Ca^{2+} and larger tonic contraction due to capacitative Ca^{2+} influx through non-voltage-gated Ca^{2+} channels.

Aboud et al (1993) investigated the subtypes of adrenoceptors involved in contractions of the rat aorta, vas deferens, and spleen.

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α-Sympatholytic Activity in the Isolated Rat Anococcygeus Muscle

Purpose and Rationale

The rat anococcygeus muscle as pharmacological tool was introduced by Gillespie (1972, 1980) and Gibson and Gillespie (1973). This smooth muscle has a dense adrenergic innervation and contracts to noradrenaline, acetylcholine, 5-hydroxytryptamine, but not to histamine. Moreover, the muscle contracts to field stimulation or stimulation of extrinsic nerves. The preparation can be used to assess the pre- and postsynaptic α-adrenoceptor-blocking activity of drugs (Doggrell 1980, 1983).

Procedure

The two anococcygeus muscles arise from the upper coccygeal vertebra close to one another in the midline of the pelvic cavity. The muscles pass caudally, lying first behind and to one side of the colon, finally joining together to form a ventral bar in front of the colon a few mm from the anus. The extrinsic nerves pass in a branch of the perineal nerve on either side to enter the deep surface of each muscle just before the formation of the ventral bar.

After sacrifice, the abdomen of rats is opened in the midline, the pelvis split, and the bladder and urethra removed. Care is required in clearing the lower part of the urethra to avoid damage to the ventral bar of muscle, the only region lying ventral to the colon. The colon is then cut through at the pelvic brim, the pelvic portion pulled forward, and the delicate connective tissue behind cleared until the anococcygeus muscles come into view. The muscles are isolated, in some instances with the extrinsic nerve intact. The extrinsic nerves on either side run in the posterior scrotal branch of the perineal nerve and leave it to enter the deep surface of the anococcygeal muscles as they lie on the lateral surface of the colon. The ventral bar is cut through and each muscle mounted in a 100-ml bath containing Krebs solution at 36 °C. The solution is gassed with 95 % O_2 +5 % CO_2. Tension is measured with isometric transducers and displayed on a polygraph. Field stimulation of the intramural nerves is applied after drawing the muscles through a pair of electrodes similar to those described by Burn and Rand (1960); when the muscles are stimulated through their extrinsic nerves, the nerves are drawn through similar electrodes. Stimulation of either intramural or extrinsic nerves is with 1-ms pulses at 20 Hz and at a supramaximal voltage.

Dose–response curves are established with doses of 2×10^{-7} to 4×10^{-6} M noradrenaline and 4×10^{-7} to 4×10^{-5} M acetylcholine or with graded frequencies of electrical stimulation.

The effect of noradrenaline is abolished by α-adrenergic antagonists, such as 10^{-6} M phentolamine. Dose–response curves show a parallel shift characteristic of competitive antagonism.

Evaluation

Contractions of the anococcygeus muscle induced by an α-adrenergic agonist after addition of the

test compound are compared with the initial values and expressed as percentage thereof. For in-depth analysis, full dose–response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

Modifications of the Method

Gibson et al. (1990) found L-N^G-nitroarginine to be a potent inhibitor of non-adrenergic, non-cholinergic relaxations in the rat anococcygeus muscle.

Oliveira and Bendhack (1992) found that dopamine has a dual effect in the rat anococcygeus muscle: a partial effect due to an indirect sympathomimetic action and a partial effect due to the interaction with postjunctional receptors.

Brave et al. (1993) investigated the interaction between motor sympathetic and inhibitory non-adrenergic, non-cholinergic nerves in the rat anococcygeus muscle using L-N^G-nitroarginine, an inhibitor of L-arginine/NO synthase.

Cakici et al. (1993) described a coaxial bioassay system consisting of the guinea pig trachea as the donor organ for epithelial-derived relaxing factors and phenylephrine-precontracted rat anococcygeus muscle as assay tissue.

Iravani and Zar (1993) found differential effects of nifedipine on nerve-mediated and noradrenaline-evoked contractions of rat anococcygeus muscle.

Rand and Li (1993) studied the modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves.

Mudumbi and Leighton (1994) investigated the mechanisms of action of relaxation induced by bradykinin and by electrical field stimulation in isolated rat anococcygeus muscle, where contractile tone had been elevated with clonidine.

Gwee et al. (1995) investigated the prejunctional and postjunctional inhibition of adrenergic transmission in the rat isolated anococcygeus muscle by cimetidine.

Najbar et al. (1996) found that smooth muscle cells in the rat anococcygeus muscle are endowed with two distinct P-2-purinoreceptors which subserve contractions.

De Godoy et al. (2003) evaluated the inhibitory effects of atropine and hexamethonium on the angiotensin II-induced contraction of rat anococcygeus muscles.

Pettibone et al. (1993) examined the inhibitory potency and selectivity of an oxytocin antagonist against oxytocin-stimulated contractions of the mouse anococcygeus muscle.

Dehpour et al. (1993) and Radjaee et al. (1996) used isolated anococcygeus muscles from rabbits and found an extremely regular activity induced by methoxamine or clonidine.

Zhang et al. (2011) used anococcygeus muscle to test isoindolinone- and isobenzofuranone-containing phenoxylalkylamines as potent α(1)-adrenoceptor antagonists.

Toque and colleagues (2009) compared the effect of the phosphodiesterase 5 inhibitors sildenafil, tadalafil, and vardenafil on rat anococcygeus muscle.

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β_1-Sympatholytic Activity in Isolated Guinea Pig Atria

Purpose and Rationale

The β-agonist isoprenaline (isoproterenol) induces an increase in the frequency and force of contraction of spontaneously beating isolated

right atria and potentiates contractions of electrically stimulated isolated left atria. Drugs with β-sympatholytic activity inhibit these isoprenaline-induced effects. The β-receptor-blocking activity of drugs can be evaluated in isolated right (a) and left (b) guinea pig atria. Since the heart contains predominantly β_1-adrenoreceptors, β_1-blocking activity is assessed by this test.

Procedure

Pirbright White guinea pigs of either sex weighing 250–300 g are used. The animal is sacrificed by stunning and exsanguination. The heart is removed, and the right or the left atrium is cut off and mounted in a 50-ml organ bath with a preload of 100 mg. The Krebs–Henseleit solution is maintained at 32 °C and aerated with 95 % O_2/5 % CO_2. Contractions are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg).

Right Atrium

After an equilibration period of 30 min, isoprenaline is administered into the organ bath to potentiate inotropy and frequency of the isolated right atrium. Cumulative doses of isoprenaline are added starting from a concentration of 0.05 µg/ml; consecutive doses are administered at 3-min intervals.

When a stable maximum plateau of the effect is achieved, the organ bath is thoroughly flushed for 1 min; flushing is repeated twice, 5 and 20 min later. The whole procedure is repeated with the same isoprenaline concentrations (control baseline values = 100 %).

The test compound is then added into the organ bath, and 5 min later, again isoprenaline is given at cumulative doses.

If the test compound has β-receptor-blocking activity (β-sympatholytic):

1. Higher isoprenaline concentrations are necessary to induce the same potentiation of inotropy and frequency.
2. At the same isoprenaline concentrations added as before, the increase in inotropy and frequency is reduced.

At the end of the experiment, again a cycle without test drug is performed.

Left Atrium

The left atrium is stimulated by a square wave stimulator with 2 impulses/s at a voltage of 15 V and an impulse duration of 1 ms. After an equilibration period of 30 min, the β-agonist isoprenaline is added at concentrations of 0.05–0.1 mg/ml. The organ bath is then thoroughly flushed for 1 min. Flushing is repeated twice, 5 and 20 min later. The whole procedure is repeated with the same cumulative isoprenaline concentrations (control baseline values = 100 %) and flushing procedure.

When a stable plateau of contractions is achieved, the test compound is added into the organ bath, and 3 min later, isoprenaline is added again at cumulative concentrations.

If the test compound has β-receptor-blocking activity (β-sympatholytic), the isoprenaline-induced effects are inhibited.

In addition, refractory period is determined before and after drug administration.

Evaluation

(a) Percent inhibition of (a) isoprenaline-induced or (b) electrically induced and isoprenaline-potentiated increased inotropy and frequency by test drug is calculated as compared to predrug activity (=100 %).
(b) Percent change in refractory period is calculated.

IC_{50} values are determined from the individual dose–response curves.

Statistical evaluation is performed by means of the paired *t*-test.

Standard compounds:

- Propranolol HCl
- Amrinone
- Nifedipine
- Milrinone

Modifications of the Method

A detailed description of the use of isolated atrial preparations has been given by Levy (1971).

Instead of the right atrium, Doggrell and Hughes (1986) used the isolated right ventricle of the rat for the assessment of the β-adrenoreceptor-blocking activity of propranolol and investigated the competitive nature of the isoproterenol antagonism at various doses with Schild plot analysis. Doggrell (1988) used the isolated left atria of the rat for simultaneous assessment of membrane-stabilizing and β-adrenoreceptor-blocking activity.

Berthold et al. (1990) described a method for testing cardiotonic sodium channel activators in isolated, electrically stimulated left guinea pig atria after potassium depolarization.

Olson et al. (1995) studied the function of isolated rat left atria and papillary muscles and quantified the voltage-response relationship between punctate and field electrical stimulation after pretreatment with reserpine or β-blockers.

Goineau and colleagues (2012) compared the effect of sodium channel blocker lidocaine on the slowing of cardiac conduction using atrial action potential model in rabbit and guinea pig left stimulated atria.

Shurayama et al. (1991) investigated the electrophysiological effects of sodium channel blockers (mexiletine, lidocaine, disopyramide, aprindine, and flecainide) on the guinea pig left atrium.

Penson et al. (2008) used rat isolated left atria and right ventricle to compare the effect of ischemic preconditioning and β-adrenoceptor-mediated preconditioning on the myocardial ischemia, providing detailed description of atrial preparations.

Gardner and Broadley (1999) analyzed the characteristics of adenosine receptors in guinea pig isolated right and left atria and papillary muscles.

Quan and colleagues (2010) described preparation of perfused beating rabbit atria, which they used to study β1-adrenergic receptor-activated mechanism of decreasing atrial natriuretic peptide release.

Boer et al. (2011) investigated involvement of different adrenoceptor subtypes in the catecholamine-induced automatism in rat left atria.

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β_2-Sympatholytic Activity in the Isolated Tracheal Chain

Purpose and Rationale

Contraction of bronchial smooth muscle is induced by the cholinergic agonist carbachol. The carbachol effect can be antagonized by the β-agonist isoprenaline (isoproterenol). A compound has β-sympatholytic activity if the spasmolytic action of isoprenaline is inhibited. The β-sympatholytic effect of drugs can be evaluated in an in vitro model. Since the trachea contains predominantly β_2-adrenoreceptors, β_2-blocking activity can be assessed by this test.

Procedure

Male Pirbright White guinea pigs weighing 250–300 g are used. The animals are sacrificed by stunning and exsanguination. The trachea is removed and cut into individual rings. Six rings are connected in series by means of short loops of silk thread. The tracheal chain is mounted in a 50-ml organ bath with a preload of 1 g for isotonic registration. To the nutritive solution (Tyrode) containing ascorbic acid and 1.0 g/l glucose, the α-receptor-blocking agent phentolamine (0.1 μg/ml) and the spasmogen carbachol (80 ng/ml) are added. The solution is maintained at 34 °C and aerated with 95 % O_2 and 5 % CO_2

Experimental Course

After an equilibration period of 30 min, cumulative doses of 10^{-10} to 10^{-7} M of the spasmolytic agent isoprenaline are added. When maximal relaxation is obtained, the organ bath is flushed and the procedure repeated. After the two control relaxations with isoprenaline, the tissue is rinsed thoroughly, and the first dose of the test compound is administered. Three minutes later, cumulative doses of isoprenaline are administered as before. Following a 10-min washout and recovery period, the next dose of the test compound is given. Up to ten drug concentrations can be tested with one organ.

Standard compounds:

- Propranolol
- Practolol

Evaluation

Percent inhibition of isoprenaline-induced relaxation under drug treatment is calculated compared

to maximal relaxation induced by isoprenaline alone (control = 100 %).

A competitive antagonism of the test compound is evaluated and can be quantitated from the dose–response curve.

Modifications of the Method

For a detailed review on pharmacological techniques for the in vitro study of airways, see publication by Fedan et al. (2001). The authors evaluated preparation of tracheal and bronchial strips and rings, also describing in detail the technique of isolated, perfused trachea preparation.

O'Donnell and Wanstall (1980) used guinea pig tracheal preparations, where K^+-depolarization was achieved by replacing all the Na^+ in Krebs solution by an equivalent amount of K^+ causing a sustained contraction of the preparations. A dose-dependent relaxation effect of isoprenaline could be obtained provided that the preparations were repolarized by washing in normal Krebs solution between curves. pA2 values were in good agreement with values obtained in other types of tracheal preparations.

The guinea pig superfused trachea and dispersed tracheal cells have been used by Buckner et al. (1995) to compare the effects of isoproterenol and forskolin on immunologic and nonimmunologic histamine release.

Lundblad and Persson (1988) found that epithelial removal has a little consequence for the pharmacology of the guinea pig tracheal open ring preparation in vitro.

Cheng et al. (2014) described preparation of rat trachea strips, which could be used as a simple and rapid test needed for screening parasympathetic mimetic agents and potential tracheal contraction agents.

Liu et al. (2014) used rat trachea strips to evaluate the synergized effect of steroids and antihistamines on airway smooth muscle contractility.

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Angiotensin-Converting Enzyme Inhibition in the Isolated Guinea Pig Ileum

Purpose and Rationale

The angiotensin-converting enzyme (ACE) is responsible for the formation of the active angiotensin II from the inactive angiotensin I (Campbell et al. 2004). The same enzyme is also responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and inhibition of the activity of bradykinin. ACE inhibition results in decreased angiotensin II activity and potentiation of the bradykinin effect. The guinea pig ileum contracts in response to both peptides, angiotensin II and bradykinin, and can be used for quantitative determination of ACE-inhibiting activity.

Procedure

Guinea pigs of either sex weighing 300–500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, and the mesentery is being cut away as necessary. When the colon is reached, the intestine is cut free. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode solution is passed through the tube and the intestine until the effluent is clear. The mesentery is cut away from the intestine that was joined to the colon. Pieces of 3-cm length are cut. Preferably, the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into an organ bath with Tyrode solution at 37 °C being oxygenated with O_2. The other end is fixed to an isometric force transducer (UC-2, Gould-Statham, Oxnard, USA). Responses are recorded on a polygraph.

Angiotensin I Antagonism

After an equilibrium time of 30 min, angiotensin I is added in a concentration of 10 ng/ml bath solution. The force of contraction is recorded, and the angiotensin I dosage is repeated once or twice until the responses are identical. Then the potential ACE inhibitor is added. After a 5-min incubation time, again angiotensin I is added. The contraction is diminished depending on the activity of the ACE inhibitor.

Bradykinin Potentiation

Pieces of guinea pig ileum are prepared as described before. After an equilibrium time of 30 min, bradykinin is added in a concentration of 15 ng/ml bath solution. The force of contraction is recorded and bradykinin additions are repeated once or twice until the response is identical. Then the potential ACE inhibitor is added. After a 5-min incubation time, again bradykinin is added. The contraction is potentiated depending on the activity of the ACE inhibitor.

Evaluation

Angiotensin I Antagonism

The contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. Using various doses of the ACE inhibitor, IC_{50} values (concentrations inducing 50 % inhibition) are calculated. As standards, ramipril, enalapril, and captopril are used.

Bradykinin Potentiation

The increase of the contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. As standard, ramiprilat is used.

Modification of the Method

Minshall et al. (2000) demonstrated that angiotensin I-converting enzyme (ACE/kininase II) inhibitors potentiated guinea pig ileum's isotonic contraction to bradykinin and its analogues.

Carmona and Juliano (1996) used isolated guinea pig ileum to assayed new retro-inverso tripeptides that preferentially inhibit angiotensin-converting enzyme or potentiate the response of smooth muscle to bradykinin.

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Contractile and Relaxing Activity on Isolated Blood Vessels Including Effects of Potassium-Channel Openers

Purpose and Rationale

The contractile process within the vascular smooth muscle results from an increase in the concentration of intracellular Ca^{2+}. Inhibition of vasoconstriction occurs by addition of calcium antagonists or by removal of extracellular calcium. The vasorelaxing effects of compounds can be tested in isolated rodent arteries (pulmonary artery, thoracic aorta). Arterial rings or strips with or without endothelial lining are contracted with different agents, e.g., extracellular K^+ and Ca^{2+}, the α-adrenoceptor agonists phenylephrine and noradrenaline, the Ca^{2+} ionophore A23187, or the thromboxane receptor agonist U46619. Compounds with vasodilating activity antagonize the induced contractions.

Potassium-channel openers such as cromakalim, nicorandil, pinacidil, or HOE 234 induce relaxation of contracted smooth musculature (Bolton et al. 1998). These effects are explained by data from the patch clamp technique and ion flux experiments as well as by antagonism against potassium-channel blockers. They indicate the potential use as antihypertensive and antiasthmatic drugs (Hamilton and Weston 1989; Edwards and Weston 1990, 1993; Weston and Edwards 1992). The studies are complicated by the high diversity of potassium channels including ATP-sensitive, voltage-sensitive, and Ca^{2+}-activated channels (Mourre et al. 1986; Blatz and Magleby 1987; Ashcroft and Ashcroft 1990; Jan and Jan 1990; Pongs 1992; Wann 1993). Since each functional channel appears to consist of four different subunits, the possibility exists that there may be hundreds of different voltage-sensitive K channels, depending on their subunit composition. Ashcroft and Gribble (2000) discussed new windows on the mechanism of action of K_{ATP} channel openers.

Glibenclamide is an antagonist of the ATP-modulated K^+ channel allowing the localization of the binding sites (Eltze 1989; French et al. 1990; Mourre et al. 1990; Miller et al. 1991).

Procedure

Male Pirbright White guinea pigs weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 250–400 g are used as donor animals. The tested vessels are the thoracic aorta or the arteria pulmonalis.

Materials and solutions

Physiological salt solutions (PSS) [mM]			
	PSS I	PSS II	PSS III
NaCl	122	112	72 (92)
KCl	5.0	5	40 (20)
$CaCl_2$	1.2	–	–
$MgSO_4$	0.56	0.56	0.56
KH_2PO_4	1.2	1.2	1.2
$NaHCO_3$	25	25	25
EDTA	–	0.2	–
Glucose	12	12	12

Contracting agents	
$K^+ + Ca^{2+}$	40 mM + 0.5 mM
$K^+ + Ca^{2+}$	20 mM + 0.5 mM
U 46619 (thromboxane A_2 analogue)	1 μM
A 23187 (calcium ionophore)	5 μM
Noradrenaline	1 μM
Phenylephrine	0.1 μM
Acetylcholine	1 μM
Oxyhemoglobin	10 μM
Methylene blue	10 μM

Animals are sacrificed by stunning and exsanguination. At least four isolated organs are tested per drug. The heart and the pulmonary artery are quickly removed and immersed in PSS I at room temperature. The artery is dissected into rings, and endothelial cells are removed by gently rubbing the intimal surface. Spirally cut strips of 15–20-mm length and 1–1.5-mm width are suspended at a resting force of 380 mg in an organ bath containing 20 ml oxygenated (95 % O_2, 5 % CO_2) PSS I at 37 °C. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg).

To test the effect of compounds on vessels with intact endothelial lining, the thoracic aorta of rats is isolated and dissected free from surrounding tissue. Rings of 3-mm width are cut and suspended in the organ bath containing PSS I. Isometric force is measured with a force transducer (UC-2, Gould-Statham, Oxnard, USA) under a resting tension of 500 mg.

The functional integrity of the endothelium is tested before drug administration. One μM acetylcholine in the organ bath should result in a transient relaxation.

After an equilibration period of 1 h, contraction of each vessel strip or ring is induced by addition of one of the contracting agents into the organ bath.

To induce contractions of potassium-depolarized vessels, three different PSS solutions are used (PSS I for 30 min, PSS II for 3 × 15 min, and PSS III). Contraction is induced in the presence of PSS III by adding 0.5 mM Ca^{2+} into the organ bath.

When a stable plateau of contraction is achieved, cumulative concentrations of the test compound are added into the organ bath to obtain drug–response curves. Consecutive concentrations are added either at 1-h intervals or when the response of the previous dose has reached a steady-state level.

In order to study the time course of relaxation and the duration of action, only one concentration is tested.

To test whether the mechanism of action of a vasorelaxing agent is related to the liberation of nitric oxide, methylene blue or oxyhemoglobin (10 μM) are added to the organ bath 15–30 min prior to the cumulative administration of the test compound. Methylene blue or oxyhemoglobin blocks selectively NO-induced relaxation.

Evaluation

Mean values of relaxation ±SEM are calculated. The height of contraction before the first drug administration is taken as 100 %.

IC^{50} values are determined from the individual dose–response curves. IC^{50} is defined as the dose of drug leading to a 50 % relaxation of the contraction induced by KCl or other agonists.

Statistical evaluation is performed by means of the *t*-test.

Modifications of the Method

Calderone et al. (1996) compared four rat aortic preparations (single ring, spiral strip, zigzag strip,

and multiple rings) on the basis of responses to noradrenaline and acetylcholine. They recommended the multiple-ring preparation as the most suitable of all four for the study of vasoactive drugs because of the reproducibility of both contracturant and relaxing responses.

Kent et al. (1982) used rat aortic strips contracted to a stable tension by either phenylephrine or barium chloride for comparison of vasodilators.

Wilson et al. (1988) studied in isolated rings of rat aorta precontracted with noradrenaline the antagonism of glibenclamide against the vasorelaxation induced by cromakalim.

Löhn et al. (2002) cannulated cerebral arteries from mice with glass cannulas on both sides, allowing an application of hydrostatic pressure to the vessel. Diameter was measured by using a videomicroscopic system (Nikon Diaphot, Düsseldorf, Germany) connected to a personal computer with appropriate software for detection of changes of vessel diameter (TSE, Bad Homburg, Germany).

Nishimura and Suzuki (1995) tested the contractile responses to 5-HT in basilar arteries, superior mesenteric arteries, and thoracic aortas from stroke-prone spontaneously hypertensive rats in comparison to normal Wistar–Kyoto rats and found that the hyperresponsiveness to 5-HT is mediated by different 5-HT receptor subtypes.

Fouda et al. (1991) used the isolated tail arteries from rats. Differences of the vasoconstrictor response to potassium and norepinephrine between tail arteries from spontaneously hypertensive, renovascular hypertensive, and various strains of normotensive rats were found.

Hamilton et al. (1986) and Dacquet et al. (1987) studied the effects of calcium entry blockers in rat portal vein.

Bråtveit and Helle (1984) studied the inhibition of vascular smooth muscle by vasoactive intestinal peptide (VIP) in the isolated rat portal vein.

Shetty and Weiss (1987) studied the inhibition of spontaneous rhythmic movements and norepinephrine-induced tension responses in the rat portal vein.

Edwards et al. (1991) compared the effects of several potassium-channel openers on the rat bladder and rat portal vein in vitro.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin gene-related peptide to relax mesenteric arteries precontracted with norepinephrine.

Chen et al. (1996) studied the contractile effects of noradrenaline and neuropeptide Y given alone or in combination on isolated rat mesenteric resistance vessels.

Gurden et al. (1993) used guinea pig aorta relaxation for functional characterization of adenosine receptor types.

Eltze (1989) studied the antagonism of glibenclamide against potassium-channel openers in the isolated guinea pig pulmonary artery.

Szentmiklósi et al. (1995) used circular segments from the proximal part of the main pulmonary artery of guinea pigs to study contractile and relaxant effects of adenosine receptors.

Pikkers and Hughes (1995) examined the effect of hydrochlorothiazide on intracellular calcium concentration $[Ca^{2+}]i$ and tone in guinea pig mesenteric arteries. Vessels were mounted on a microvascular myograph and loaded with the Ca^{2+}-sensitive fluorescent dye, Fura-2.

Nishimura et al. (1998) used isolated aorta rings from Syrian hamsters. Contractile responses were recorded with an isometric transducer (TSE, Bad Homburg, Germany) and stored (TSE data acquisition software).

Meisheri et al. (1990) recommended the use of the isolated rabbit mesentery artery as a sensitive in vitro functional assay to detect K^+-channel-dependent vasodilators.

Mironneau and Gargouil (1979) studied the influence on electrophysiological and mechanical parameters of longitudinal smooth muscle strips isolated from rabbit portal vein by means of a double sucrose-gap method associated with a photoelectric device for recording contractions.

Lauth et al. (2001) performed superfusion assays with the rabbit jugular vein. Four venous ring segments (3–4 mm long) were tested simultaneously by mounting them between force transducers and a rigid support for measurement of isometric force. Increasing doses of bradykinin were applied as bolus injections, and the ensuing constrictor response was monitored with the aid of

a PC-operated analysis system (BioSys, TSE, Bad Homburg, Germany).

McBean et al. (1986, 1988) used isolated segments of the arteria basilaris of pigs to detect compounds with antivasoconstrictive properties. Contraction is elicited by PGF2α, serotonin, or norepinephrine. Specimens are obtained from adult pigs (strain: Deutsche Landrasse) within 30 min after slaughter from the local slaughterhouse and stored in nutritive solution. The vessels are trimmed to a length of 4 mm, and the segments are suspended between 2 L-shaped metal hooks in a bath containing 20 ml modified Krebs–Henseleit solution (NaCl 148 mM, KCl 5.4 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 12 mM, glucose 12 mM). The bath solution is maintained at 37 °C and continuously gassed with carbogen to produce a resulting pH of 7.35–7.45. The preparation is incubated under a tension of 37.28 mN (optimal passive load producing the largest contractile response to 3×10^{-6}MPGF2α). Following a 60-min stabilization period, the vessels are sensitized with 30 mM KCl for 10 min. The vessels are washed for 1 min and allowed to recover for 30 min with additional 1-min washes at 15 and 30 min. Thereafter, contractions of the vessels are induced by adding PGF2α at 3×10^{-6} M. The contractile response is allowed to plateau, and then the test compound is administered at cumulative doses.

For each test compound, a dose–response curve is recorded. The EC50 is obtained graphically or by means of a Hill plot. The EC50 is defined as the dose of drug producing half maximal response.

Werner et al. (1991) studied the vascular selectivity of calcium antagonists using porcine isolated ventricular trabeculae and right coronary arteries.

Merkel et al. (1992) used isolated porcine coronary artery rings precontracted with prostaglandin F2α to demonstrate the vasorelaxant activity of an A1-selective adenosine agonist.

Miwa et al. (1993) compared the effect of a K^+-channel opener with cromakalim, nitroglycerin, and nifedipine on endothelin-1-induced contraction of porcine coronary artery.

Satoh et al. (1993) investigated in isolated porcine large coronary arteries whether or not the vasorelaxant actions of nicorandil and cromakalim would be selective using seven different vasoconstrictor agonists.

Yokoyama et al. (1994) studied the vasodilating mechanisms of several pyridinecarboximidamide derivatives in isolated porcine coronary arteries.

Makujina et al. (1995) described a procedure that facilitates the eversion of vascular smooth muscle. Vascular segments of porcine coronary artery, approximately 2 cm in length, were sutured to portions of polyethylene tubing inserted into the lumen of the vessel. After being secured and stabilized by the tubing, the vessel was everted while immersed in physiological buffer. Intracellular calcium concentrations (measured by Fura-2 AM fluorometry) and tension were registered simultaneously in everted rings denuded of endothelium.

Izumi et al. (1996) tested a K^+-channel opener and related compounds in isolated porcine coronary arteries contracted with 25 mM KCl.

Frøbert et al. (1996) described impedance planimetry as a new catheter-based technique to measure porcine coronary artery pharmacodynamics and compared the results with the commonly used wire-mounted isometric tension technique after in vitro application of nifedipine in various concentrations. A four-electrode impedance measuring system was located inside a 12-mm-long balloon which was introduced into 3–4-cm-long segments of the left anterior descending coronary artery obtained from 70- to 90-kg Danish Landrace–Yorkshire pigs.

Hamel et al. (1993) dissected segments (3–4 mm long) of temporal ramifications of the middle cerebral artery from bovine brains and mounted them between two L-shaped metal prongs in a tissue bath containing Krebs–Ringer solution at 37 °C. Changes in muscle tension were measured by a force displacement transducer and recorded on a polygraph. Several 5-HT receptor agonists were tested for their ability to induce vasoconstriction, and their potencies were compared to that of 5-HT. The authors concluded that bovine pial arteries appear to be the best available model for the human cerebrovascular 5-HT1D receptor.

De la Lande et al. (1996) used isolated segments from proximal (4.5 mm i.d.) and distal (0.5 mm i.d.) bovine coronary arteries and found a heterogeneity of response to glyceryl trinitrate.

The isolated human coronary artery was used to study the vasoconstriction by acutely acting antimigraine drugs (Saxena et al. 1996a, b, 1997).

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Isolated Guinea Pig Ureter

Purpose and Rationale

The isolated guinea pig ureter shows phasic–rhythmic contractions after addition of KCl to the organ bath. Inhibition of this effect can be explained as a modulation of potassium channels.

Procedure

Male unfasted guinea pigs weighing 400–500 g are sacrificed, and both ureters are removed immediately without the part directly connected to the pelvis in order to exclude the pacemaker region responsible for spontaneous activity. Each segment of 2-cm length is placed in a Petri dish containing Tyrode solution at 37 °C, freed of surrounding connective tissue, and then suspended at a baseline tension of 0.5 p in a 25-ml organ bath containing Tyrode solution at 37 °C being aerated with 5 % CO_2/95 % O_2, pH 7.4. Contractions are measured isometrically using Gould-Statham UC-2 transducers. After a 15-min equilibration period, KCl is added to the bath in a final concentration of 3×10^{-2} Mol/l and left in the bath for 2 min. KCl induces a constant series of phasic–rhythmic contractions without a rise in baseline tone. Subsequent washing causes the immediate disappearance of the rhythmic contractions. This addition of KCl is repeated and the values of these two experiments are used as initial values. The antagonistic activity is studied by addition of the test drug 1 min prior to the KCl challenge. Percentage of the following parameters is determined: the mean height of contractions, frequency of contractions, and product of mean height and frequency of contractions. For interaction studies, the potassium-channel blocker glibenclamide 10^{-6} mol/l is added 1 min prior to the test drug.

Evaluation

Arithmetic means and standard deviations of the data are calculated and compared with initial values using Student's *t*-test.

Critical Assessment of the Method

The isolated guinea pig ureter stimulated with KCl can be used for studies on the modulation of potassium channels.

Modifications of the Method

Yoshida and Kuga (1980) recorded electrical activities in a preparation consisting of the pelvic region and the upper ureter of the guinea pig. Train field stimulation of the pelvic region evoked a train of nerve action potentials followed by a multiphasic smooth muscle action potential after a latency of about 2.5–8.0 s. This smooth muscle response was abolished by tetrodotoxin and dibucaine and also by cholinergic blocking agents.

The effects of veratridine and of yohimbine on the efflux of norepinephrine from electrically stimulated guinea pig ureters were studied by Kalsner (1992).

Maggi and Giuliani (1994) studied the excitability and refractory period of the guinea pig ureter to electrical field stimulation.

Roza and Laird (1995) and Laird and Cervero (1996) studied the pressor responses to distension of the ureter in anesthetized rats as a model of acute visceral pain.

A simple method for measurement of ureteric peristaltic function in vivo in anesthetized rats was published by Kontani et al. (1993).

Al-Aown et al. (2011) proposed using of porcine model of isolated ureteric smooth muscle for the evaluation of the effect of different substances on the ureter, specifically the effects of phosphodiesterase type 5 inhibitors

Jered and colleagues (1999) have used ureters from adult domestic swine and compared four different methods of ureteric suspension to determine the optimal contractile response to electric field stimulation and carbachol.

Villa et al. (2013) compared the effect of α1A-adrenoceptor antagonists silodosin, tamsulosin, and prazosin on contraction of the human and rat isolated ureter.

Kobayashi et al. (2009) have showed that α1A-adrenoceptor antagonist silodosin inhibited α-adrenoceptor agonist-induced contraction in both mouse and hamster ureters.

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Isolated Corpus Cavernosum

Purpose and Rationale

The isolated corpus cavernosum of rabbits has gained interest as pharmacological model since selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (PDE5) were found to be effective in the treatment of erectile dysfunction in men (Ballard et al. 1996; Jeremy et al. 1997; Chuang et al. 1998; Liu et al. 1998; Turko et al. 1999; Wallis 1999; Wallis et al. 1999; Stief 2000; Aydin et al. 2001; Thompson et al. 2001; Lin et al. 2002).

Procedure

Male New Zealand White rabbits weighing 3–4 kg are sedated with an intramuscular injection of 25 mg/kg ketamine +6 mg/kg xylazine. Anesthesia is maintained by intravenous injection of 25 mg/kg Nembutal. The penis is removed at the level of the attachment of the corporal bodies to the ischium. The corpus cavernosum (total length about 20 mm) is sharply dissected from the tunica albuginea and two longitudinal strips with unstretched length about 10 mm are made from the proximal, more muscular portion.

Corporal strips are placed in organ baths containing 10 ml Tyrode buffer (NaCl 124.9 mmol/l, KCl 12.5 mmol/l, $MgCl_2 \cdot 6H_2O$ 0.5 mmol/l, NaH_2PO_4 H_2O 0.4 mmol/l, $CaCl_2$ 1.8 mmol/l, and glucose 5.5 mmol/l) at 37 °C. Each tissue is equilibrated with a mixture of 95 % O_2 and 5 % CO_2 at pH 7.4. One end of each strip is connected to a force displacement transducer, and changes in muscle tension are measured and recorded with a polygraph. After zeroing and balancing transducers and strip chart, 2.0 g of tension is placed on each strip, and the strips are allowed to equilibrate for 30 min.

Each strip is prestimulated with 10 μM phenylephrine and then relaxed by electrical field stimulation with square wave pulses of 80 V, 1-ms duration at 2–16-Hz frequency. Then sodium nitroprusside (0.01–100 μM) is added as NO donor. Finally, the standard (sildenafil 1 nM to 1 μM) or the test compound is added.

Evaluation

The dose-dependent increase of relaxation after test compound and standard is measured. From dose–response curves, activity ratios can be calculated.

Modifications of the Method

Wallis et al. (1999) studied the inhibition of human phosphodiesterases PDE1 to PDE6 by sildenafil in various tissues, such as the cardiac ventricle, corpus cavernosum, skeletal muscle, and retina.

Park et al. (1997) reported functional characterization of angiotensin II receptors in rabbit corpus cavernosum.

Yildirim et al. (1997) investigated the effects of castration and testosterone on the constricting effect of phenylephrine and endothelium-dependent and endothelium-independent relaxing effects of different agonists in the corpus cavernosum of male rabbits.

Liu et al. (1998) analyzed the pharmacological effects of in vitro ischemia on rabbit corpus cavernosum.

Gupta et al. (1998) found that activation of G_i-coupled postsynaptic α_2-adrenoceptors causes contraction of smooth muscles in the corpus cavernosum of rabbits.

Teixeira et al. (1998) used a bioassay cascade to study the effect of *Tityus serrulatus* scorpion venom on the rabbit isolated corpus cavernosum.

Cellec and Moncada (1998) used the clitoral corpus cavernosum of female rabbits to study the role of nitrergic neurotransmission in non-adrenergic, non-cholinergic relaxation responses.

The isolated corpus cavernosum of **rats** has been used by Tong and Cheng (1997), Gemalmaz et al. (2001), and Wingard et al. (2003), of **mice** by Gocmen et al. (1997) and Mizusawa et al. (2001), of **dogs** by Hayashida et al. (1996) and Comiter et al. (1997), of **monkeys** by Okamura et al. (1998), and of **horses** by Recio et al. (1997).

Studies in isolated **human** corpus cavernosum were performed by Holmquist et al. (1991), Bush et al. (1992), Rajfer et al. (1992), Cellek and Moncada (1997), Ballard et al. (1998), Omote (1999), Wallis et al. (1999), Lin et al. (2000), and Stief et al. (1998, 2000).

In vivo studies measuring intracavernous pressure in **rats** were performed by Ari et al. (1996), Chan et al. (1996), Moody et al. (1997), Reilly et al. (1997), Chang et al. (1998), Mills et al. (1998), Gemalmaz et al. (2001), Takagi et al. (2001), Rajasekaran et al. (2005), and Wingard et al. (2003).

Cashen et al. (2002) measured intracavernous pressure in anesthetized mice.

In vivo studies on penile erection were performed in **cats** by Champion et al. (1997).

Intracavernous pressure was measured in vivo in anesthetized **dogs** by Ayajiki et al. (1997), Sarikaya et al. (1997), Carter et al. (1998), and Noto et al. (2000).

Bischoff and Schneider (2000) described a simple and quantitative model to study agents that influence penile erection in **conscious rabbits**. Erection was assessed by measuring the length of uncovered mucosa before and after the intravenous administration of agents. Animals did not require anesthesia during the course of the study.

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Isolated Guinea Pig Ureter

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Cardiovascular Analysis In Vivo

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Hemodynamic Screening in Anesthetized Rats

Purpose and Rationale

The test is used to detect the effect of compounds on blood pressure and heart rate of anesthetized rats and to check for possible interference with adrenergic receptors. Antihypertensive agents with different mechanisms of action can be detected with this test.

Procedure

Male Sprague–Dawley rats weighing 250–400 g are used. At least two animals are necessary for screening of one compound. The rats are anesthetized by intraperitoneal injection of 8 ml/kg of a solution of 8 % urethane and 0.6 % chloralose. The trachea is cannulated to facilitate spontaneous respiration. Body temperature is maintained at 38 °C by placing the animal on a heating pad.

The left femoral vein is cannulated for drug administration, which is standardized to injections of 0.2 ml/100-g body weight over a period of 1 min. For measurement of hemodynamic parameters and for intra-arterial administration of test compound, a cannula is inserted retrogradely into the right carotid artery. The tip of the catheter is positioned close to the origin of the subclavian artery. This allows most of the injected substances to reach the CNS via the vertebral artery before going into the general circulation.

For continuous monitoring of blood pressure (systolic and diastolic pressure) and heart rate, the catheter is connected to a pressure transducer (Statham DB 23).

When stable hemodynamic conditions are achieved for at least 20 min (control values), test boli of adrenaline (1 mg/kg) and isoprenaline (0.25 mg/kg) are administered. When baseline values are again established, increasing doses of the test substance (0.01, 0.1, 3.0 mg/kg) are given intra-arterially. In case of no effect, the interval between successive doses is 15 min, otherwise 60 min. To check for α- or β-blocking activity,

adrenaline and isoprenaline administration is repeated after injection of the highest dose of test compound. If the test compound shows no effect, a standard antihypertensive compound is administered for control purpose.

Hemodynamic parameters are recorded continuously during the whole experiment.

Evaluation

Changes in blood pressure and heart rate after drug administration are compared to control values obtained during the 20-min predrug period.

Maximal changes in BP and HR and duration of the effect are reported.

The results are scored relative to the efficacy of standard compounds for the degree of the effect and the duration of the effect.

Statistical significance is not tested because of the small number of animals used ($n = 2$, sometimes 3 or 4), but larger numbers of animals have to be used for quantitative evaluation.

Critical Assessment of the Method

Due to the administration of the test compounds via the right common carotid artery, not only peripherally acting vasodilators and neuron blockers but also compounds affecting the blood pressure regulating mechanisms in the CNS are detected. Bolus injections of adrenaline and isoprenaline reveal possible α- or β-antagonistic effects.

Standard data:

The following compounds at the doses indicated lead to a strong decrease in blood pressure:

- Clonidine 0.008 mg/kg
- Dihydralazine 1.0 mg/kg
- Phentolamine 3.0 mg/kg
- Prazosin 0.1 mg/kg
- Propranolol 1.0 mg/kg
- Urapidil 1.0 mg/kg
- Verapamil 0.1 mg/kg

Modifications of the Method

Several authors (Mervaala et al. 1999; Wallerath et al. 1999; Rothermund et al. 2000; Baltatu et al. 2001) monitored arterial pressure and heart rate using a pressure transducer system and continuously recorded on a computer-based registration system (TSE, Bad Homburg, Germany).

A procedure for differential intra-arterial pressure recordings from different arteries in the rat was described by Pang and Chan (1985).

DeWildt and Sangster (1983) described the evaluation of derived aortic flow parameters measured by means of electromagnetic flowmetry as indices of myocardial contractility in anesthetized rats.

Using a special Millar ultraminiature catheter pressure transducer and a thermodilution microprobe, Zimmer et al. (1987, 1988) measured right ventricular functional parameters in anesthetized, closed-chest rats.

Veelken et al. (1990) published improved methods for baroreceptor investigations in chronically instrumented rats.

Salgado and Krieger (1988), de Abreu and Salgado (1990), and Da Silva et al. (1994) studied the function of the **baroreceptor reflex** in thiopental anesthetized rats. The left aortic nerve was isolated and supported by a bipolar stainless steel electrode and carefully insulated with silicone rubber. Carotid pressure was recorded simultaneously with aortic nerve discharges on an oscilloscope and monitored with a loudspeaker.

King et al. (1987) developed a cross circulation technique in rats to distinguish central from peripheral cardiovascular actions of drugs. The right common carotid arteries were ligated, and the left common carotid arteries and left and right external jugular veins of two phenobarbital-anesthetized rats were connected with polyethylene tubing so that peripheral blood from one rat, A, supplied the head of another rat, B, and then returned to the body of A, and vice versa, for peripheral blood from rat B. Each rat was artificially ventilated with O_2, the chest was opened, and both subclavian arteries were ligated. Prior to the ligation of the subclavian arteries, blood flow from rat A supplied its own brain and both brain

hemispheres but not the brain stem of rat B. Following subclavian artery ligation, blood flow from rat A did not supply A's brain, but supplied both hemispheres and brain stem of rat B. The head of each rat was, therefore, rendered dependent on the carotid arterial blood supply from another rat. This rat cross circulation preparation can be used to separate the central and peripheral cardiovascular actions of drugs.

Zavisca et al. (1994) studied the hypertensive responses to defined electrical and mechanical stimuli in anesthetized rats. Rats were given etomidate, 3.8 mg/kg/h intravenously following carotid artery and jugular vein cannulation. At 15 min after beginning the infusion, four types of noxious stimuli were administered sequentially at 1-min intervals: type 1 (square electrical waves 125 cps, 1.6 ms, 2-s duration, varying current from 0.4 to 12 mA), type 2 (a single 10-mA electrical stimulus, 5-s train duration), type 3 (tail clamping), and type 4 (skin incision). After each stimulus, maximum change in systolic blood pressure was measured. Graded electrical stimulation allowed the best quantitative evaluation of the hypertensive response to noxious stimuli.

Hyman et al. (1998) described a novel catheterization technique for the in vivo measurements of **pulmonary vascular responses** in rats. Male Charles River rats weighing 26–340 g were anesthetized and strapped in supine position to a fluoroscopic table. They breathed air enriched with oxygen through an endotracheal tube inserted by tracheostomy. Catheters were inserted into the femoral blood vessels. The venous catheters were passed to the right atrium under fluoroscopy. A F-1 thermistor–catheter was passed from the left carotid artery into the ascending aorta under fluoroscopy, and a PE-50, 150-mm plastic catheter with a specially constructed curved tip was passed fluoroscopically from the left jugular vein into the main pulmonary artery. A plastic radiopaque 22-gauge catheter 100 mm in length with a curved tip was passed with a 0.025-mm soft-tip coronary guiding catheter from the right jugular vein through the right atrium to the inferior vena cava. The coronary soft-tip guide was then withdrawn. A specially curved 102.5-mm transseptal needle, 0.4 mm in diameter, was then passed though the catheter. Both the needle and catheter were withdrawn into the superior portion of the right atrium under fluoroscopic guidance so that the needle and catheter both rotated freely. With the rat in a slight left anterior oblique position, the catheter and needle were carefully rotated anteriorly to the intra-atrial septum. With gentle pressure, the catheter and needle can be felt and seen fluoroscopically to pass through the atrial septum. As the needle was withdrawn, the curve of the catheter permitted passage of the tip into the vein draining either the left or right lower lobe. The catheter was carefully positioned near the pulmonary venoatrial junction and fixed in place. Mean pressures in the femoral artery, pulmonary artery, and pulmonary vein at the venoatrial junction were measured with pressure transducers and recorded on a polygraph. Cardiac output was obtained in triplicate by delivering 0.1 ml normal saline at room temperature into the femoral venous catheter at the right venoatrial junction and determining thermodilution cardiac output with the thermistor–catheter in the ascending aorta.

Hayes (1982) described a technique for determining contractility, intraventricular pressure, and heart rate in the **anesthetized guinea pig** by inserting a needle, attached to a pressure transducer, through the chest wall into the left ventricle.

Williams et al. (1965) used **castrated male ferrets** anesthetized by intramuscular injection of a mixture of 55 mg/kg ketamine and 4 mg/kg xylazine to measure the effects of a nonpeptidyl endothelin antagonist on endothelin-induced pressor responses.

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Blood Pressure in Pithed Rats

Purpose and Rationale

The pithed rat has been proposed for assessing pressor substances by Shipley and Tilden (1947). The preparation is frequently used to evaluate drug action on the cardiovascular system since this preparation is devoid of neurogenic reflex control that may otherwise modulate the primary drug effect.

Procedure

Male rats weighing 250–350 g are prepared for pithing under halothane anesthesia. The left carotid artery is cannulated for blood pressure monitoring and blood sampling. Furthermore, the trachea and the right jugular vein are cannulated. The rats are pithed inserting a steel rod, 2.2 mm in diameter and about 11 cm in length, through the orbit and foramen magnum down the whole length of the spinal canal. Via the tracheotomy tube, the animals are ventilated with a small animal ventilation pump. Inspirated air is oxygen-enriched by providing a flow of oxygen across a T-piece attached to the air intake of the ventilation pump (Harvard Apparatus model 680). The rats are ventilated at a frequency of 60 cycles/min with a tidal volume of 2 ml/100 g body weight. Thirty minutes after pithing, a 0.3-ml blood sample is withdrawn from the carotid cannula and immediately analyzed for pO_2, pCO_2, pH, and derived bicarbonate concentration using an automatic blood gas analyzer. By alterations of the respiratory stroke volume of the pump, the values are adjusted to: pCO_2 30–43 mmHg, pH 7.36–7.50, and pO_2 87–105 mmHg.

Continuous registration of blood pressure and cardiac frequency (Hellige He 19 device and Statham P23 DB transducer) is performed via the left carotid artery.

In order to measure α_1- and α_2-antagonism, first dose–response curves are registered using doses of 0.1–30 μg/kg i.v. phenylephrine (a selective α_1-agonist) and 1–1,000 μg/kg i.v. BHT 920 (a selective α_2-agonist). The test drug is administered intravenously and the agonist dose–response curves are repeated again 15 min later.

Evaluation

If the curve of blood pressure response to the agonists is shifted, dose–response curves are plotted on a logarithmic probit scale and potency ratios are calculated.

Modifications of the Method

Gillespie and Muir (1967) described a method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat by coating those parts of the pithing rod which lay in the sacral and cervical region of the spinal cord with high resistance varnish to restrict stimulation to the thoracolumbar region. The steel rod is insulated with an adhesive throughout its length except for a 5-cm section which provides a sufficient stimulation area of the lower thoracolumbar nerves. For stimulating nerve fibers supplying exclusively the heart, a pithing rod is used which is insulated throughout its length except for a 0.5-cm section 7 cm proximal to the tip. The spinal cord is stimulated electrically using the pithing rod as the cathode and a hypodermic needle which is inserted under the skin near the right hind limb, as the anode. Varying the intensity and/or the duration of the stimulation, dose–response curves can be registered which are altered after treatment with drugs.

Curtis et al. (1986) described an improved pithed rat method by mounting the preparation vertically with the head pointing downwards resulting in considerably higher blood pressure and heart rate.

MacLean and Hiley (1988) studied the effect of artificial respiratory volume on the cardiovascular responses to an α_1- and α_2-adrenoceptor agonist in the air-ventilated pithed rat using microsphere technique and analysis of arterial blood gases and pH.

Trolin (1975) used decerebrated rats to study the clonidine-induced circulatory changes.

Balt et al. (2001) compared the angiotensin II type 1 (AT_1) receptor blockers losartan, irbesartan, telmisartan, and the ACE inhibitor captopril on inhibition of angiotensin-II-induced facilitation of sympathetic neurotransmission in the pithed rat.

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Antihypertensive Vasodilator Activity in Ganglion-Blocked, Angiotensin II-Supported Rats

Purpose and Rationale

The method is used to demonstrate direct vasodilator activity of potential antihypertensive agents. The experimental model is an anesthetized, ganglion-blocked rat whose blood pressure is maintained by an intravenous infusion of angiotensin II. The test allows to differentiate between centrally acting antihypertensives and peripheral vasodilators.

Procedure

Male Wistar rats weighing 275–450 g are anesthetized with a combination of urethane (800 mg/kg) and chloralose (60 mg/kg) administered intraperitoneally in a volume of 10 ml/kg. Following induction of anesthesia, chlorisondamine (2.5 mg/kg) is injected into the peritoneal cavity to abolish sympathetic and parasympathetic nerve activity. The right femoral artery is cannulated to monitor blood pressure (Statham pressure transducer P23 DB) and heart

rate. Both femoral veins are cannulated to administer drugs or infuse angiotensin II. The trachea is intubated and animals are allowed to breathe spontaneously. Following a stabilization interval of 10–15 min, angiotensin II is infused at a rate of 0.25 or 3.5 μg/min in a volume equivalent to 0.05 ml/min (Harvard infusion pump).

After an increase of blood pressure, a new elevated steady-state pressure is established within 15–20 min. Drugs are subsequently injected intravenously over an interval of 3 min in a volume of 2 ml/kg. Mean arterial pressure is recorded on a polygraph at 5, 10, 15, 20, and 30 min after initiation of drug administration. Seven to nine animals are used for each drug and dose level studied.

***α*-Adrenoreceptor blockade** can be determined in ganglion-blocked rats. Pressor responses to graded doses of phenylephrine injected intravenously are obtained before and 15 min after administration of test compounds. Sufficient concentrations of phenylephrine have to be given to ensure a rise in mean arterial blood pressure of 50 mmHg or more. Data obtained from five or six animals are averaged and resultant dose–response curves plotted. The dose of phenylephrine required to elicit a 50-mmHg increase in mean arterial blood pressure is interpolated from dose–response curves.

Standard data:

The following compounds are used as standards and, at the doses indicated, lower mean arterial blood pressure by about 50 mmHg:

- Cinnarizine 3.0 mg/kg, i.v.
- Hydralazine 1.0 mg/kg, i.v.
- Minoxidil 10.0 mg/kg, i.v.
- Saralasin 0.03 mg/kg, i.v.
- Molsidomine 0.1 mg/kg, i.v.

Evaluation

Mean values ± SEM are given for mean arterial blood pressure and heart rate. Changes of these parameters after drug administration are compared to control values obtained immediately before the application of the test compound. Statistical significance is assessed by means of the paired *t*-test.

Critical Assessment of the Method

A hypotensive response in this model appears to correlate more closely with antihypertensive activity in DOCA-salt hypertensive rats than does a vasodilator response in the perfused hind limb of anesthetized dogs and allows a distinction between central antihypertensive and vasodilators.

Modifications of the Method

Santajuliana et al. (1996) developed a standard ganglionic blockade protocol to assess neurogenic pressor activity in conscious rats. Rats were instrumented with arterial and venous catheters for measurement of arterial pressure and heart rate and for administration of three different ganglionic blockers (trimethaphan, hexamethonium, and chlorisondamine).

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Blood Pressure in Conscious Hypertensive Rats (Tail-Cuff Method)

Purpose and Rationale

Rats with spontaneous or experimentally induced hypertension are widely used for screening of potentially antihypertensive compounds. The indirect tail-cuff method allows the determination of systolic blood pressure according to the following principle: The cuff is quickly inflated to well above suspected systolic blood pressure; the pulse

will then be obliterated. Thereafter, pressure in the cuff is slowly released and, as the pressure falls below systolic blood pressure, the pulse will reappear. The method is analogous to sphygmomanometry in human and can be applied not only at the tail of awake rats but also in dogs and small primates. The indirect tail-cuff method is widely used to evaluate the influence of antihypertensive drugs in spontaneously and experimentally hypertensive rats.

Procedure

Male spontaneous hypertensive rats (Charles River) weighing 300–350 g or rats with experimentally induced hypertension are used.

Surgical Procedure to Induce Renal Hypertension

Male Sprague–Dawley rats weighing 80–100 g are anesthetized by intraperitoneal injection of 0.8 ml 4 % chloral hydrate solution. Both kidneys are exposed retroperitoneally. To induce renal hypertension, a silver clip (0.2 mm diameter, 4 mm length) is placed onto both renal arteries, the kidneys are reposed, and the wound is closed by suture.

Within 5–6 weeks, operated animals attain a renal hypertension with a systolic blood pressure (BPs) of 170–200 mmHg (mean normal physiological BPs for rats is 100 mmHg). Only animals with a BPs = 180 mmHg are used for the tests.

Test Procedure

The procedure is the same for spontaneously and experimentally hypertensive rats. Groups of six animals are used per dose. The control group receives saline only. To reduce spontaneous variations in blood pressure, animals are adjusted to the experimental cage by bringing them into the restraining cage which is enclosed in a 31–32 °C measuring chamber three to four times before the start of the experiment for a period of 30–60 min.

To measure blood pressure, a tubular inflatable cuff is placed around the base of the tail and a piezoelectric pulse detector is positioned distal to the cuff. The cuff is inflated to approximately 300 mmHg. As the pressure in the cuff is slowly released, the systolic pressure is detected and subsequently recorded on a polygraph.

The test substance is administered intraperitoneally or by gavage once per day over a period of 5 days. The usual screening dose of a new compound is 25 mg/kg. Blood pressure and heart rate measurements are taken at the following times:

Day 1: predose and 2 h postdrug
Day 3: predose and 2 h postdrug
Day 5: predose, 2 h postdrug, and 4 h postdrug

Between measurements, animals are returned to their home cages.

Standard compounds:

- Endralazine (3 mg/kg p.o.)
- Nifedipine (3 mg/kg p.o.)
- Urapidil (5 mg/kg p.o.)

Evaluation

Mean values in systolic blood pressure before and after drug administration and the duration of the effect are determined. Percent decrease in systolic blood pressure under drug treatment is calculated. Statistical significance is assessed by the Student's *t*-test.

Scores for % decrease in systolic blood pressure and for the duration of the effect are allotted.

Critical Assessment of the Method

The indirect tail-cuff method is being used in many laboratories with many modifications of the devices. Pfeffer et al. (1971) found a good correlation between values obtained with the indirect tail-cuff method and values measured directly with indwelling carotid arterial cannulae, whereas Buñag et al. (1971) reported a lack of correlation

between direct and indirect measurements of arterial pressure in unanesthetized rats, and Patten and Engen (1971) found difficulties to measure accurate systolic values at higher blood pressure. A good correlation between direct blood pressure data from the carotid artery in rats and readings with the tail-cuff method was found by Matsuda et al. (1987) who developed a six-channel automatic blood pressure measuring apparatus with a highly sensitive photoelectric sensor for the detection of tail arterial blood flow and a microcomputer system for automatic measurement of systolic blood pressure and heart rate and for data acquisition and processing.

Modifications of the Method

Details of the tail-cuff method in rats have been discussed by Stanton (1971).

Special equipment for measuring blood pressure in rats is commercially available (e.g., TSE GmbH, Bad Homburg, Germany).

Widdop and Li (1997) described a simple versatile method for measuring tail-cuff systolic blood pressure in conscious rats. A tail cuff consisting of a metal T-piece tube with latex rubber inside the tube is placed around the tail at the proximal end. A piezoelectric transducer (model MLT1010) is strapped to the ventral surface of the tail to record the pulse signal from the caudal artery and connected directly to a MacLab data-acquisition system (ADInstruments Pty Ltd.).

The tail-cuff method for measurement blood pressure has been adapted for dogs, monkeys (Wiester and Iltis 1976), and cats (Mahoney and Brody 1978).

Blood pressure can be measured from the hind leg of the rat using a leg cuff and a photoelectric cell situated at the dorsal surface of the foot (Kersten et al. 1947). When the leg is occluded, the foot swells and the amount of light striking the photocell is reduced. When the pressure in the cuff is released, the arterial blood flow is restored, the increase of foot volume is decreased, and the amount of light transversing the paw increases.

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Direct Measurement of Blood Pressure in Conscious Rats with Indwelling Catheter

Purpose and Rationale

The method first described by Weeks and Jones (1960) allows the direct measurement of arterial pressure in conscious rats eliminating the influence of anesthesia on cardiovascular regulation.

Procedure

Preparation of Cannulae

In order to prepare the cannulae, 7-cm- and 12-cm-long pieces are cut from PE-10 and PE-20 tubings, respectively. A stylet wire is inserted into the PE-10 tubing and the PE-20 tubing is also slipped over the stylet wire. The ends of the tubings are heated in a current of hot air and fused together. Ridges are made to anchor the cannula in the animal's tissue. In order to make a ridge, the stylet wire is left inside the cannula and the cannula is heated in a fine jet of hot air. When the polyethylene at the point of heating becomes soft, the cannula is pressed slightly and thus a ridge is formed. One ridge is formed at the PE-20 tubing, about 0.5 cm away from the junction with the PE-10 tubing, and 3 more ridges are formed on the PE-20 tubing at a distance of about 1 cm from each other, first one being situated about 3 cm away from the free end of the PE-20 tubing. The stylet wire is then removed from the cannula, and the PE-10 portion of the cannula near the junction with the PE-20 tubing is wound around a glass rod with a diameter of 4 mm. Two rounds are made. Then it is dipped in a boiling water bath for about 5 s. When taken out of the bath, the cannula retains its circles, forming a spring-like structure.

Implantation of Cannulae

Male Sprague–Dawley rats weighing about 300 g are used. The rat is anesthetized with 45 mg/kg pentobarbital i.p. The area of the neck and the abdomen are shaved and cleaned with 70 % alcohol. The viscera are exposed through a midline abdominal incision. A segment of the abdominal aorta is exposed just above the bifurcation. A trocar is passed through the psoas muscles adjacent to this segment of the aorta, through the muscles of the back and under the skin until it emerges from the skin of the neck. Then the cannula is inserted into the trocar and the trocar is withdrawn from the body. The end of the cannula thus comes out from the neck, being anchored by silk sutures to the neck skin and to the psoas muscle. The cannula is filled with heparin solution and the end which is projecting out from the neck skin is blocked with a tight fitting stainless steel needle. Then the other end of the cannula is implanted into the aorta. The aorta is wiped with a cotton-tipped applicator stick above the bifurcation, occluded above this segment, and punctured with a bent 27-gauge hypodermic needle. The tip of the PE-10 catheter is inserted through the needle and advanced up the aorta. The intestines are replaced and the wound sutured. The rats are allowed to recover for one week.

Measurement of Blood Pressure

The occluding stainless steel needle is removed and the cannula flushed with diluted heparin solution. The rat is placed in a small cage to restrict its movements, even so it is free to move. The cannula is connected to a Statham P23 DB pressure transducer, and blood pressure is recorded on a polygraph. Test drugs or standards are administered either subcutaneously or orally. Recordings are taken before and after administration of drug over a period of 1 h.

Evaluation

Changes of blood pressure are measured for degree and duration. Five rats are used for each dose and

compound. The maximal changes of each group are averaged and compared with the standard.

Critical Assessment of the Method

Direct measurement of arterial blood pressure in unanesthetized rats originally introduced by Weeks and Jones (1960) has become a valuable and widely used tool in cardiovascular research.

Modifications of the Method

A detailed description of a slightly modified Week's method has been given by Stanton (1971).

Improvements of the method for continuous direct recording of arterial blood pressure and heart rate in rats have been described by Buñag et al. (1971), Laffan et al. (1972), Buckingham (1976), Garthoff and Towart (1981), and Garthoff (1983). A detailed description of permanent cannulation of the iliolumbar artery was given by Remie et al. (1990).

Wixson et al. (1987) described a technique for chronic catheterization of the carotid artery in the rat. Prepared cannulae are commercially available (IRC Life Science, Woodland Hills, CA).

A newer modification uses the access to the aorta via the common carotid artery (Linz et al. 1992). Rats are prepared under thiopental anesthesia with arterial PE-50 lines (Intramedic from Clay Adams, USA). The lines are introduced into the ascending aorta via the right carotid artery for direct measurement of arterial blood pressure and into the jugular vein for i.v. application of test compounds. Both lines, filled with saline containing heparin, are surfaced on the neck. The animals are allowed to recover for at least 2 days. Blood pressure is monitored through Statham R P23DB transducers connected to a recording device. During measurements, the lines are kept open with countercurrent saline infusion at a rate of 1 ml/h.

Bao et al. (1991) placed one catheter via the right femoral artery in the abdominal aorta in rats for recording mean arterial pressure and two additional catheters via the left carotid artery into the descending aorta for application of bradykinin and bradykinin antagonists.

Arterial pressure was recorded in unanesthetized rats after induction of severe hypertension by complete ligation of the aorta between the origin of the renal arteries by Sweet and Columbo (1979).

Hilditch et al. (1978) described a device for the direct recording of blood pressure in conscious dogs.

Akrawi and Wiedlund (1987) described a method for chronic portal vein infusion in unrestrained rats. Hepatic drug metabolism can be studied by infusion into the portal vein and blood collection from the femoral vein.

Robineau (1988) described a method for recording electrocardiograms in conscious, unrestrained rats. Electrodes were implanted subcutaneously and a socket connector was sutured on the head of the animal. A flexible cord leading to a swivel collector was linked to an ECG amplifier.

Kurowski et al. (1991) reported on an improved method to implant, maintain, and protect arterial and venous catheters in conscious rats for extended periods of time.

Schenk et al. (1992) measured cardiac left ventricular pressure in conscious rats using a fluid-filled catheter.

Tsui et al. (1991) recommended a reliable technique for chronic carotid arterial catheterization in the rat.

Hagmüller et al. (1992) described a tail-artery cannulation method for the study of blood parameters in freely moving rats.

Liebmann et al. (1995) described an in vivo long-term perfusion system which is based on 195 automated, computer-controlled high-frequency heparin (10 U/ml) flushing of a cannula inserted into the tail artery of freely moving rats.

Santajuliana et al. (1996) used conscious rats instrumented with arterial and venous catheters to assess neurogenic pressor activity after administration of ganglionic blockers.

Rezek and Havlicek (1975) described simple cannula systems for the infusion of experimental substances in chronic, unrestrained animals. A cannula with a removable cap is used for

infusions into various parts of the digestive tract. Intravenous infusions can be performed through a closed system cannula which avoids a possible introduction of air into the circulation.

Kimura et al. (1988) described a method for chronic portal venous, aortic, and gastric cannulation to determine portal venous and aortic glucose and lactate levels in conscious rats.

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Cannulation Techniques in Rodents

Purpose and Rationale

Cardiovascular pharmacology requires special techniques for catheterization and permanent cannulation of vessels. A few methods are described below.

A comprehensive literature survey on methods for vascular access and collection of body fluids from the laboratory rat was written by Cocchetto and Bjornsson (1983).

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Permanent Cannulation of the Jugular Vein in Rats

Purpose and Rationale

Permanent cannulation of the jugular vein in rats in combination with a head attachment apparatus allowing easy connection of cannulae was first introduced by Steffens (1969). Modifications were described by Brown and Hedge (1972), by Nicolaidis et al. (1974), and by Dons and Havlik (1986). A detailed description was given by Remie et al. (1990).

Procedure

Rats are anesthetized with N_2O_2/O_2/halothane. The shaven neck of the animal on the right side is disinfected with chlorhexidine solution. The incision is made just above the right clavicle. Connective and adipose tissue are pushed aside with blunt forceps and the jugular vein is exposed. The external jugular vein is followed and the division into the maxillary vein, the facial, and the linguofacial vein identified. The largest vein is chosen and mobilized for a distance of about 5 mm. Small artery forceps are used to clamp the vessel. The vein is then ligated rostral to the clamp with 6–0 silk, and a second ligature is put loosely around the vessel, but not tightened. Using iridectomy scissors, a V-shaped hole is cut in the vein 2 mm rostral from the bifurcation. Prior to its insertion into the vessel, a sterile cannula is connected to a 1-ml syringe filled with a heparinized saline solution. The vessel is dilated by means of a sharp pointed jeweler's forceps, the cannula slit between the legs of the forceps and gently pushed into the vessel until the tip is at the level of the right atrium. Then the forceps is removed, the caudal ligature gently tied, and the rostral ligature used to anchor the cannula to the vessel. The cannula is tunneled to emerge at the top of the head. While the skin in the neck is held firmly, the artery forceps is inserted subcutaneously in caudal direction over a distance of about 3 cm, then turned anticlockwise in the direction of the incision in the neck. The cannula is grasped with the forceps. Then the forceps is pulled back until the cannula emerges at the crown of the head and closed by a small microvascular clamp. The cannula is slid over the short end of a 20G stainless steel needle bent to a 90° angle. The catheter is flushed with saline and filled with polyethylene/heparin solution. The long end of the L-shaped stainless steel adapter is closed with a piece of heat-sealed PE tubing, and the wounds are closed with sutures.

Modifications of the Method

Hutchaleelaha et al. (1997) described a simple apparatus for serial blood sampling from the external jugular vein which permits simultaneous measurement of locomotor activity in freely moving rats.

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Permanent Cannulation of the Renal Vein in Rats

Purpose and Rationale

A detailed description for permanent cannulation of the renal vein in rats was given by Remie et al. (1990).

Procedure

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. This provides an excellent view to the vena cava. At its confluence with the vena cava, the right renal vein is stripped of its adipose tissue and the peritoneum is opened. Using small anatomical forceps, the peritoneum is detached from the vena cava by making small spreading movements with the forceps just beneath the peritoneum. Subsequently, the vena cava and the renal vein are mobilized for approximately 1.5 cm, to allow for clamping of the vessel. A four or five fine-stitch purse string is placed in the vessel at the confluence of the vena cava and the right renal vein. Using a Barraquer needle holder and a cotton-wool stick, the 7–0 silk suture, armed with a BV-1 needle, is guided through the vessel. After each stitch, any bleeding has to be immediately arrested by applying light pressure using a cotton-wool stick. Having completed the suture, a single knot is made with the drawstrings. Three microvascular clips are then placed on the vena cava and the renal vein: first the proximal clip on the vena cava, followed by the clip on the renal vein, and finally the distal vena cava clip. A small aperture is cut immediately inside the purse-string suture using iridectomy scissors and jeweler's forceps. The cannula, which is filled with a heparinized saline solution, is pushed into the opening as far as possible. The purse-string suture is pulled taut and the clip of the renal vein removed, while pushing the cannula further. The proximal clip on the vena cava is now removed as quickly as possible. The patency of the cannula is checked and the drawstrings of the purse-string suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head.

The cannula together with a L-shaped adapter is fixed to the skull.

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Permanent Cannulation of the Portal Vein in Rats

Purpose and Rationale

Several techniques have been described for cannulation of the portal vein in rats (Hyun et al. 1967; Pelzmann and Havemeyer 1971; Suzuki et al. 1973; Sable-Amplis and Abadie 1975; Helman et al. 1984). A detailed description for permanent cannulation of the portal vein in rats was given by Remie et al. (1990). After additional application of platinum electrodes around the portal vein in close proximity to the catheter tip, this model can also be used to study the presynaptic regulation of neurotransmitter release from nonadrenergic nerve terminals (Remie and Zaagsma 1986; Remie et al. 1988, 1989).

Procedure

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. Using a microneedle holder and a cotton-wool stick, a four or five fine-stitch purse-string suture (7–0 silk suture armed with a BV-1 needle) is placed in the wall of the portal vein at the side opposite the gastroduodenal vein. The diameter of the purse string should be about 1 mm. After the suture has been completed, a single knot is made with the drawstrings. The portal vein is clamped with a small curved hemostatic bulldog clamp. Using iridectomy scissors and a pair of jeweler's forceps, the center of the purse string is cut; a cannula filled with heparinized saline is inserted into the vessel and pushed upwards. The purse string is gently tightened taking care not to obstruct the cannula. The drawstrings of the suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

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Permanent Cannulation of the Thoracic Duct in Rats

Purpose and Rationale

Collection of lymph is rather difficult and has performed mainly in dogs (Biedl and Offer 1907; Gryaznova 1962, 1963; Vogel 1963). Some techniques have been described for the rat (Bollman et al. 1948; Girardet 1975). Remie et al. (1990) did not obstruct the duct by placing a purse-string suture in the wall of the duct, by which the cannula is secured. The animal's lymph can be collected during the experiment, and after refilling the cannula, the lymph flow remains undisturbed.

Procedure

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal cavity, the intestines are placed in gauze moistened with warm saline and laid to the left of the animal. The suprarenal abdominal artery is located and mobilized by gently tearing the connective tissue. Using blunt dissection technique, the thoracic duct is mobilized along the dorsolateral surface of the aorta. A small three to four fine-stitch purse-string suture is placed in the wall of the duct, using a 9–0 Ethilon suture. A hole is cut inside the purse string with a very fine pair of scissors, while holding the wall with angled jeweler's forceps. The cannula is filled with heparinized saline solution and is inserted into the duct using anatomical forceps. After the tip of the cannula has been inserted into the thoracic duct, the curved forceps are removed and the total tip is pushed into the duct. The ligature is then closed and some lymph will flow into the cannula. The cannula is secured within the abdominal cavity by attaching it to the abdominal muscle near the xiphoid cartilage with a 7–0 silk suture. Following the closure of the abdominal wall and the tunneling of the cannula to the crown of the head, an L-shaped adapter is placed on the cannula, filled with PVP solution and closed with a heat-sealed polyethylene cap.

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Portacaval Anastomosis in Rats

Purpose and Rationale

In 1877 the Russian surgeon Eck reported the achievement of successful portacaval shunts in dogs. Lee and Fischer (1961), Funovics et al. (1975), and de Boer et al. (1986) described

portacaval shunt in the rat. A detailed description of surgery for portacaval anastomosis in rats was given by van Dongen et al. (1990).

Procedure

Rats are anesthetized with N_2O_2/O_2 in combination. After opening the abdominal wall, the intestines are placed left to the animal on gauze moistened with warm saline solution. Proximally and distally to the animal's right renal vein, the vena cava is then stripped of its adipose and connective tissue, and the retroperitoneal cavity is opened. Using anatomical forceps, the peritoneum is dissected from the vena cava by making small spreading movements with the forceps just above the vena cava. The portal vein is pulled slightly to the left using straight anatomical forceps and freed from the hepatic artery and the gastroduodenal artery with curved anatomical forceps. Rostral to the celiac artery, the abdominal artery which is covered with peritoneum is freed from its lateral muscle bed over a length of approximately 5 mm providing enough space for placing a small bulldog clamp at a later stage of the operation. Without occlusion, a six fine-stitch purse string is placed in the wall of the vena cava close to its confluence with the right renal vein. Using a Barraquer needle holder and a cotton-wool stick, the 7–0 silk suture armed with a BV-1 needle is guided through the vessel. After each stitch, bleeding has to be arrested immediately, by applying light pressure on the area, again using the cotton-wool stick. After the suture has been completed, a single knot is made with the drawstrings. The drawstrings should come together at the rostral part of the purse string. A bulldog clamp, modified to resemble a Satinsky vascular clamp, is then placed on the vena cava.

Before clamping the abdominal aorta rostral to the celiac artery with a small bulldog clamp, a ligature (7–0 silk) is placed around the portal vein as close as possible to the hilus of the liver. Subsequently, the clamp is placed on the aorta and the ligature tightened. A Heifetz clip is then placed transversely onto the portal vein at its confluence with the gastroduodenal vein. The portal vein is cut just distally from the ligature. A prepared button is slipped over the left-hand straight small anatomical forceps, while the right-hand forceps are used to pass the portal vein to the left-hand anatomical forceps. The vein is then grasped and pulled through the button. Subsequently, the button is pushed as close as possible to the Heifetz clip and clamped to the clip using a Pilling bulldog clamp.

Using small straight and curved anatomical forceps, the portal vein is reversed around the button and fixed with a previously prepared 7–0 silk suture. The Pilling bulldog clamp is then removed and replaced at the end of the Satinsky clamp for reasons of stability. The vena cava is then somewhat elevated, bringing it into closer contact with the portal vein button.

A longitudinal cut is made in the purse-string suture using iridectomy scissors and jeweler's forceps. One drawstring of the suture is clamped with a small hemostat and put under slight tension in a rostral direction. The button manipulated by its grip is pushed into the vena cava. The purse string is tightened with the left hand while the right hand still holds the button in position. The button is released and two additional knots tied. The Satinsky clamp is removed first followed by the Heifetz clamp and the bulldog clamp on the aorta. After replacing the intestines, the abdominal wall is closed in two layers.

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Cardiovascular Analysis in Anesthetized Mice

Purpose and Rationale

To fully utilize the potential of mouse models with specific gene mutations, it is necessary to study the functional consequences of genetic manipulations in fully intact mice. Lorenz and Robbins (1997) developed and validated a methodology to study cardiovascular parameters in closed-chest mice.

Procedure

Adult mice of either sex weighing 25–35 g are anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 100 mg/kg thiobutabarbital. After the mice are placed on a thermally controlled surgical table with body temperature continually monitored via a rectal probe, a tracheotomy is performed with a short length (<1 cm) of PE-90 tubing. The right femoral artery is then cannulated with polyethylene tubing which is pulled over a flame to a small diameter (~0.4 mm OD). The catheter is advanced ~1 cm, near the level of the aorta, and connected directly to a low-compliance COBE CDXIII fixed-dome pressure transducer for the measurement of arterial blood pressure. The right femoral vein is then cannulated with the same type of small-diameter tubing and connected to a microinjection pump for the infusion of experimental drugs. To assess myocardial performance, the right carotid artery is cannulated with a 2 F Millar MIKROTIP transducer (Model SPR-407, Millar Instruments, Houston TX). This high-fidelity transducer, which has a tip diameter of ~0.67 mm, has a reported frequency response that is flat up to 10,000 Hz and therefore can be used to accurately monitor the high frequency of the mouse ventricular pulse pressure. During continual monitoring of the blood pressure wave to ascertain the anatomic position of the catheter, the tip of the transducer is carefully advanced through the ascending aorta and into the left ventricle. When the stable waveform of the ventricular pressure profile is achieved, the transducer is anchored in place with 7–0 silk sutures. After completion of the surgery, all wounds are closed with cyanoacrylate to minimize evaporative loss of fluid, and the animals are allowed to stabilize for 30–45 min.

Evaluation

Blood pressure signals from the COBE transducer and from the Millar transducer are amplified, and the output is recorded and analyzed with a MacLab 4/s data-acquisition system connected to a Macintosh 7100/80 computer which allows the calculation of the following parameters:

dP/dt first derivative of the ventricular pressure wave
MAP mean arterial pressure
HR heart rate
LVP systolic and diastolic left ventricular pressure
LVEDP left ventricular end-diastolic pressure

Further indices of ventricular performance can be calculated from d*P*/d*t*.

Modifications of the Method

Champion et al. (2000) described a **right-heart catheterization technique** for in vivo measurement of vascular responses in lungs of intact mice. CD1 mice weighing 25–38 g were anesthetized with thiopentobarbital (85–95 mg/kg i.p.) and ketamine (3 mg/kg i.p.) and were strapped in

supine position to a thermoregulated fluoroscopic table. The trachea was cannulated and the animals breathed with room air enriched with 95 % O_2/5 % CO_2. A femoral artery was cannulated for the measurement of systemic arterial pressure. Heart rate was electronically monitored from the systolic pressure pulses with a tachometer (Grass model 7P44A). The left jugular vein was cannulated for the administration of agonists and antagonists.

For measuring pulmonary arterial pressure, a special single lumen catheter was constructed. The catheter was 145 mm in length and 0.25 mm in outer diameter, with a specially curved tip to facilitate passage through the right heart, main pulmonary artery, and the left or right pulmonary artery. Before the catheter was introduced, the catheter curve was initially straightened with a 0.010-in. straight angioplastic guide wire to facilitate passage from the right jugular vein into the right atrium at the tricuspid valve under fluoroscopic guidance. As the straight wire was removed, the natural curve facilitated entry of the catheter into the right ventricle. A 0.010-in. soft-tip coronary artery guide wire was then inserted, and the catheter was passed over the guide wire into the main pulmonary artery under fluoroscopic guidance. Pressure in the main pulmonary artery was measured with a pressure transducer, and mean pulmonary artery pressure was derived electronically and recorded continuously.

Cardiac output was measured by the thermodilution technique. A known volume (20 µl plus catheter dead space) of 0.9 % NaCl solution at 23 °C was injected into the right atrium, and changes in blood temperature were measured at the root of the aorta. A cardiac output computer equipped with a small animal interface was used. The thermistor microprobe was inserted into the right carotid artery and advanced to the aortic arch, where changes in aortic blood temperature were measured. A catheter placed in the right jugular vein was advanced to the right atrium or main pulmonary artery for rapid bolus injection of saline. The saline solution was injected with a constant-rate syringe to ensure rapid and repeatable injection of the saline indicator solution. Thermodilution curves were recorded on a chart recorder and pulmonary and systemic blood pressure monitored continuously. Catheter placement was verified by postmortem examination.

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Blood Pressure in Anesthetized Cats

Purpose and Rationale

Cats are the most sensitive species of cardiovascular regulation. They were used extensively for cardiovascular screening. Recently, experiments in dogs are preferred since this species can be bred more easily in homogeneous strains.

Procedure

Adult cats of either sex weighing 2.5–4 kg are anesthetized by intraperitoneal injection of 35 mg/kg sodium pentobarbital. Tracheotomy is performed and a tracheal cannula is inserted so that the cat can be mechanically ventilated with room air. A femoral artery and two femoral veins are cannulated for measurement of arterial blood pressure and systemic administration of drugs. The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler. Rectal temperature is monitored and maintained between 37 °C and 38 °C with a heating pad.

The following drugs are injected i.v. as challenges:

- Epinephrine 0.1, 0.3, 0.5 µg/kg
- Norepinephrine 0.1, 0.3, 0.5 µg/kg
- Isoproterenol 0.1, 0.2, 0.4 µg/kg
- Carbachol 0.1, 0.2, 0.5 µg/kg

At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline.

Test drugs are injected at various doses followed by injections of the challenging drugs.

Evaluation

Dose–response curves of challenging drugs are established before and after injections of the test drugs.

Critical Assessment of the Method

Blood pressure experiments in anesthetized cats are very valuable as screening techniques for cardiovascular agents. Moreover, potentiation of norepinephrine response has been used as screening procedure for antidepressants with norepinephrine uptake inhibiting activity.

Modifications of the Method

Sander (1965) investigated the vasoconstrictor and vasodilator effects of procaine in spinal cats. The animals were anesthetized with ether and ventilated with a positive pressure pump via a tracheal cannula. The spinal cord was then cut between the second and third vertebrae, and ether administration stopped. The remaining portion of the spinal cord above the transection was destroyed by passing a curette through the spinal canal.

Yardley et al. (1989) studied cardiovascular parameters in spinal cats. The animals were anesthetized with 80 mg/kg intravenously administered α-chloralose. The spinal cord was transected or crushed at the first cervical segment after tetracaine hydrochloride (0.125 mg in 0.1 ml) had been injected into this region of the cord. Systemic blood pressure was supported at a level sufficient to maintain constricted pupils (mean value 45 ± 5 mmHg) by volume expansion with blood from a donor (10–20 ml) or an infusion of dextran.

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Cardiovascular Drug Challenging Experiments in Anesthetized Dogs

Purpose and Rationale

Sympathomimetic and cholinomimetic compounds as well as angiotensin II and carotid occlusion exert characteristic responses in blood pressure of anesthetized dogs. Antagonism or potentiation of these responses allows to characterize the cardiovascular activity of a new compound.

Procedure

Adult Beagle dogs of either sex weighing between 8 and 15 kg are anesthetized with 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital, and 75 mg/kg sodium pentobarbital. Additional doses of sodium pentobarbital are given as needed. The dogs are intubated with a cuffed endotracheal tube and placed on a Harvard respirator (20 ml/kg, 10–15 cycles/min). A femoral vein and artery are cannulated using polyethylene tubing for drug administration and determination of arterial blood pressure, respectively. The animals are bilaterally vagotomized.

The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler.

Drug challenges: One of the following combinations of drugs is administered i.v. to the dogs. The challenges are given in a fixed order: at least twice prior to test-drug administration to insure consistent responses and again starting 15 min posttest drug. Epinephrine and norepinephrine (1 μg/kg), isoproterenol (0.25 μg/kg), carbachol (0.25 μg/kg), tyramine (100 μg/kg), bilateral carotid occlusion (45 s), phenylephrine (10 μg/kg), isoproterenol (0.25 μg/kg), angiotensin II (0.2 μg/kg), and carbachol (0.25 μg/kg). At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline. Challenge drug doses are sometimes varied to keep the mean arterial pressure within the following limits: epinephrine (+30 to +60 mmHg), norepinephrine (−30 to +70 mmHg), tyramine (+30 to +70 mmHg), isoproterenol (−30 to −50 mmHg), carbachol (−30 to −50 mmHg), phenylephrine (−30 to +70 mmHg), angiotensin II (+30 to +50 mmHg), and bilateral carotid occlusion (+30 to +70 mmHg).

Evaluation

The recordings are studied to detect any changes in the arterial pressure response to the challenge drug before and after test-drug administration and to observe any changes in blood pressure and heart rate. Results are expressed as the percentage change from the predrug response.

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Hemodynamic Analysis in Anesthetized Dogs

Purpose and Rationale

The hemodynamic effects of compounds supposed to affect the cardiovascular system are evaluated by measuring preload and afterload

of the heart, contractility, heart rate, cardiac output, and peripheral or coronary flow. To measure these cardiovascular parameters accurately, the use of larger animals such as dogs or pigs is necessary.

This experimental model allows the classification of test drugs according to their action as having:

- Positive inotropic effects
- Negative inotropic effects (Ca^{2+}-antagonist, antiarrhythmic?)
- Hypertensive effects
- Hypotensive effects
- Coronary-dilating effects
- β-Blocking effects
- α-Blocking effects
- Antianginal effects
- Peripheral vasodilating effects

Procedure

Male or female inbred Beagle or Labrador–Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital and continued with an infusion of 4–6 mg/kg/h. A catheter is placed into the cephalic vein for intravenous injections. Another catheter is placed into the duodenum for enteral administration. Respiration is maintained with room air through a tracheal tube using a positive pressure respirator, e.g., Bird Mark 7 respirator. Blood gas analyses are performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannula inserted into the left femoral artery and connected to a Statham pressure transducer (Statham P23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left common carotid artery into the left ventricle. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/dt_{max} is differentiated and heart rate is counted. The LVP signal also triggers a cardiotachometer.

Cardiac output, pulmonary artery pressure (PAP), and stroke volume are measured by a thermodilution technique using a cardiac output computer (Gould/Statham SP 1245) and a balloon-tip triple-lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

Myocardial oxygen consumption (MVO_2) is calculated as pressure-work index according to Rooke and Feigl (1982).

Femoral blood flow and coronary flow are measured with electromagnetic flow probes attached to the femoral artery and the circumflex branch of the left coronary artery (LCX), respectively.

Experimental Course

When stable hemodynamic conditions and blood gas values of $pO_2 > 100$ mmHg and $pCO_2 < 35$ mmHg are achieved for at least 20 min (control values), the test substance is administered through a catheter inserted into a cephalic vein in doses of 0.1, 0.3, 1.0, and 3.0 mg/kg or into the duodenum in doses of 0.3, 1.0, 3.0, and 10.0 mg/kg.

All parameters are recorded continuously during the whole experiment.

Characteristics

- Blood pressure
  - Systolic, BPs
  - Diastolic, BPd
- Left ventricular pressure, LVP
- Left ventricular end-diastolic pressure, LVEDP
- Maximal rate of pressure rise, dp/dt max
- Heart rate, HR
- Peripheral blood flow in A. femoralis, PF
- Blood pressure in A. pulmonalis, PAP
- Coronary flow, CF
- Cardiac output, CO
- Stroke volume, SV
- Total peripheral resistance, TPR
- Left ventricular stroke work, LVSW
- Left ventricular minute work, LVMW
- Left ventricular myocardial oxygen consumption, MVO_2

Calculation of Results and Evaluation

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formulae:

- Stroke volume [ml/beat],

$$SV = \frac{CO}{HR}$$

- Total peripheral resistance [dyns/cm^5],

$$TPR = \frac{BPm}{CO} \times 79.9$$

- Left ventricle stroke work [J/beat],

$$LVSW = (BPm - LVEDP) \times SV \times 0.333 \times 10^{-3}$$

- Left ventricular minute work [J/min],

$$LVMW = LVSW \times HR$$

- Left ventricular myocardial oxygen consumption [ml O_2/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \times \frac{(0.8BPs + 0.2BPd) \times HR + SV}{BW} + 1.43$$

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25.08 \times 10^{-4}$
BPs = systolic blood pressure [mmHg]
BPd = diastolic blood pressure [mmHg]
BPm = mean blood pressure [mmHg]
HR = heart rate [beats/min]
CO = cardiac output [ml/min]
SV = stroke volume [ml/beat]
$LVEDP$ = left ventricular end-diastolic pressure [mmHg]
BW = body weight [kg]

Changes in parameters measured after drug administration are compared to control values obtained during the 20-min predrug period.

Results are presented as mean ± SEM with $n > 3$.

Statistical significance is assessed by means of the paired *t*-test.

Modifications of the Method

The effect of drugs on the carotid artery occlusion effect can be studied in anesthetized dogs. The occlusion of right and left common carotid arteries is performed by squeezing them between a polyethylene tubing and a twine which is passed inside the tubing and around the carotid artery. An occlusion of the carotid arteries for 30 s causes an increase of systolic blood pressure by 40–50 mmHg. Inhibition of this effect by drugs is tested.

Studies in anesthetized dogs can be used to determine the influence of cardiotonic drugs on propranolol-induced cardiac insufficiency (Rajagopalan et al. 1993).

Instead of dogs, **pigs (German landrace)** weighing between 20 and 35 kg can be used. They are pretreated with ketamine 500 mg/5 ml i.m., metomidate hydrochloride 200 mg/4 ml i.p., and xylazine 60 mg/3 ml i.m. and anesthetized with 15–20 mg/kg sodium pentobarbital, followed by continuous infusion of 12 mg/kg/h. The parameters are evaluated similarly to the experiments in dogs.

Measurement of cardiac output by the thermodilution method in **rats** was described by Richardson et al. (1962) and Müller and Mannesmann (1981).

Thermodilution methods were used by Rosas et al. (1964) in anesthetized rats and by Carbonell et al. (1985) and by Salyers et al. (1988) in conscious rats to determine hemodynamic parameters.

Oxygen pressure, carbon dioxide pressure, and pH in coronary venous and common carotid arterial blood of anesthetized dogs have been measured using a blood gas analyzer (Aisaka et al. 1988).

Acute ischemic left ventricular failure can be induced in anesthetized dogs by repeated injections of plastic microspheres into the left coronary artery (Smiseth and Mjøs 1982; Sweet et al. 1984; Schölkens et al. 1986).

A coronary catheter was introduced through the right femoral artery and advanced under fluoroscopy to the left coronary ostium, guided by injection of small amounts of contrast medium. After reaching baseline values, acute left ventricular failure was induced by subsequent intracoronary injections of plastic microspheres (52.9- ± 2.48-μm nonradioactive tracer microspheres). The microspheres were suspended in saline with a drop of Tween 80 and sonified before use, 1 mg microspheres/1 ml saline corresponding to approximately 12,000 microspheres (13–16 injections of microspheres or 3.4–5.0 mg/kg). Microspheres were injected every 5 min for 70–90 min. Each microsphere injection effected an immediate and stepwise increase in LVEDP. With this procedure, LVEDP can be increased to a desired level in a very controlled manner. In the 30 min following embolization, LVEDP continued to increase by approximately 5 mmHg. Animal with arrhythmias had to be excluded from the study. Thirty min after the end of embolization, when hemodynamic parameters had stabilized, drug administrations were started.

Valdes-Cruz et al. (1984) developed an **open-chest preparation in dogs** to validate the accuracy of a two-dimensional Doppler echocardiographic method for estimating pressure drops across discrete stenotic obstructions.

In order to assess the potential of a single-breath technique (using Freon 22) as an effective way to estimate cardiac output noninvasively, Franks et al. (1990) measured simultaneously with the single-breath technique the aortic flow using an electromagnetic flowmeter in anesthetized dogs.

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Hemodynamic Measurements in Conscious Dogs

Purpose and Rationale

The potency of a cardiovascular drug depends on the direct effects at the cellular level and on the response of the cardiovascular control mechanisms. The latter are often markedly influenced by anesthesia. The chronically instrumented conscious dog with renal hypertension is therefore a more realistic test model to evaluate the effects of antihypertensive, antianginal, and cardiotonic compounds. The test is used to evaluate hemodynamic drug effects in conscious dogs, an experimental model with chronic arterial and ventricular catheterization and renal artery constriction.

Procedure

Male or female Labrador–Harrier dogs weighing 15–25 kg are used. They are anesthetized with 1 mg/kg xylazine i.m., followed by 1 mg/kg xylazine i.v. and 18 mg/kg sodium pentobarbital i.v. For chronic instrumentation and induction of renal hypertension, fluid-filled catheters are implanted into the abdominal aorta and into the left ventricle. The catheters are tunneled subcutaneously and exteriorized on the nape of the neck dorsally. Renal hypertension is induced by placing silastic constrictors around both renal arteries. Hemodynamic measurements are performed after a two-week recovery period or later.

To familiarize the dogs to the test surroundings, they are brought into the laboratory two to three times before the start of the study. Thus, drug testing is possible without sedation. During the experiment, the animal rests quietly on a laboratory table.

Experimental Protocol

Hemodynamic measurements are performed by connecting the two implanted catheters to Statham pressure transducers. Pressure signals, electronically differentiated LVP dp/dt max and heart rate are recorded with a polygraph.

After reaching stable hemodynamic conditions for at least 20 min (control baseline values), the test compound is administered either orally in a gelatin capsule or by intravenous injection into the cephalic vein.

Hemodynamic parameters are recorded continuously starting 30 min before to 120 min after drug administration and thereafter at 1-h intervals until 6 h after dosage.

Evaluation

The following parameters are monitored:

- Systolic blood pressure [mmHg]
- Diastolic blood pressure [mmHg]
- Left ventricular end-diastolic blood pressure, LVEDP [mmHg]
- Left ventricular pressure at dp/dt max [mmHg/s]
- Heart rate [beats/min]

Mean values ± SEM are calculated with $n > 3$ as differences to predrug control values.

Modifications of the Method

Mann et al. (1987) described a simple procedure for direct blood pressure measurement in conscious dogs using the Vascular Access Port™, consisting of a 33 × 13-mm reservoir body affixed to a silicone rubber catheter.

Müller-Schweinitzer (1984) described a method for the assessment of vasoconstrictor agents by recording venous compliance in the conscious dog. Changes in the diameter of the canine saphenous vein, produced by inflation to 45 mmHg of a sphygmomanometer cuff placed on the upper hind leg, were recorded.

Hintze and Vatner (1983) compared the effects of nifedipine and nitroglycerin in conscious dogs, instrumented for instantaneous and continuous measurements of coronary arterial and left ventricular diameters with an ultrasonic dimension gauge, arterial and left ventricular pressure with implanted miniature gauges, and coronary blood flow with an electromagnetic flowmeter or a Doppler ultrasonic flowmeter.

Shimshak et al. (1986) studied the recovery of regional myocardial contractile function after a 10-min coronary artery occlusion in chronically instrumented conscious dogs.

Wright et al. (1987) described a minimally invasive technique which allows assessment of histamine H_1-receptor antagonist activity in conscious dogs based on the inhibition of tachycardia caused by intravenous administration of the H_1-receptor agonist, 2-pyridylethylamine.

Hashimoto et al. (1991) studied the coronary effects of nicorandil in comparison with nitroglycerin in chronic conscious dogs instrumented with ultrasonic crystals and electromagnetic flowmeters in the circumflex coronary artery.

Hartman and Warltier (1990) described a model of multivessel coronary artery disease using conscious, chronically instrumented dogs. A hydraulic occluder and an ameroid constrictor were implanted around the left anterior descending and the left circumflex coronary arteries. Pairs of piezoelectric crystals were implanted within the subendocardium of the left anterior descending and the left circumflex coronary artery perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion.

Hof et al. (1990) used the Doppler method for measuring cardiac output in **conscious rabbits**.

Grohs et al. (1993) simultaneously assessed cardiac output with pulsed Doppler and electromagnetic flowmeters during cardiac stimulation.

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Hemodynamic Studies in Monkeys

Purpose and Rationale

Prior to studies in human beings, studies of cardiovascular effects of new drugs in monkeys are necessary in some instances.

Procedure

Rhesus monkeys of either sex weighing between 5 and 8 kg are anesthetized with 20 mg/kg ketamine hydrochloride followed by 50 mg/kg pentobarbital-Na given slowly i.v. A small side branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure recordings using a blood pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Compounds are administered either intravenously or via a gastric fiberscope, e.g., Olympus XP 10, into the duodenum under visual control. The cardiovascular parameters are registered for a pretest period of 30 min and then during 60 min after intravenous administration or 2 h after intragastric administration of the test drug. Three to six animals are used for evaluation.

Evaluation

Mean values ± SD are calculated for the pretest period and for the cardiovascular effects every min for 5 min after i.v. administration and then every 5 min. After intragastric administration, the values are registered every 5 min up to 30 min and then every 10 min. The values after administration of the test compound are compared statistically with the pretest values using the Student's *t*-test.

Modifications of the Method

Lacour et al. (1993) studied cardiovascular parameters in conscious **cynomolgus monkeys** (*Macaca fascicularis*). A silicone catheter (internal and external diameter 0.64 and 1.19 mm, respectively) was implanted under aseptic conditions into the thoracic aorta via a carotid artery after the monkeys had been anesthetized with 40 mg/kg ketamine and 0.5 mg/kg acepromazine intramuscularly. The vascular catheter (filled with an aqueous solution of 40 % polyvinylpyrrolidone and 20 % heparin) was inserted into a carotid artery. A patch of silicone was sewn around the artery to maintain the catheter in position, the latter being routed subcutaneously and exteriorized at the top of the head into a stainless steel connector. This connector was fixed to the skull with screws and dental cement and sealed with a plug to protect the catheter from damage. The monkeys were permitted a 3-week minimum recovery period. Before the experiment was performed, the monkeys were placed in a primate-restraining chair on several occasions, of gradually increasing duration, for experiment acclimatization.

Pulsatile arterial pressure was recorded by connecting the arterial catheter to a polygraph

via a Statham P23 ID pressure transducer. Mean arterial pressure and heart rate were derived from the pulse pressure signal and recorded. A catheter was inserted acutely into a saphenous vein for administration of compounds.

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Measurement of Cardiac Output and Regional Blood Flow with Microspheres

Purpose and Rationale

The microsphere technique allows the measurement of cardiac output and regional blood flow. Using different radionuclides, repeated determinations are possible. The method is applicable not only for dogs, cats, and minipigs (Hof et al. 1980) but also for rats (McDevitt and Nies 1976; Bonnacrossi et al. 1978; Ishise et al. 1980; Stanek et al. 1985) using microspheres of appropriate size.

Procedure

Male Sprague–Dawley rats weighing 265–375 g are anesthetized with 35 mg/kg i.p. pentobarbital. The right carotid and right femoral arteries are cannulated. Using pressure monitoring, a carotid cannula is manipulated into the left ventricle. Carbonized microspheres (15 ± 5 μ diameter) labeled with ^{85}Sr are drawn into a glass injection chamber and suspended in 0.3 ml 6 % dextran so that each chamber contains 60,000–80,000 microspheres. The radioactivity in each chamber is determined by gamma scintillation counting before and after microsphere injection, the difference being the amount of radioactivity injected. The microspheres are injected into the left ventricle in a total volume of 0.8 ml 6 % dextran over 20 s. Simultaneously, arterial blood from the femoral artery is withdrawn at 0.8 ml/min for 90 s with a syringe withdrawal pump.

Evaluation

This reference blood sample is used to calculate the cardiac output by the formula:

$$\text{Cardiac output} = \text{counts injected} \times \frac{\text{reference sample withdrawal rate}}{\text{reference sample counts}}$$

After obtaining the reference sample, the animals are sacrificed with pentobarbital and the organs dissected, placed in counting vials, and counted for 5 min. Regional distribution of the cardiac output is calculated by comparing the radioactivity in each organ with the total injected radioactivity. Organ flow is determined by multiplying the cardiac output by the fractional distribution of the cardiac output to the organ.

Critical Assessment of the Method

Problems associated with the microsphere technique in rats are the hemodynamic effects of the solutions used to inject the microspheres and the effects of blood withdrawal after repeated determinations (Stanek et al. 1985).

Modifications of the Method

For repeated determinations, other nuclides have been used, such as ^{46}Sc, ^{51}Cr, ^{141}Ce, and ^{125}I (Hof et al. 1980).

Kovách et al. (1992) used up to five radiolabeled microspheres (^{57}Co, ^{113}Sn, ^{85}Sr, ^{95}Nb, and ^{46}Sc) for measurement of regional cerebral blood flow in cats.

Faraci and Heistad (1992) measured blood flow with radioactive microspheres (15 μ diameter) labeled with ^{46}Sc, ^{95}Nb, ^{153}Gd, ^{85}Sr, and ^{141}Ce in anesthetized rabbits.

Grover et al. (1990) and Gross et al. (1992) measured myocardial blood flow in dogs with the radioactive microsphere technique.

Kowallik et al. (1991) measured regional myocardial blood flow with multiple colored microspheres. The method yielded values very similar to those obtained with radioactive microspheres.

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Carotid Artery Loop Technique

Purpose and Rationale

The carotid loop method, originally described by van Leersum (1911) for rabbits, has been used by several authors (e.g., Child and Glenn 1938; Valli et al. 1967; O'Brien et al. 1971; Meyer et al. 1989a, b) for measurement of blood pressure or blood sampling in conscious dogs and sheep (Lagutchik et al. 1992).

Procedure

Male or female inbred Beagle or Labrador–Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital and continued with an infusion of 4–6 mg/kg/h. The animal is placed on a heated operating table. The skin on the ventral side of the neck is carefully shaved and disinfected. The course of the carotid artery is outlined by palpation along the tracheal border. About 2 cm of skin is taken on each side marking the width of the flap. The medial incision is made slightly above the

thyroid cartilage and is extended caudal to a point about 1 cm lateral and 1 cm above the manubrium sterni. The lateral incision again lies about 2 cm from the line of the carotid artery and parallel to it. The lateral incision is only half as long as the medial one. The incisions are made down to the subcutaneous tissue over the platysma muscle. Between the skin and the muscle, the flap is undermined. All bleeding points are carefully clamped and tied.

The subcutaneous tissue, the platysma myoides muscle, and the anterior fascia of the neck are incised in the course of the midline incision down to the plane of cleavage between the sternohyoid and sternomastoid muscles. By blunt dissection, these muscles are separated, disclosing at their depth the neurovascular bundle over which lies the internal jugular vein. The floor of the space so isolated is formed by the longus capitis muscle. By careful dissection, these muscles are separated at least 1 cm above and below the limits of the incision in the skin. The superior thyroid artery marks the uppermost portion of the carotid artery suitable for exteriorization. The plane of cleavage is followed caudal to the origin of the sternocleidomastoid muscle at the manubrium sterni. Throughout the limits of the incision, the artery is dissected free from the internal jugular vein and then from the vagus nerve.

The first step in the exteriorization of the artery is the reapproximation of the muscle borders beneath the vessel by mattress sutures. In order to prevent tension on the completed loop due to contraction of the sternomastoid and sternohyoid muscles, it is important to reapproximate these muscles throughout their course. Sutures are placed at the edges of skin. The tubular flap of skin is then approximated loosely around the carotid artery. It is essential that the skin flap fits loosely around the artery. A continuous suture of fine silk is started at the place where the vessel emerges from the muscle borders. The suture is so placed as to include the artery in a sling of skin which isolates the vessel from the line of suture of the underside of the completed loop. Finally, the proximal and distal quarters of the flap are closed with sutures, while the skin tube is closed with a continuous suture. Antibiotics are given locally and systemically.

One thickness of gauze is placed beneath the loop and along each border a strip of gauze in order to relieve the loop from the pressure caused by the remainder of dressings. Around the neck is wrapped a gauze bandage several turns of which have passed behind the forelimbs in order to prevent the dressing from riding upwards on the animal's neck. Over this is placed a plaster roll protecting the loop from the animal's efforts of scratching. The dressings are changed on the fifth and seventh day when the sutures can be removed.

Blood pressure measurements can be made according to Riva-Rocci's principle by placing a inflatable cuff around the loop.

Critical Assessment of the Method

The carotid artery loop method needs some surgical experience and very meticulous caretaking of the animals.

Modifications of the Method

Lewis et al. (1980) placed a CO_2 sensor using mass spectrometry and its through flow cuvette in a common carotid artery-to-jugular vein loop in anesthetized cats.

Meyer et al. (1989a, b) studied pulmonary gas exchange in panting dogs with an exteriorized carotid artery loop.

Kaczmarczyk et al. (1979) used conscious, chronically instrumented dogs with electric flow probes around the left renal artery and a carotid loop to study postprandial volume regulation.

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Measurement of Heart Dimensions in Anesthetized Dogs

Purpose and Rationale

The measurement of the heart dimensions allows to localize the effect of a drug on the activity of the heart. An ultrasonic technique is used for continuous measurement of left ventricular dimensions. Compounds are tested with potential antianginal activity due to the reduction of left ventricular diameter. The test is used to evaluate the influence of drugs on left ventricular external and internal diameter in anesthetized dogs.

Procedure

Male or female Beagle or Labrador–Harrier dogs weighing between 15 and 25 kg are used for the test. The dog is anesthetized by intravenous injection of 35–40 mg/kg sodium pentobarbital followed by subcutaneous injection of 2 mg/kg morphine. Respiration is maintained through a tracheal tube with N_2O/O_2 (3:1) using a positive pressure respirator.

Implantation of Ultrasonic Transducers

Ultrasonic transducers are constructed and implanted as described by Stinson et al. (1974).

To measure left ventricular external diameter (LVED), two ultrasonic transducers are fixed to the left ventricular wall. One crystal is sutured to the posterior wall within the rectangular area formed by the left circumflex coronary artery and the left posterior descending artery. The other one is placed near the first diagonal branch of the left anterior descending coronary artery. Exact positioning is assured with an oscilloscope.

To measure left ventricular internal diameter (LVID), the transducers are placed in the same anatomical area as for the epicardial crystals. However, they are pushed through the wall of the left ventricle through stab wound incisions. The crystals are positioned across the greatest transverse diameter of the left ventricle, one on the anterior and the other on the posterior endocardial wall.

Bleeding during the implantation procedure is controlled by umbilical tapes around the cranial and caudal veins and by purse-string sutures at the implantation sites. The pericardial incision and the chest are closed by sutures, and the transducer wires are connected to the recording equipment.

In each dog, either LVED or LVID is measured together with the other hemodynamic parameters.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannulated femoral artery by a pressure transducer (Statham P23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left A. carotis communis. LVEDP is measured on a high-sensitivity scale. From the pressure curve, d*p*/d*t*max is differentiated and heart rate is calculated.

Hemodynamic parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 30 min (control values), the test substance is administered by intravenous or intraduodenal injection.

Readings are taken at times 0, 15, 30, 45, 60, 75, 90, and 120 after drug administration. Left ventricular dimensions are measured at the end of the diastole and systole.

Characteristics:

- Blood pressure
  - Systolic blood pressure
  - Diastolic blood pressure
- Left ventricular pressure, LVP
- Left ventricular end-diastolic pressure
- Left ventricular contractility, d*p*/d*t*
- Heart rate, HR
- Left ventricular external diameter, LVED
- Left ventricular internal diameter, LVID

Evaluation

Hemodynamic parameters, LVED, and LVID [mm] are determined.

Changes in parameters after drug administration are compared to control values obtained during the 30-min predrug period.

Statistical significance is assessed by means of the paired *t*-test.

Since a change in the diameter of the left ventricle is a reasonable accurate index of left ventricular volume, a reduction of LVED or LVID with no change in d*p*/d*t* and HR can be considered as a strong indicator for "venous pooling" and thus an antianginal activity of a compound.

Scores are allotted relative to the efficacy of standard compounds assessing the intensity as well as the duration of the effect.

Standard data:

		LVED [mm]		LVID [mm]	
Nitroglycerin	0.005 mg/kg, i.v.	−0.9	20 min	−1.2	30 min
Isosorbide dinitrate	0.1 mg/kg, i.v.			−0.6	120 min
Molsidomine	0.2 mg/kg, i.v.	−2.1	>60 min	−1.4	>120 min
Nifedipine	0.1 mg/kg, i.v.			+1.2	120 min

Modifications of the Method

Novosel et al. (1992) measured the dimensions of the right ventricle with microsonometry in anesthetized rabbits.

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Telemetric Monitoring of Cardiovascular Parameters in Rats

Purpose and Rationale

Radiotelemetry allows the recording of cardiovascular parameters in conscious, free-moving animals. Several authors (Brockway et al. 1991; Mattes and Lemmer 1991; Guiol et al. 1992; Morimoto et al. 1992; Basil et al. 1993; Brockway and Hassler 1993; Lemmer et al. 1993, 1994, 1995; Calhoun et al. 1994; Diamant et al. 1993; Kramer et al. 1993a, 1995; Griffin et al. 1994; Kuwahara et al. 1994; Sato et al. 1994, 1995; van den Buuse 1994; Kinter 1996; Becker et al. 1997; Witte et al. 1998) used commercially available systems with some modifications to study the circadian rhythm of blood pressure and the influence of drugs on heart rate, blood pressure, and motility in rats.

Procedure

The telemetry and data-acquisition system (e.g., Data Sciences International, Inc., St Paul, MN) consists of four parts:

1. The implantable transmitter, which measures the pressure. This device contains a highly stable ion implant, semiconductor, strain-gauge sensor, and battery-powered electronics to process the information from the pressure sensor and to telemeter it from within the animal. Arterial pressure is transmitted to the sensor via a 0.7-mm-diameter, fluid-filled catheter.
2. The receiver which detects the signal from the implanted transmitter and converts it to a form readable by computer.
3. The pressure reference module, which measures atmospheric pressure to allow for the telemetered absolute pressure to be converted to a gauge pressure.
4. The data-acquisition software, which accepts data from the reference module and the receivers, filters corrupt samples from the incoming data stream, converts the telemetered pressure to millimeters of mercury, subtracts atmospheric pressure from the telemetered pressure, and stores the data for retrieval, plotting, and analysis.

Under pentobarbital anesthesia, the telemetry transmitter is implanted into rats. The descending aorta is exposed between the renal arteries. A vascular clamp is made by putting two surgical threads on the proximal and distal part of the artery. The catheter tip is inserted through an incision in the vessel. A drop of cyanoacrylate glue is applied to the dried entry point. The transmitter is sutured to the abdominal musculature.

Evaluation

Data from individual animals are recorded over long periods of time which allow the investigator to follow the circadian rhythm under several experimental conditions.

Modifications of the Method

Hess et al. (1996) monitored pulmonary arterial pressure in freely moving **rats** by inserting the sensing catheter of a telemetric system through a small hole and pushing it into the pulmonary artery.

Further cardiovascular studies in rats using the telemetric system were reported by Sgoifo et al. (1998) and Webb et al. (1998).

Kramer et al. (1993b) used telemetry to record electrocardiogram and heart rate in freely moving **mice**.

Carlson and Wyss (2000) used small telemetry probes for long-term recording of arterial pressure and heart rate in mice after implantation to the carotid artery or the abdominal aorta.

DePasquale et al. (1994) used radiotelemetry to monitor cardiovascular function in conscious **guinea pigs**.

Telemetric ECG recordings in **cardiomyopathic hamsters** were reported by Desjardins et al. (1996).

Van den Buuse and Malpas (1997) studied cardiovascular parameters in **rabbits** by radiotelemetry.

Astley et al. (1991) and Smith et al. (1993) used telemetric systems to monitor cardiovascular responses in **baboons**.

Schnell and Wood (1993) measured blood pressure and heart rate by telemetry in conscious, unrestrained **marmosets**.

An ultrasonic blood flowmeter telemetry system for **cats** and rabbits has been described by Yonezawa et al. (1989, 1992).

Telemetry was used by Symons et al. (1992) to monitor the severity of events representing myocardial dysfunction in **miniswine**.

Savory and Kostal (1997) applied the telemetric system for chronic measurement of cardiovascular and other parameters in **chicken**.

Radiotelemetry has also been used for other pharmacological experiments, such as field potential analysis by radioelectroencephalography (see section "EEG Analysis from Rat Brain by Telemetry" in chapter "► Effects on Behavior and Muscle Coordination"), step-through passive avoidance (see section "Step Through" in chapter "► Behavioral Methods Used in the Study of Learning and Memory"), shock-prod burying test in rats (see section "Progressive Ratio Procedure" in chapter "► Tests for Anxiolytic Activity"), measurement of body temperature (see section "Antipyretic Testing in Rats" in chapter "► Anti-Pyretic Activity"), and motility in rats and mice (Clement et al. 1989; Guillet et al. 1990; Diamant et al. 1993; van den Buuse 1994).

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Cardiovascular Effects After Intracerebroventricular Administration

Purpose and Rationale

Several drugs, like α_2-adrenergic agonists, act primarily at central sites. Their effects can be most clearly demonstrated after injection into the cerebroventricular system. The first experiments have been performed in cats. The method has been adapted to rats.

Procedure

Rats of either sex weighing 250–350 g are anesthetized with 100 mg/kg hexobarbital i.p. The scalp is cut in a sagittal line. With a dental drill, a hole of 1–1.5 mm diameter is drilled through the cranial bone 1 mm lateral and 2 mm caudal of the bregma. A PVC is introduced perpendicular to the bone to a depth of 3 mm in order to reach the lateral cerebral ventricle. The catheter is fixed with dental cement and the wound closed. Test substances are administered through the catheter. To measure blood pressure, one catheter is placed in one carotid artery and connected to a Statham transducer. Blood pressure and heart rate are recorded on a polygraph over a period of at least 30 min. For long-acting drugs, registration periods up to 2 h are necessary. After the experiment, the animal is sacrificed and the brain removed to confirm the site of injection.

Evaluation

Systolic and diastolic blood pressure as well as heart rate after intracerebroventricular injection are expressed as percentage of pretreatment values. The response is compared with the standard clonidine which is effective in doses of 4–60 μg.

Modifications of the Method

Based on the work of Feldberg and Sherwood (1954) and Hayden et al. (1966), Mastrianni et al. (1986) developed an intracerebroventricular perfusion system for the study of centrally acting antihypertensive drugs in the rat. The antihypertensive effect of clonidine could be observed over several hours.

Methods used to detect central hypotensive activity of drugs have been reviewed by Timmermans (1984).

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Influence on Orthostatic Hypotension

Purpose and Rationale

Orthostatic hypotension with dizziness up to unconsciousness is a syndrome occurring in many human individuals. Moreover, several drugs are known to cause orthostatic hypotension. In several animal species, such as rabbit, cat, and dog, this syndrome can be evoked by changing the usual horizontal position into a vertical position with the head upwards using a tilting table.

Procedure

Cats of either sex weighing 2.0–3.0 kg are temporarily anesthetized with ether. Anesthesia is maintained by intravenous injection of 70 mg/kg chloralose. The animal is fixed with its legs on a heated operating table which can be tilted by 90°. The carotid artery is cannulated for measuring blood pressure through a Statham P23 DB transducer on a 6-channel Hellige recorder. The femoral vein is cannulated for injection of the test compound. After the blood pressure is stabilized for 30 min, the animal is quickly tilted to a vertical position for 1 min. Due to the change of position and gravitational force, there is a rapid fall in blood pressure which recovers as soon as the animal is restored to its original position. After taking the control reading, the test compound is administered intravenously and the same procedure is repeated. The fall in blood pressure is recorded.

Evaluation

A significant increase in postural hypotension with respect to the control would indicate that the test compound may produce orthostatic hypotension in human. Moreover, some compounds, like sympathomimetics, can reduce or prevent postural hypotension.

Modifications of the Method

Sponer et al. (1981) described a method for evaluating postural hypotension in conscious **rabbits** placed on a tilting table whereby blood pressure was measured from the central artery of the ear.

Takata et al. (1999) reported a rabbit model for evaluation of chlorpromazine-induced orthostatic hypotension.

Humphrey and McCall (1982) described a model for predicting orthostatic hypotension during acute and chronic antihypertensive drug therapy in **rats** anesthetized with chloralose, urethane, and pentobarbital using a heated tilting table.

Lee et al. (1982) evaluated postural hypotension induced by drugs in conscious restrained normotensive rats. Blood pressure was recorded after cannulation of the femoral artery under light ether anesthesia. A special tilting table was build for simultaneous studies in four rats.

Martel et al. (1996, 1998) studied the phenomenon of cardiovascular deconditioning observed in crew members of space flights in rats after tail suspension.

Socci et al. (2000) studied cardiovascular responses to simulated microgravity in Sprague–Dawley rats. Microgravity is known to induce orthostatic intolerance and baroreflex impairment in astronauts. The authors used 30° head-down tilt, 24-h whole-body suspension, or 7-day tail suspension to mimic microgravity and to find treatment ameliorating the symptoms.

Baum et al. (1981) studied antihypertensive and orthostatic responses to drugs in conscious **dogs**. A catheter was placed in the subclavian artery for measurement of blood pressure and exteriorized at the back of the neck some days prior to the experiment. The animals were placed into a sling and tilted to the 90° upright position for periods of 60 s every hour by lifting their forelimbs. Blood pressure response before and after treatment with test drugs was measured.

A nonhuman **primate** model for evaluating the potential of antihypertensive drugs to cause orthostatic hypotension was described by Pals and Orley (1983) Polyvinyl catheters were implanted in the abdominal aorta and the vena cava via an external iliac artery and vein to **cynomolgus monkeys** during ketamine anesthesia. The catheters were routed subcutaneously from the groin area to the top of the head and exteriorized. After recovery the animals were placed in restraining chairs allowing the change from vertical to horizontal position.

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Bezold–Jarisch Reflex

Purpose and Rationale

The circulatory collapse after intravenous injection of veratrine has been first described in cats and is known as Bezold–Jarisch reflex (von Bezold and Hirt 1867; Jarisch and Richter 1939a, b; Jarisch 1940; Aviado and Guavera-Aviado 2001).

Fleckenstein et al. (1950) recommended this as a suitable animal model of shock.

The original observation was a triphasic blood pressure response in cats or dogs characterized by a short-lasting fall in blood pressure accompanied by bradycardia, followed by a short-lasting increase and then a long-lasting decrease of blood pressure after intravenous injection of veratridine or other veratrum alkaloids.

Kalkman et al. (1984) showed that three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin observed in the Bezold–Jarisch reflex.

The Bezold–Jarisch reflex has been studied in several species, such as **cats** (Takei et al. 1995; Vayssettes-Couchay et al. 1997), **dogs** (Zucker and Cornish 1981; Barron and Bishop 1982; Harron and Kobinger 1984; Giles and Sander 1986; Baugh et al. 1989; Watson et al. 1995), **ferrets** (Andrews and Bhandari 1993), **rabbits** (Chen 1979), guinea pigs, **rats** (Fozard 1984; Gylys et al. 1988; Cohen et al. 1989; Blower 1990; Miyata et al. 1991; Turconi et al. 1991; Matsumoto et al. 1992; Meller et al. 1992; Robertson et al. 1992; Kishibayashi et al. 1993; Geissler et al. 1993; Haga et al. 1994; Hegde et al. 1995; Eglen et al. 1995; Göthert et al. 1995; Delagrange et al. 1996; De Vries et al. 1997; Malinowska et al. 2001; Godlewski et al. 2003), and **mice** (Eglen et al. 1994; Middlefell et al. 1996), whereby species differences have been observed (Yamono et al. 1995).

In cats and dogs, the Bezold–Jarisch reflex was elicited by veratrine and veratridine but also by capsaicin and the 5-HT_3 receptor agonists 2-methyl-5-HT, phenylbiguanide, chlorophenylbiguanide, and serotonin itself.

In rats, mostly 5-HT or 2-methyl-5-HT were used as stimuli to characterize 5-HT_3 receptor antagonists.

Procedure

Male Sprague–Dawley rats weighing 250–380 g are given food and water ad libitum, except those used for intraduodenal drug administration; these rats are deprived of food overnight. The animals are anesthetized by intraperitoneal injection of 1.5 g/kg urethane. Body temperature is maintained at 37 °C by placing the animal on a heating pad. The left jugular vein or duodenum, trachea, and left femoral vein are cannulated for drug administration (i.v. or i.d.), facilitation of respiration, and injection of 2-methyl-5-HT, respectively. Heart rate is derived from a limb lead II ECG monitored via subdermal platinum electrodes and is recorded with amplifiers on a polygraph. A dose–response curve to 2-methyl-5-HT (5–100 μg/kg, i.v.) is constructed in each rat to establish a submaximal dose (usually 10 or 20 μg/kg, i.v.) which elicits a reproducible bradycardic response. Each rat receives then a single dose of test drug or standard and is then challenged with 2-methyl-5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min post dosing. A separate group of rats receiving vehicle (saline for i.v., deionized water for i.d.) is similarly tested in each study.

Evaluation

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls. Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's multiple comparison test.

Modifications of the Method

Harron and Kobinger (1984) used capsaicin to elicit the Bezold–Jarisch reflex in anesthetized artificially respirated dogs pretreated with a beta-adrenoceptor antagonist to evaluate the activity of clonidine-like drugs on central alpha$_2$-adrenoceptors after intracisternal administration.

The Bezold–Jarisch reflex in rats has been used for evaluation of 5-HT_3 receptor agonists (Rault et al. 1996; López-Tudanca et al. 2003).

Rocha et al. (2003) found an enhancement of the Bezold–Jarisch reflex in the acute phase of myocardial infarction of the anesthetized rabbit.

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Endotoxin-Induced Shock

Purpose and Rationale

Many bacterial infections as well as allergic reactions are known to induce pathophysiological events that may lead to shock in man. When experimental animals are injected with endotoxin and galactosamine, shock and death occur in all untreated animals 5–7 h after injection. The endotoxin-induced shock is marked by pulmonary embolism, bronchospasm, and renal failure. Bacterial lipopolysaccharides (endotoxins) play an important role in the pathogenicity of Gram-negative infections.

The reactivity of animals to endotoxin may be enhanced by simultaneous administration of galactosamine. Galactosamine is a specific hepatotoxic agent that leads to early metabolic alterations and consequent cellular liver damage. The following procedure is used to detect compounds that prevent the occurrence of endotoxin-induced shocks.

Cardiovascular parameters of endotoxin-induced shock are greatly influenced by various anesthetics. For this reason, a model was proposed by Brackett et al. (1985) and Schäfer et al. (1987) to study the circulatory shock pattern after endotoxemia in conscious unrestrained rats.

Procedure

Male Sprague–Dawley rats weighing 300 ± 10 g are anesthetized with 5 % enflurane. A tracheal cannula is connected to a rodent respirator delivering 2 % enflurane. Via the right jugular vein, the tip of one catheter is placed just adjacent to the right atrium for injection of endotoxin, monitoring of central venous pressure, and rapid injection of room-temperature saline to produce thermodilution curves for calculation of cardiac output. The right carotid artery is cannulated with a thermistor–catheter combination for measurement of thermodilution cardiac output curves and aortic blood pressure. The thermistor tip is placed in the aortic arch just distal to the aortic valve. The catheters are guided under the skin exiting through the back of the neck just below the base of the skull.

The animals are allowed to regain consciousness and are then placed in cages that allow unrestrained movements about the cage at all times throughout the study with no further handling. The experimental animals receive a 20-s infusion of 40 mg/kg endotoxin (*E. coli*, Difco) being paired with sham animals with identical catheters but receiving an equal volume of saline. Test compounds are injected intravenously 10 min prior to endotoxin injection. Cardiac outputs are measured using the thermodilution technique by rapidly injecting a volume calculated to deliver 100 µl of room temperature saline to the circulatory system. Central venous and aortic blood pressure and heart rate are continuously monitored for the following 4 h. Cardiac output measurements are made 5, 15, 30, 60, 120, 180, and 240 min after endotoxin. At the end of the study, the animals are sacrificed and the catheters checked visually to ensure proper placement.

Evaluation

Central venous pressure, arterial pressure, and cardiac output of drug-treated animals receiving endotoxin are compared with animals receiving endotoxin only and saline–sham-treated animals. Furthermore, cardiac index, total peripheral resistance, and stroke volume are calculated. The small intestines of all rats are examined for severity of hemorrhage using a five-point scale. Repeated-measures analysis of variance is used to analyze the data.

Modifications of the Method

Lindenbaum et al. (1990) studied the effect of *E. coli* endotoxin on cardiovascular parameters of anesthetized **dogs**. Inhibition of the deterioration of metabolic functions and improvement of cardiovascular parameters were found after cocarboxylase treatment.

Endotoxin-induced shock has been tested in **mice** (Galanos et al. 1979). Groups of 10 male C57BL/6 mice weighing 20–22 g are injected intravenously with a mixture of 0.01 μg of *Salmonella* abortus equi lipopolysaccharide and 7.5–15 mg galactosamine in 0.02 ml phosphate-buffered saline. The test compound is administered either intravenously at the same time or orally 45 min prior challenge. Twenty-four hours later, the number of surviving mice is determined.

Metz and Sheagren (1990) reviewed the effects of ibuprofen in animal models of septic shock.

Von Asmuth et al. (1990), Luongo et al. (1998), and Cuzzocrea et al. (2004) described a zymosan-induced shock model in **mice**. Male CD mice (20–22 g) were treated intraperitoneally with zymosan (500 mg/kg, suspended in saline solution) or with zymosan and drug (rosiglitazone 3 mg/kg, intraperitoneally) at 1 and 6 h after zymosan. Eighteen hours after administration of zymosan, animals were assessed for nonseptic shock. Clinical severity of systemic toxicity was scored for the whole experimental period (12 days) in the mice after zymosan or saline injection on a subjective scale ranging from 0 to 3: 0 = absence, 1 = mild, 2 = moderate, and 3 = serious. The ranging scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhea, and lethargy) observed in the animals. The final score was the sum of the single evaluation (maximum value 12).

Overbergh et al. (2003) studied acute shock induced by antigen in NOD mice. The 8-week-old NOD, BALB/c, and C57BL/6 mice were immunized by injection of 100 μg antigen [hen egg-white lysozyme (HEL), GAD65 (p524–542) (SRLSKVAPVIKARMMEYGTT), bovine insulin B (ins-B) chain, heat shock protein (hsp)-65, PLP peptide (amino acids 135–151), ovalbumin, keyhole limpet hemocyanin (KLH), and tetanus toxin] emulsified at a 1:1 concentration in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) in the hind footpads. NOD–*SCID* mice were injected with 100 μg HEL antigen suspended in CFA. All mice were reinjected with the same antigen 3 weeks later in a similar manner. Clinical evolution and survival rate after sensitization with various peptides were monitored in different mouse strains. Shock was characterized by piloerection; prostration; erythema of the tail, ears, and footpads; and dyspnea with shallow breathing. Serum for antibody measurement and spleens for quantification of mRNA levels were collected before immunization and again before booster administration.

Baldwin et al. (1991) tested the effect of polymyxin B on experimental shock from meningococcal lipooligosaccharide and *Escherichia coli* lipopolysaccharide endotoxins in anesthetized **rabbits**.

Muacevic and Heuer (1992) tested the effect of platelet-activating factor antagonists in anesthetized rats.

Otterbein et al. (1993) tested the effects of peptides on survival of mice injected with 50 mg/kg lipopolysaccharide endotoxin in mice and on survival of rats with fecal peritonitis.

Mountz et al. (1995) reported an increased susceptibility of fas mutant **MRL-Ipr/Ipr mice** to staphylococcal enterotoxin B-induced septic shock.

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Hemorrhagic Shock

Purpose and Rationale

Hemorrhagic shock is one of the most severe consequences of accidents. Several animal models in various species have been developed to resemble the conditions in man and to test therapeutic or prophylactic measures (Lamson and de Turk 1945; Selkurt and Rothe 1961; Mills 1967). A method for hemorrhagic shock in anesthetized as well as in unanesthetized rats has been described by van der Meer et al. (1987). Experimental hemorrhagic shock is defined as a situation in which the cardiovascular system, after a period of hypovolemia followed by complete reinfusion of the shed blood, gradually deteriorates ending in the death of the animal.

Procedure

Female rats weighing 170–190 g are anesthetized by i.p. injection of sodium pentobarbital, 25 m/kg, followed after 20 min by 20 mg/kg, and kept in a chamber at 30 °C and relative humidity

over 80 %. The left femoral vein is cannulated for application of the test drug. The right common iliac artery is cannulated and the cannula (polyvinyl chloride, 14 cm long, inner diameter 2 mm) is filled with heparin and exteriorized in the neck. After intra-arterial injection of 0.2 ml heparin 500 IU/ml, the cannula is connected to a siliconized calibrated glass reservoir (inner diameter 18 mm), the height of which can be changed to adjust the surface of the shed blood to a fixed level.

The test drug is injected i.v. 5 min prior to bleeding. Bleeding is performed against (at heart level) 30 mmHg for 1 h, 25 mmHg for 0.5 h, 30 mmHg for 1 h, 25 mmHg for 0.5 h, and finally 30 mmHg for 1 h. The shed blood is partially taken up again spontaneously. After 4 h, reinfusion is started by increasing the pressure to 60 mmHg for 5 min, to 80 mmHg for 5 min, and (if necessary) to 100 mmHg. During the hypovolemic phase, respiration becomes gradually slower. If respiration arrest is imminent, 0.5 ml 5 % glucose is injected intra-arterially, thus avoiding death during the period of hypovolemia. Practically all rats die at an average of 4 h after complete reinfusion.

Evaluation

Survival time is taken as the time between complete reinfusion and death. Average survival time of treated animals is compared with that of controls. Furthermore, after autopsy the number of gastrointestinal lesions, subendocardial hemorrhage, kidney tubular necrosis, and liver cell necrosis are registered by histological examination.

Critical Assessment of the Method

In spite of the fact that hemorrhagic shock does not reflect the situation of traumatic shock in man in every aspect, the condition is close enough to use the model for testing compounds which potentially inhibit or ameliorate shock in man.

Modifications of the Method

A method to study hemorrhagic shock in dogs has been described in detail by Mills (1967). Large dogs weighing 20–30 kg are anesthetized by an i.v. injection of 25 mg/kg sodium pentobarbital. The animals are respirated by means of a Harvard respirator set at a stroke volume of 400 ml and a rate of 20 respiration/min. Blood pH is regulated between 7.37 and 7.42 by varying the gas flow between 100 % O_2 and a mixture of 95 % O_2 and 5 % CO_2. Central arterial blood pressure is recorded by inserting a catheter through one femoral artery to the aortic arch. Pulmonary artery pressure is measured by inserting a PE-50 catheter through a small neck vein, reaching the right ventricle and allowing to float into the pulmonary artery. The right atrial catheter is also inserted through a small neck vein. After the chest is opened, the left atrial catheter is tied in place through a small opening in the left atrial appendage. Blood flow is measured in the ascending aorta (cardiac output), carotid, superior mesenteric, renal, and femoral arteries using electromagnetic flowmeters. Furthermore, pulse rate is monitored from the electrocardiogram.

The test drug is injected i.v. 10 min prior to bleeding. Blood is removed either at a specific volume or until a selected reduction of blood pressure has occurred. The cardiovascular parameters of treated animals are compared with those of controls.

Shock associated with hemoconcentration was produced in dogs (Davis 1941) by bleeding from the carotid artery and injections of 25 % sodium chloride solution subcutaneously in doses of 25 ml.

The effect of insulin on glucose uptake in the soleus muscle of rats during hemorrhagic shock was studied by Chaudry et al. (1975).

Bauer et al. (1995) used hemorrhagic shock in rats to evaluate the influence of interleukin-1 on leukocyte–endothelial cell interactions and the microcirculation in the liver by means of intravital microscopy after application of an interleukin-1 receptor antagonist.

Kitajima et al. (1995) studied gastric mucosal injury induced by hemorrhagic shock in rats.

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Tourniquet Shock

Purpose and Rationale

Compression of extremities in man by heavy objects for periods of several hours results in the so-called crush syndrome. The rescued individual shows immediately a favorable response to therapy, but within a few hours, symptoms of shock develop followed by signs of progressive renal damage leading to death (Duncan and Blalock 1942). Moreover, arterial bleeding after accidents needs the applications of tourniquets. During surgical procedures on extremities, a tourniquet may be necessary (Wilgis 1971), the time of which has to be limited in order to avoid fatal consequences. The pathophysiological mechanisms of tourniquet-induced shock remain still to be elucidated. Nevertheless, animal models in rats (Chandra and Dave 1970), rabbits (Little 1974), and dogs (Goto et al. 1988) had to be developed to evaluate drugs capable to inhibit the fatal consequences of crush and tourniquet shock.

Procedure

Wistar rats of either sex weighing 250–280 g are anesthetized with phenobarbital. The tourniquets consist of rubber tubes (internal diameter 4 mm, external diameter 5.8 mm). Both tights are fastened by the rubber tubes, and the pressure which is monitored by a miniature pressure sensor and an amplifier (e.g., Kyowa Electronic Instruments Co, Tokyo) is adjusted to 1.5 kg/cm^2. The rubber tubes are knotted and the sensor removed. After 3 h the animals are treated with the test compound or the control solution. The tourniquet is left in place for 6 h while the animals remain under pentobarbital anesthesia. Then, the rubber tubes are removed, and the rats are returned to their cages. Within a few min, the reperfused hind limbs, which have been pale blue, turn pink. The animals are then allowed free access to food and water. Blood is withdrawn at different intervals during the tourniquet and afterwards for measurement of hematocrit, transaminases, urea nitrogen, and total protein. Time to death is registered.

Evaluation

Statistical evaluation of the survival intervals is performed with the log-rank test according to

Peto et al. (1976). Blood chemical data are analyzed using the Kruskal and Wallis (1952) rank-sum test. Multiple comparisons are corrected by the Bonferroni's method (Dunn 1961).

Critical Evaluation of the Method

These methods are valuable to find drugs effective in this life-threatening situation.

Modifications of the Method

Ghussen et al. (1979) studied the effect of methylprednisolone on the experimental tourniquet shock in **dogs**.

Haugan and Kirkebo (1984) used a model in anesthetized **rats** with tourniquet shock by bilateral hind-limb occlusion for 3 1/2 h and burn shock by scalding the hind 50 % of the body surface for 30 s in 90 °C water.

Horl and Horl (1985) investigated the effect of tourniquet ischemia on carbohydrate metabolism in **dog** skeletal muscle

Sáez et al. (1982) followed the time course of appearance of lactic dehydrogenase enzymes in the serum of **rats** after different periods of ischemia by bilateral application of rubber band tourniquets to the hind legs.

Sáez et al. (1986) studied the effects of allopurinol on biochemical changes of the gastrocnemius muscle in rats subjected to tourniquet shock followed by reperfusion.

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Heatstroke

Purpose and Rationale

Heatstroke is a medical emergency where quick diagnosis and treatment of victims are essential for positive prognosis. Several animal models have been established by investigators in heat-related studies. Rats (Francesconi and Mager 1978; Hubbard et al. 1977, 1979; Kielblock et al. 1982), rabbits (Shih et al. 1984), dogs (Bynum et al. 1977), and sheep (Tayeb and Marzouki 1990) are considered to be the most suitable models because of their similarity to man in response to high temperature.

Procedure

Male Sprague–Dawley rats weighing 450–550 g are fasted 18–24 h before the experiment. For prevention studies, the animals are treated subcutaneously 1 h before either being restrained in an appropriate wire cage which is placed into an environmental chamber set at 41.5 °C ambient temperature or being exercised in a motor-driven treadmill. Core temperature (rectal probe inserted 6.5 cm) is measured using copper/constantan thermocouples in conjunction with a thermocouple reference oven and a 10-channel data-acquisition system with a teletype printout. After reaching exhaustion or a predetermined core temperature, all rats are monitored at 26 °C ambient temperature while resting in plastic cages lined with wood shavings. After recovery, animals are returned to their cages and allowed water but no food for 24 h.

Evaluation

LD_{50} values are determined in treated and control animals.

Modifications of the Method

Kielblock et al. (1982) analyzed cardiovascular function by direct recording of arterial blood pressure and ECG analysis.

Francesconi and Mager (1978) studied pathochemical indices, such as serum lactate concentration, potassium levels, and plasma creatine phosphokinase.

Kregel et al. (1988) investigated peripheral vascular responses to hyperthermia in the rat by implantation of Doppler flow probes on the superior mesenteric, left iliac or left renal, and external caudal arteries. They concluded that a selective loss of compensatory vasoconstriction triggers the cascade of events that characterize heatstroke.

Shido and Nagasaka (1990) studied thermoregulatory responses to acute body heating in rats acclimated to continuous heat exposure. Indirect external warming was performed by raising the jacket water temperature surrounding the calorimeter from 24 °C to 39 °C. Intraperitoneal heating was made through an electric heater implanted chronically in the peritoneal cavity.

Chiu et al. (1995) reported an increased survival in rat heatstroke by reducing hypothalamic serotonin release after administration of interleukin-1 receptor antagonist.

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α- and β-Adrenoreceptors in the Mouse Iris

Purpose and Rationale

A simple method to test mydriatic substances is the test on the mouse pupil as described by Pulewka (1932). The diameter of the pupil is narrowed by intensive light illumination. A dose-dependent increase of pupil diameter can be achieved by intraperitoneal application of atropine and synthetic mydriatics (Ing et al. 1945; Burn et al. 1950). The mydriatic effect of hexamethonium analogues has been measured by Blackman et al. (1956). Mydriasis is induced by norepinephrine, epinephrine, and isoproterenol and can be antagonized by α- or β-blockers (Freundt 1965).

Procedure

Male mice weighing 15–20 g are used. They are kept for at least 30 min in separate beakers under bright illumination before the pupil diameters are measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice are placed beneath long low-power fluorescent tubes and on top of glossy white paper. The pupil diameter is measured in mm before and at various time intervals after treatment. Groups of 5–10 mice are used for each dose of compound and for vehicle control.

To test sympatholytic activity, various doses of the α- or β-blocker are injected subcutaneously 30 min prior to intravenous injection of 0.1 mg/kg norepinephrine, or 0.05 mg/kg epinephrine, or 20 mg/kg isoproterenol. The effect of norepinephrine is blocked by α-blockers, but not by β-blockers; the effect of epinephrine by both α- and β-blockers; and the effect of isoproterenol by β-blockers, but not by α-blockers.

Evaluation

The mean values of diameters in the groups treated with α- or β-blockers are compared with those of animals treated with norepinephrine, epinephrine, or isoproterenol only.

Modifications of the Method

Edge (1953) used mydriasis in the mouse as a quantitative method of estimating parasympathetic ganglion block.

Håkanson et al. (1987) used the isolated iris sphincter of pigmented rabbits to test multiple

tachykinin pools in sensory nerve fibers. The eyes were taken out within 1 min after sacrifice and opened by an incision 2–3 mm posterior to the limbus, followed by excision of the iris from the ciliary margin. The iris sphincter muscle was then opened, cut in half, and mounted vertically on a Perspex holder in a 7-ml tissue bath maintained at 35 °C. The mechanical activity after electrical stimulation was recorded isometrically using a force displacement transducer and a polygraph.

Kern (1970) used isolated sphincter and dilator muscles from human eyes obtained at autopsy for studies on sympathomimetics and adrenergic blocking agents. Cholinotropic and α- and β-adrenergic receptors were identified.

Responses to bradykinin and/or capsaicin of the isolated iris sphincter were considered to be mediated by substance P released from the trigeminal nerve (Ueda et al. 1984).

Pupillary dilatation can be used as an index for central nervous system α_2-adrenoreceptor activation (Koss 1986).

Clonidine induces mydriasis which is mediated by α_2-adrenoreceptors located in the brain (Berridge et al. 1983; Hey et al. 1985). Blockade of presynaptically located α_2-adrenoreceptors is considered as a possible mechanism for antidepressant drugs. Mianserin was able to antagonize clonidine-induced mydriasis in the rat.

Gower et al. (1988) studied a large number of psychotropic drugs in this model with the aim to reveal in vivo α_2-adrenoreceptor blocking effects of new compounds.

Male Wistar rats weighing 230–300 g were anesthetized with pentobarbital, 60 mg/kg i.p., and a polyethylene catheter was inserted into the femoral vein for drug administration. The rat's head rested on the base platform of a binocular Olympus microscope positioned so that the pupil diameter of the right eye could be measured by means of a micrometer inserted into one eyepiece of the microscope. A constant light intensity was maintained throughout the experiment. Rats were first injected with saline 25 min after anesthesia induction. The pupil diameter was measured 1 min after injection. Five min after measurement, mydriasis was induced by clonidine (0.1 mg/kg, i.v.) and the diameter was measured 1 min after injection. This was followed by the test compound, injected at 6-min intervals at increasing doses. The pupil was measured at 1 min after each injection. The dose inhibiting 50 % of the clonidine-induced mydriasis (ID_{50}) was determined per rat from the cumulative dose–response curve.

Savontaus et al. (1997) studied the effect of an imidazoline derivative against detomidine-induced mydriasis in anesthetized rats.

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α_2-Adrenoreceptor Blockade Measured In Vivo by Clonidine-Induced Sleep in Chicks

Purpose and Rationale

In young chicks, clonidine causes a loss of righting reflex which is antagonized by mianserin (Pommier et al. 1982). This phenomenon was used to measure α_2-adrenoceptor blockade in vivo by Gower et al. (1988).

Procedure

Male white Leghorn chicks are used either a few hours after hatching or 1 or 2 days later. Clonidine-induced loss of righting reflex (sleep) is determined with eight animals at a time. Two animals are treated with placebo and 2 with each of 3 dose levels of the test compound. Tests with groups of 8 animals are continued until 10 animals are tested per dose level or placebo treatment. The chicks are marked with ink and injected intraperitoneally with placebo or the test compound. Ten min later, 1.2 mg/kg clonidine is injected into a leg muscle, and the animals are placed individually in small Makrolon cages. The beginning of sleep time is defined as the moment at which the animals can be placed on their back and remain in this position. Sleep time is recorded until they return to their feet spontaneously or another attempt to put them on their back fails. Sleep time is recorded for a maximum period of 30 min.

Evaluation

Statistical evaluations of differences in median sleeping times are done with the Mann–Whitney *U*-test. Dose–response relations for various drugs can be calculated.

Critical Assessment of the Method

Compounds with known α_2-adrenoceptor blocking activity antagonize clonidine-induced sleep in chicks dose dependently. Yohimbine is one of the most active compounds. However, also other centrally active compounds of which their main effect is not α_2-blockade reduce clonidine-induced sleeping time. One of the most potent is apomorphine acting on dopamine D_2-receptors. Therefore, the clonidine-induced sleeping test in chicks cannot be regarded as highly specific for α_2-adrenoceptors.

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Activity at β_1- and β_2-Adrenoreceptors in the Rat

Purpose and Rationale

The relative potency of catecholamines as stimulants of β-adrenoreceptor-mediated responses varies in different tissues indicating the existence of two subtypes of β-receptors (β_1 and β_2) (Lands et al. 1967a, b). β-Adrenoreceptors in the heart have been classified as being of the β_1-subtype. β-Adrenoreceptors in the uterus, diaphragm, bronchioles, and small intestine have been classified as being of the β_2-subtype, since in these tissues, epinephrine is more potent than norepinephrine. These observations led to the development of selective agonists and antagonists. Isolated organs (see below) having predominantly one receptor subtype, such as the isolated heart and the isolated atrium for β_1, and the isolated uterus or the isolated tracheal chain for β_2, are used to test compounds for selective activity. Assessing both activities in the same animal in vivo results in the advantage that pharmacokinetic and metabolic influences of the drug being tested are the same for both parameters.

Procedure

Female Sprague–Dawley rats (200–220 g) are anesthetized with 60 mg/kg pentobarbital i.p. prior to pithing (Gillespie and Muir 1967). The animals are artificially respired with room air using a Harvard small animal ventilator (90 strokes/min at a pressure of 7 cm H_2O). Body temperature is maintained by placing the animals on a heated operating table. The left carotid artery is cannulated for continuous monitoring of blood pressure via a Statham P23 ID pressure transducer. The blood pressure signal is used to trigger an instantaneous rate meter for continuous monitoring of heart rate. A femoral vein is cannulated for intravenous administration of drugs.

A midline incision is made to expose one horn of the uterus. The ovarian artery is cut, tied, and one horn dissected free from the ovary leaving the myometrial blood supply intact. A cotton thread is attached to the free end of the uterine horn, passed through a glass-jacketed organ bath and connected to an isometric (Pioden UF1) transducer for measurement of spontaneous contractions. A cannula is inserted into the peritoneal cavity for administration of drugs by the i.p. route. The organ bath is positioned such that it surrounds the uterine horn without touching it. The tissue is perifused with Krebs–Henseleit solution being gassed with 95 % O_2/5 % CO_2 and maintained at 37 °C. A resting tension of 0.2 g is applied to the tissue, which is allowed to stabilize until spontaneous contractions are constant over a period of 5–10 min. All recordings are made on a polygraph.

Evaluation of Agonists

Dose–response curves after i.v. injection are established for isoprenaline (nonselective between β_1- and β_2-adrenoreceptors), salbutamol (selective for β_2-adrenoreceptors), and noradrenaline (selective for β_1-adrenoreceptors) in increasing heart rate (beats/min) and decreasing the height of uterine contraction (calculated as percentage of the original amplitude). Animals given noradrenaline are pretreated with phenoxybenzamine (3.3 mmol/kg i.v.) in order to antagonize irreversibly the α-adrenoreceptors. Agonist dose–response curves ($n > 4$) on heart

rate and uterine relaxation are carried out by assessing the activity of at least three doses of each agonist. New synthetic compounds can be tested after intraperitoneal administration additionally.

Evaluation of Antagonists

The ability of a nonselective β-blocker, such as propranolol (1 mmol/kg i.v.); a β_1-selective β-blocker, such as atenolol; and a β_2-selective β-blocker to inhibit responses to isoprenaline on both heart rate and uterine relaxation is assessed by comparing the log-linear portion of the dose–response curve to isoprenaline in the absence and in the presence of the β-adrenoreceptor antagonist in the same animal. Dose ratios for each antagonist are calculated.

Critical Assessment of the Method

The method described by Piercy (1988a, b) has the advantage to measure both agonistic and antagonistic activity and to differentiate between effects on β_1- and β_2-adrenoreceptors. Compared to tests in isolated organs, in vivo activity can be determined after intraperitoneal or intraduodenal administration.

Modifications of the Method

Härtfelder et al. (1958) studied the influence of various agents on the contractions of electrically stimulated **isolated uteri of rabbits and guinea pigs**.

Nathason (1985) evaluated the activity of beta-blockers to inhibit the cardioacceleratory effect of systemically administered isoproterenol in **unanesthetized, restrained albino rabbits** together with the effect on membrane-bound adenylate cyclase in homogenized ciliary process villi in order to find compounds selectively lowering intraocular pressure.

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β_1- and β_2-Sympatholytic Activity in Dogs

Purpose and Rationale

Intravenous administration of isoprenaline (isoproterenol) stimulates β_1-receptors of the heart which can be detected as an increase in contractility (dp/dt max). Intra-arterial injection of isoprenaline stimulates β_2-receptors of peripheral blood vessels leading to an increased peripheral blood flow.

Therefore, a β_1- or β_2-blocking activity of a compound is revealed by the inhibition of the effects of isoprenaline. The following tests are used to evaluate β-blocking activity of drugs. A β-blocker screening is done in anesthetized dogs (a); in addition, the test allows a differentiation between β_1- and β_2-receptor activity and the determination of ED_{50} values (b).

Procedure

Male or female Beagle dogs weighing about 20 kg are used. Animals are premedicated with 1 g Inactin (i.v.) and anesthetized by intravenous administration of 20 mg/kg chloralose and 250 mg/kg urethane. In addition, they receive a subcutaneous injection of 2 mg/kg morphine 1 h after the start of anesthesia. Animals are heparinized. Respiration is maintained through a tracheal tube using a positive pressure respirator. End-expiratory CO_2 content is measured continuously; respiratory rate and depth of respiration are adjusted to 4.5–6 vol.% end-expiratory CO_2. For administration of isoprenaline, a peripheral vein is cannulated.

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a cannula inserted into a femoral artery is connected to a pressure transducer (Statham P23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted via the left arteria carotis communis. LVEDP is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted. Peripheral blood flow in the femoral artery is measured with an electromagnetic flow probe.

Screening for β-Blocking Effects in Anesthetized Dogs

Following a steady-state period of 30–60 min, isoprenaline is administered intravenously two to three times to the anesthetized animal and hemodynamic parameters are recorded (control values = 100 %). Then, the test substance is injected intravenously at cumulative doses (final concentrations of 0.01, 0.05 and 0.15 mg/kg). For each dose, 10-min "drug effects" are monitored by measuring hemodynamic parameters. Then the effect of isoprenaline is tested again (three times).

In other experiments, a single dose of the drug is administered to determine the duration of action.

If a test compound does not show an inhibitory influence on isoprenaline effects, a second test compound is administered.

All hemodynamic parameters are registered continuously during the whole experiment.

Testing for β_1- and β_2-Blocking Effects: Determination of ED_{50}

Following a steady-state period of 30–60 min, isoprenaline is administered for i.v. administration (β_1-test) twice at a dose of 0.5 μ g/kg and for intra-arterial administration (β_2-test) twice at a dose of 0.05 μ g/kg. Hemodynamic parameters are recorded (control values = 100 %). Then, the test substance is injected intravenously at cumulative doses. Consecutively increasing doses are given at 15-min intervals. For each dose, 10-min "drug effects" are monitored by measuring hemodynamic parameters. Thereafter, isoprenaline is given intravenously and 5 min later intra-arterially.

All hemodynamic parameters are registered continuously during the whole experiment.

Characteristics:

- Blood pressure
  - Systolic, BPs
  - Diastolic, BPd
- Heart rate
- Left ventricular pressure, LVP
- Left ventricular end-diastolic pressure, LVEDP
- dp/dt max
- Peripheral flow, A. femoralis
- ECG, lead II

Standard compounds:

- Propranolol HCl
- Practolol
- Metoprolol tartrate

Evaluation

β_1-Receptor antagonism is measured as a decrease in contractility (dp/dt max).

Inhibition of the isoprenaline-induced elevation of heart rate is considered as an indicator for nonselective β-blockade. For cardioselective β-receptor blockers, the increase in dp/dt max is inhibited with lower doses of test drug than the rise in heart rate.

β_2-receptor blockade by a test drug is measured as inhibition of the isoprenaline-induced increase in peripheral blood flow.

The different hemodynamic parameters are determined.

Percent inhibition of the isoprenaline-induced effects by a test compound is calculated and compared to the isoprenaline effects before drug administration (=100 %).

ED_{50} values for $\beta 1$- and $\beta 2$-antagonism are calculated by log-probit analyses. ED_{50} is defined as the dose of drug leading to a 50 % inhibition of the isoprenaline effects.

An $ED_{50}\ \beta_1/ED_{50}\ \beta_2$-ratio of <1 indicates that a β-blocking agent predominantly influences β_1-receptors (cardioselectivity).

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Intrinsic β-Sympathomimetic Activity in Reserpine-Pretreated Dogs

Purpose and Rationale

β-Blocking agents can be classified as:

- β-Blocking agents with intrinsic sympathomimetic activity (ISA)
- β-Blocking agents with membrane stabilizing activity (MSA)
- β-Blocking agents with organ selectivity (high affinity to heart β_1-receptors)

In the following procedure with reserpine-pretreated dogs, β-blocking agents with intrinsic sympathomimetic activity can be identified. Reserpine administration 24 h before the start of the experiment leads to a depletion of catecholamine depots. Thus, it is possible to differentiate between indirectly acting sympathomimetics such as tyramine and directly acting ones such as noradrenaline.

This test is used to identify β-blocking drugs with intrinsic sympathomimetic activity.

Procedure

Male or female Beagle dogs weighing about 15 kg are used. Twenty-four h before the test, dogs receive an intramuscular injection of 0.3 mg/kg reserpine. At the day of the experiment, the animals are anesthetized by intravenous administration of 10–20 mg/kg sodium pentobarbital. Respiration through a tracheal tube using a positive pressure respirator is controlled by measuring end-expiratory CO_2 concentrations (4–5 vol.%).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a femoral artery is cannulated and connected to a pressure transducer (Statham P23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted into the left ventricle via the left common carotid artery. LVEDP is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted.

Experimental Course

The test substance is administered by continuous intravenous infusion of 0.02 mg/kg (1 ml/min) until a cumulative dose of 3 mg/kg is achieved (within approximately 150 min). Thereafter, the velocity of infusion is doubled (0.04 mg/kg, 2 ml/min). The test is finished when a cumulative dose of 7 mg/kg is achieved (after a total time of approximately 250 min).

Hemodynamic parameters are registered continuously during the entire experiment.

Characteristics:

- Blood pressure
  - Systolic blood pressure
  - Diastolic blood pressure
- Left ventricular pressure, LVP
- Left ventricular end-diastolic pressure, LVED
- d*p*/d*t* max
- Heart rate, HR

Evaluation

The different hemodynamic parameters are determined. As a measure for intrinsic sympathomimetic activity (ISA), the increase in d*p*/d*t* max and in heart rate are evaluated. Absolute and relative differences of these parameters in drug-treated animals are compared to vehicle control values.

Statistical evaluations are performed by means of the Student's *t*-test if $n > 4$.

Scores are allotted relative to the efficacy of standard compounds for intensity as well as for duration of the effect.

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Cat Nictitating Membrane Preparation (Ganglion-Blocking Activity)

Purpose and Rationale

Nicotinic acetylcholine receptors are involved in the ganglionic neurotransmission. Various subtypes are described for nicotinic acetylcholine receptors (Sargent 1995; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

The nictitating membrane of the cat has been used extensively in pharmacological studies to evaluate ganglion-blocking activity because of the ease with which its movements can be recorded, because of the simplicity of its innervation (the purely adrenergic fibers have their cell bodies in the easily accessible superior cervical ganglion of the same site), and because its blood supply (via the external carotid artery) is accessible for intra-arterial injections. Preganglionic and postganglionic stimulation allow the interpretation of the mode of action of vasoactive drugs.

Procedure

The animal is anesthetized with 35 mg/kg sodium pentobarbital i.p. Tracheostomy is performed and a tracheal cannula is inserted. On one side, the sympathetic nerve is exposed, separated from the vagus nerve, and prepared in order to place electrodes for preganglionic and postganglionic stimulation. Preferably, the vagus nerve at this site is severed at the central end. The head of the animal is fixed in a head holder to prevent head movements. A linear transducer is fixed at the mid of the border of the nictitating membrane allowing the registration of the contractions on a polygraph. Preganglionic and postganglionic stimuli are exerted by a square wave stimulator, with a pulse width of 0.3–0.5 ms, an amplitude of 1–3 V, and a frequency of 20/min. The amplitude and pulse width varies from animal to animal. The sympathetic nerve is stimulated before and after the administration of the compound, and the changes in the contraction of the nictitating membrane are noted. Furthermore, the response of the nictitating membrane to exogenous adrenaline is registered.

Evaluation

The decrease of the response after drug application is expressed as percentage of the control

before drug. Ganglionic blockers decrease the response to preganglionic stimulation but have no influence on postganglionic stimulation or exogenous adrenaline. Neuronal blockers decrease the response to both preganglionic and postganglionic stimulation but do not affect the response to exogenous adrenaline which may even be enhanced. α-receptor blockers decrease the response to both preganglionic and postganglionic stimulation as well as decrease the effect of exogenous adrenaline. Catecholamine uptake inhibitors increase the response to both preganglionic and postganglionic stimulation as well as enhance the response to exogenous adrenaline.

Critical Assessment of the Method

The nictitating membrane preparation has been widely used for differentiation of cardiovascular effects. Since the use of higher animals such as cats has been limited to a great extent, this model is now being used only exceptionally.

As alternative, the contraction of the inferior eyelid of anesthetized **rats** after preganglionic electrical stimulation of the superior cervical ganglion has been recommended (Gertner 1956; Steinbrecher and Schmid-Wand 1986). In the modification used by Steinbrecher and Schmid-Wand (1986), the method is suitable for testing compounds with potential adrenergic and antiadrenergic activity but not for testing ganglion-blocking activities.

Male Sprague–Dawley rats are anesthetized with 100 mg/kg thiobutabarbital i.p. and kept on a heated operation table at a rectal temperature of 37 °C. One femoral vein is cannulated and filled with 4 % heparin solution. One femoral artery is cannulated for registration of blood pressure. Tracheotomy is performed and a polyethylene catheter of 5 cm length inserted. The head of the animal is fixed carefully. The vibrissae at the lower eyelid on the right side are cut, a thread attached at the margin of this eyelid and attached to a strain gauge. To immobilize the musculature of the face, the mouth of the animal is sutured and the head support attached. The right sympathetic nerve is exposed, separated from the vagus nerve, and prepared in order to place electrodes for preganglionic stimulation. For calibration, stimulation is performed twice with an interval until contraction is back to baseline. Furthermore, a dose of 0.001 mg/kg adrenaline is given as bolus injection. Eyelid contraction and blood pressure increase are recorded. Then the putative adrenergic blocker or the standard 1.0 mg/mg phentolamine is injected intravenously. Eyelid contraction after electrical stimulation or after adrenaline is reduced dose dependently.

Modifications of the Method

Quilliam and Shand (1964) assessed the selectivity of drugs by comparing the effects on ganglionic transmission and on the pre- and postganglionic nerves in the isolated superior cervical ganglion preparation of the rat.

Langer and Trendelenburg (1969) performed experiments with normal nictitating membranes of pithed cats as well as with isolated normal nictitating membranes.

Koss and Hey (1992) used frequency-dependent nictitating membrane responses by sympathetic nerve stimulation in anesthetized cats to determine the potential role of prejunctional histamine H_3 receptors.

Gurtu et al. (1992) used contractions of the cat nictitating membrane to explore the effects of calcium channel blockers on neurotransmission in vivo, by comparing the effects of verapamil and nifedipine on contractions of nictitating membrane following either electrical stimulation of the superior cervical ganglion or intravenous injection of phenylephrine.

Koss (1992) compared the peripheral and central nervous system sympatholytic actions of prazosin using the cat nictitating membrane. Submaximal contractions of the nictitating membranes were evoked by electrical stimulation of the preganglionic cervical sympathetic nerve

trunk and by stimulation of the posterior hypothalamus in anesthetized cats.

Badio et al. (1996) evaluated spiropyrrolizidines, a new structural class of blockers of nicotinic receptor channels with selectivity for ganglionic-type receptors in rat pheochromocytoma PC12 cells (with an $\alpha_3\beta_{4(5)}$-nicotinic receptor) and human medulloblastoma TE671 cells (with an $\alpha_1\beta_1\gamma\delta$-nicotinic receptor).

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Assessment of Ganglion-Blocking Activity in the Isolated Bovine Retractor Penis Muscle

Purpose and Rationale

The use of the bovine retractor penis muscle for the assessment of ganglion-blocking activity of neuromuscular blocking drugs has been recommended by Alaranta et al. (1990) and Klinge et al. (1993). Klinge and Sjöstrand (1974) performed not only extensive studies on the physiology and pharmacology of the retractor penis in the bull but also discussed the various hypotheses on inhibitory and excitatory innervation of this muscle, which is present in many vertebrates such as horses, cats, dogs, and rats, but not in men and rabbits. They also found that the effects on the isolated retractor penis muscle and on penile arteries are rather similar. The excitatory innervation was found to be predominantly α-adrenergic (Klinge et al. 1970; Klinge and Sjöstrand 1977), whereas other transmitters such as histamine and bradykinin were effective only in some species. Relaxation of the isolated retractor penis muscle could be elicited by nicotine and other nicotinic agonists (Klinge et al. 1988). In the studies on ganglion-blocking activity, strips of the retractor penis muscle are precontracted by 5-hydroxytryptamine. Relaxation induced by nicotine is antagonized by ganglion blockers.

Procedure

Retractor penis muscles are obtained from bulls of different breeds weighing 250–500 kg. Samples are dissected 10–30 cm distal to the points where the paired muscle bundles pass the anal orifice. Immediately after slaughter, the samples are freed from fat and other surrounding tissue and placed into Tyrode solution at 2–4 °C. Strips, 15–25 mm in length and 2–3 mm wide, are prepared and mounted in 20-ml organ baths containing Tyrode solution at 35 °C aerated with 95 % O_2 and 5 % CO_2. An equilibrium time of 2–4 h is allowed. During the equilibrium period, washing is performed at about 60-min intervals. Changes in tension are recorded by means of Grass FT03 force displacement transducers coupled to a polygraph.

A high-enough tone for studying the nicotine-induced relaxation, usually 8–15 g, is generated by adding 5-HP in a concentration between 0.1 and 6 μM to the organ bath. Washing is performed 2 min after application of nicotine; 60–80 min later, the tone is again raised and the application of nicotine is repeated. The effect of a neuromuscular blocking drug is studied only if the relaxations caused by nicotine in two consecutive controls are equal in size.

Evaluation

The blocking activity of a certain concentration of a drug is expressed as % reduction in the relaxation of the muscle strip, according to the following equation:

$$\frac{A - B}{A} \times 100$$

where A is the size of the control relaxation in millimeters and B is the size of the relaxation of the blocking drug. In order to construct regression lines, the activity of four or five dose levels from the assumed linear part of the concentration–effect curve is studied. The activity of each dose level is studied in at least five strips obtained from different animals. IC_{50} values are calculated from the regression lines. The parallelism of the regression lines is tested by covariance analysis.

Critical Assessment of the Method

Molar potency ratios of known ganglion-blocking agents obtained with this method were

compared with the results of other methods, such as inhibition of contraction of cat nictitating membrane evoked by preganglionic sympathetic stimulation (Bowman and Webb 1972; see section "Cat Nictitating Membrane Preparation" in this chapter), inhibition of nicotine-induced contraction of the isolated guinea pig ileum (Feldberg 1951), inhibition of contraction of guinea pig vas deferens evoked by preganglionic stimulation of the hypogastric nerve in vitro (Birmingham and Hussain 1980), depression of postganglionic action potentials evoked by preganglionic stimulation of the superior cervical ganglion of the rat in vitro (Quilliam and Shand 1964), and induction of mydriasis in mouse by blocking the ciliary ganglion (Edge 1953). A fair but not a complete agreement between the results obtained with various methods was found.

Modifications of the Method

Gillespie and Sheng (1990) studied the effects of pyrogallol and hydroquinone on the response to nonadrenergic, non-cholinergic nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles.

Parkkisenniemi and Klinge (1996) used samples of retractor penis muscles and penile arteries from bulls for functional characterization of endothelin receptors.

La et al. (1997) studied the inhibition of nitrergic nerve-induced relaxations in rat anococcygeus and bovine retractor penis muscles by hydroxycobalamin.

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Angiotensin II Antagonism

Purpose and Rationale

Angiotensin II antagonists can be tested in rats after elimination of cardiovascular reflexes by vagotomy and ganglionic blockade. Several angiotensin II antagonists possess intrinsic agonistic activity. This can be tested by injection of various doses to the vagotomized, ganglion-blocked animal. The antagonistic activity of the angiotensin II antagonist can be evaluated by antagonism against graded doses of angiotensin II. The duration of activity can be tested during continuous infusion of angiotensin II.

Procedure

Male Sprague–Dawley rats weighing about 300 g are used. They are anesthetized with 60 mg/kg sodium pentobarbital i.v. One carotid artery is cannulated and connected with a Statham P23 DB transducer. Blood pressure is recorded on a polygraph. Both jugular veins are cannulated for application of test compounds and for infusion. Both vagal nerves are cut 3 mm dorsal of the larynx. For ganglionic blockade, 10 mg/kg pentolinium tartrate is injected intravenously. At least five animals are used for evaluation of one test drug.

Intrinsic Agonistic Activity

After the blood pressure has reached a constant value, doses of 1, 2, 4, and 16 μg/kg of the test compound are injected via the jugular vein. Blood pressure is recorded.

Antagonistic Activity

In 10-min intervals, doses of 0.5, 1.0, and 2.0 μg/kg angiotensin II are injected to establish dose–response curves. After 10 min, continuous infusion is started of the potential angiotensin II blocker in a dosage of 10 μg/kg/0.1 ml/min. Ten minutes after beginning of the infusion, again doses of 0.5, 1.0, and 2.0 μg/kg angiotensin II are injected.

Duration of Activity

In this setup, angiotensin II is administered as continuous infusion at a dosage of 1 μg/kg/0.02 ml/min. When blood pressure has reached an elevated steady-state level, 0.1 mg/kg of the angiotensin II antagonist is administered.

Intensity and duration of the fall of blood pressure are recorded.

Evaluation

Intrinsic Agonistic Activity

An increase of blood pressure indicates the intrinsic agonistic activity.

Antagonistic Activity

Increases of blood pressure after graduated doses of angiotensin II during the infusion is expressed as percentage of the increase before infusion. The results are compared with known angiotensin II antagonists.

Critical Assessment of the Method

In this test, not only the potency and duration of activity but also the intrinsic agonistic activity of an angiotensin II antagonist can be tested.

Modifications of the Method

Various other pharmacological models have been used to test angiotensin II antagonists:

Blood pressure in conscious unrestrained rats with chronically implanted catheters with normal

blood pressure, spontaneous hypertension, and chronic renal hypertension (Vogel et al. 1976; Chiu et al. 1989; Brooks et al. 1992; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Nagura et al. 1995; Nozawa et al. 1997; Renzetti et al. 1995; Wong et al. 1995; Junggren et al. 1996)

Blood pressure in conscious spontaneously hypertensive and in anesthetized ganglion-blocked rats (Olins et al. 1993)

Blood pressure in pithed and in conscious renovascular hypertensive rats (Criscione et al. 1993; Wienen et al. 1993; Deprez et al. 1995; Kivlighn et al. 1995a; Kushida et al. 1995)

Blood pressure in rats after intracerebroventricularly injected angiotensin II (Vogel et al. 1976; Batt et al. 1988)

Blood pressure in conscious angiotensin I-infused and renin-dependent **hypertensive dogs** (Brooks et al. 1992; Cazaubon et al. 1993; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Keiser et al. 1995; Wong et al. 1995)

Blood pressure and heart rate in conscious sodium-depleted and sodium-repleted **cynomolgus monkeys** (Lacour et al. 1993; Cazaubon et al. 1993; Keiser et al. 1995)

Angiotensin II-induced pressor responses in **marmosets** (Nagura et al. 1995)

Blood pressure and heart rate in conscious **rhesus monkeys** and anesthetized **chimpanzees** (Gabel et al. 1995; Kivlighn et al. 1995b; Kivlighn et al. 1995c)

Inhibition of angiotensin II-induced contraction in isolated **aorta** rings or strips from **rabbits** (Chiu et al. 1989; Chui et al. 1990; Criscione et al. 1993; Cazaubon et al. 1993; Olins et al. 1993; Wienen et al. 1993; Aiyar et al. 1995; Caussade et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Kushida et al. 1995; Nagura et al. 1995; Renzetti et al. 1995; Wong et al. 1995), from **rats** (Nozawa et al. 1997), from **neonatal rats** (Keiser et al. 1993), and from **guinea pigs** (Mizuno et al. 1995)

Inhibition of angiotensin II-induced contraction in isolated rat pulmonary artery (Chang et al. 1995)

Antagonism against angiotensin II in isolated strips of rabbit aorta, rabbit jugular vein, rabbit pulmonary artery, rat portal vein, rat stomach, rat urinary bladder, human urinary bladder, human colon, and human ileum (Rhaleb et al. 1991)

Contractions of **guinea pig ileum** in situ (Khairallah and Page 1961)

Antagonism against angiotensin II in the **isolated rat uterus** (Wahhab et al. 1993)

Contractile force and prostaglandin E synthesis in electrically stimulated **rabbit isolated vas deferens** (Trachte et al. 1990)

Antagonism against angiotensin II-induced aldosterone release in **bovine adrenal glomerulosa cells** (Criscione et al. 1993) and in rat dispersed adrenal capsular cells (Chang et al. 1995)

Antagonism against angiotensin II-induced inhibition of guanylate cyclase activity in the **rat pheochromocytoma cell line** PC12W (Brechler et al. 1993)

Brooks et al. (1995) compared the cardiovascular and renal effects of an angiotensin II receptor antagonist and captopril in **rats with chronic renal failure** induced by 5/6 nephrectomy. Under sodium pentobarbital anesthesia, the right kidney was removed and approximately two thirds of the left kidney was infarcted by ligating two or three branches of the left renal artery.

Kim et al. (1997) studied the effects of an angiotensin AT1 receptor antagonist on volume overload-induced cardiac gene expression in rats. An abdominal aorta-caval shunt was prepared in 9-week-old male Wistar rats under sodium pentobarbital anesthesia. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two thirds caudal to the renal artery and one third cephalic to the aortic bifurcation with a 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating the vena cava. After the aorta was clamped, the needle was withdrawn, and a drop of cyanoacrylate glue was used to seal the aortic puncture point. The patency of the shunt was verified visually by swelling of the vena cava and mixing of arterial and venous blood. The rats were treated either with vehicle

or the angiotensin antagonist. Four days after the preparation of the AC shunt, 24-h urine volume, electrolytes, and aldosterone were measured. Six days after the AC shunt, blood was collected by puncture of a tail vein and plasma renin activity and aldosterone were measured. Seven days after AC shunt, hemodynamic studies were performed in pentobarbital anesthesia. Afterwards, the heart was rapidly excised and the left and right atria and ventricles were separated and frozen in liquid nitrogen for the extraction and measurement of cardiac tissue RNA.

Shibasaki et al. (1997) tested the effect on the renin–angiotensin–aldosterone system in **conscious rats** after cannulation of the abdominal aorta under anesthesia 3–4 days before the experiment. After oral dosing of the angiotensin II receptor antagonist, blood samples were withdrawn and plasma renin and aldosterone determined by radioimmunoassay.

Similar to the effects of ACE inhibitors, lifespan of hypertensive rats could be doubled by long-term treatment with an angiotensin II type 1 receptor blocker (Linz et al. 2000).

Ledingham and Laverty (1996) treated **genetically hypertensive New Zealand rats** with a specific AT_1 receptor antagonist via osmotic minipumps for several weeks and measured the effects on blood pressure, cardiac hypertrophy, and the structure of resistance arteries.

Transgenic animals were recommended for further studies to influence the human renin–angiotensin system (Müller et al. 1995; Wagner et al. 1996; Bohlender et al. 1996).

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ACE Inhibition Measured In Vivo in the Rat

Purpose and Rationale

The angiotensin-converting enzyme (ACE) is responsible for the cleavage of angiotensin I to the active angiotensin II. ACE is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and diminution of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The cardiovascular system is sensitive to both peptides, reacting with an increase of blood pressure to angiotensin II and with a decrease to bradykinin. These reactions can be used for quantitative determination of ACE-inhibiting activity.

Procedure

Male Sprague–Dawley rats weighing 300–400 g are used. The animals are anesthetized by i.p. injection of 70 mg/hg pentobarbital. After intubation of the trachea, they are artificially respirated with 30 strokes/min and a stroke volume of 6–8 ml. The right carotid artery is cannulated and blood pressure registered with a Statham element (P23 DB) and a polygraph. One jugular vein is cannulated for i.v. injections. After laparotomy a catheter is inserted into the duodenum for enteral administration and the wound closed again. Blood pressure is stabilized 30 % below the normal level by i.m. injection of 5 mg/kg pentolinium. In order to prevent excessive mucus production in the bronchial system, 40 μg/kg atropine sulfate is injected intramuscularly.

Inhibition of Angiotensin I Cleavage

After stabilization of blood pressure, 310 ng/kg angiotensin I is injected intravenously in 0.1 ml saline. The injection is repeated in 5-min intervals until an identical pressure reaction occurs. The test compounds are administered at doses of 1 and 10 mg/kg intravenously or 25 mg/kg intraduodenally. Three minutes after i.v. injection or 10 min after i.d. administration, again 310 ng/kg angiotensin I is injected. Standards are ramipril, enalapril, or captopril.

Potentiation of Bradykinin-Induced Vasodepression

A low dose of bradykinin has to be chosen in order to visualize the bradykinin potentiation. One μg/kg, eventually 3 μg/kg bradykinin are injected intravenously at 5-min intervals until a stable reaction is achieved. Three minutes after i.v. injection or 10 min after intraduodenal administration of the test substance, the bradykinin injection is repeated.

Evaluation

Inhibition of Angiotensin I Cleavage

The diminution of the pressure reaction to angiotensin I after administration of a potential ACE inhibitor is the parameter for the activity of the new compound. The inhibition is calculated as percent of controls. Using various doses of the ACE inhibitor, dose–response curves can be established and ID_{50} values be calculated.

Potentiation of Bradykinin-Induced Vasodepression

Potentiation of bradykinin-induced vasodepression is expressed as percentage of controls. Using various doses of the test compound and the standard, dose–response curves can be established and potency ratios calculated.

Critical Assessment of the Method

Both parameters, inhibition of angiotensin I response and potentiation of bradykinin-induced vasodepression, have been proven as reliable parameters for evaluation of ACE inhibitors.

Modifications of the Method

Natoff et al. (1981) used the ratio of responses to angiotensin I and angiotensin II in spontaneously hypertensive rats, either pithed or anesthetized with urethane, to determine the degree and the duration of effect of captopril.

Blood levels of angiotensin II can also be measured by radioimmunoassay.

Several studies in rats showed the beneficial effects of prolonged treatment with ACE inhibitors. Postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction was decreased by ACE inhibition (Linz et al. 1996).

Inhibition of angiotensin I-induced pressure response by administration of ACE inhibitors can be measured not only in anesthetized rats but also in anesthetized dogs, conscious rats, and conscious dogs (Becker et al. 1984).

Lifelong ACE inhibition doubles the life-span of hypertensive rats not only if the treatment is started at the age of 1 month (Linz et al. 1997), but ramipril also increases survival in old spontaneously hypertensive rats if treatment is started at the age of 15 months (Linz et al. 1999).

Panzenbeck et al. (1995) reported that captopril-induced hypotension is inhibited by the bradykinin blocker HOE 140 in Na^+-depleted **marmosets**.

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Evaluation of Renin Inhibitors in Dogs

Purpose and Rationale

Highly specific inhibitors of the enzyme renin are considered to be potential antihypertensive agents. These agents cause a fall in blood pressure of sodium-deficient dogs and decrease plasma renin activity as well as angiotensin II level.

Procedure

Animal Experiment

Adult mongrel dogs (8–14 kg) of either sex are given water ad libitum and maintained on a low sodium diet for 1–2 weeks before the experiment. A single intramuscular injection of 5 mg/kg furosemide is given 48 h before the experiment. On the day of the experiment, the dogs are anesthetized with sodium pentobarbital (30 mg/kg i.v.) and a cuffed endotracheal tube is positioned to allow artificial respiration. To measure arterial blood pressure, a femoral artery is catheterized with polyethylene tubing. The right and left femoral veins are catheterized for drug administration and delivery of a maintenance infusion of sodium pentobarbital (5 mg/kg/h). Blood pressure is measured directly through the catheter, which is connected to a Gould–Statham pressure transducer. Blood samples are collected from the arterial catheter.

Increasing doses of the potential renin inhibitor are infused over 30 min followed by a 30-min recovery period. Immediately after the last recovery period, the dogs are given an i.v. infusion of the angiotensin receptor antagonist saralasin (20 µg/kg/min) for 30 min. For measurement of plasma renin activity and angiotensin II levels, the dogs are infused over a period of 30 min with the test compound and blood is withdrawn at 0, 15, 30, 60, 90, 120, 180, and 240 min after the start of the infusion. After the final blood drawing, 20 µg/kg/min saralasin is infused for 30 min.

Analytical Procedures

The antibody-trapping method is preferred to measure plasma renin activity (PRA). In this procedure, PRA is determined at pH 7.4 by RIA quantification of angiotensin I (ANG I) generated and then trapped by excess anti-ANG I antibody (Poulsen and Jørgensen 1973; Nussberger et al. 1987). In tubes coated with rabbit anti-ANG I antibody (Gamma Coat™ ^{125}I plasma renin activity RIA kit; Baxter Travenol Diagnostics) and incubated in an ice-water bath, 75 µl plasma is mixed with 7 µl 3 M TRIS base buffer (pH 7.2) containing 200 mM EDTA and 3 µl 0.2 M TRIS base (pH 7.5) containing 3 g/L human serum albumin (fraction V, Sigma). Tubes are vortexed and incubated at 37 °C for 60 min. The incubation is terminated by placing the tubes in an ice-water bath. Next, 75 µl of the TRIS albumin buffer are added, followed by 1 ml phosphate RIA buffer (Gamma Coat™) containing 15,000 cpm of ^{125}I ANG I. Standard ANG I (0.2–50 ng/ml) is also incubated at 37 °C for 60 min with 10 10 µl mu;l TRIS/albumin buffer. In an ice-water bath, low renin plasma (75 µl) is added to the standards before the addition of a 1 ml tracer solution. Samples and standards are incubated for 24 h at 4 °C. Tubes are then aspirated and counted in a gamma counter.

Levels of immunoreactive angiotensin II (ir-ANG II) are measured using a procedure described by Nussberger et al. (1985). Two to three ml of whole blood is collected in prechilled glass tubes containing 125 µl of the following "inhibitor" solution: 2 % ethanol, 25 mM

phenanthroline, 125 mM EDTA, 0.5 mM pepstatin A, 0.1 mM captopril, 2 g/l neomycin sulfate, and 0.1 mM of the renin inhibitor CGP 38560. The tubes are then centrifuged and the plasma quickly frozen in liquid nitrogen and stored at −70 °C. For extraction of angiotensin peptides, Bond Elut cartridges (Bond Elut pH) containing 100 mg phenylsilica are used, along with a Vac Elut SPS 24 vacuum manifold (Analytichem; Harbor City, CA). Each cartridge is preconditioned with 1.0 ml methanol (HPLC grade) followed by 1.0 ml of water (HPLC grade) at a vacuum pressure of 5 mmHg. One milliliter of the thawed sample is then applied to the cartridge and washed with 3 ml HPLC grade water. The angiotensin peptides retained at the columns are eluted with 0.5 ml methanol (HPLC grade, vacuum pressure less than 5 mmHg) into polypropylene tubes coated with a buffer containing 0.2 M TRIS, 0.02 % NaN_3, and 2.5 mg/ml fatty acid-free bovine serum albumin (pH 7.4 with glacial acetic acid). The methanol is evaporated at 40 °C and ir-ANG II measured using an antibody (IgG Corp., Nashville, TN) with greater than 1,000-fold selectivity for ANG II.

Evaluation

All data are expressed as mean ± SEM. The hypotensive responses after various doses of the renin antagonist are compared with the inhibition of plasma renin activity and the decrease of immunoreactive angiotensin II.

Critical Assessment of the Method

The antibody-trapping method, reported here, gives a better correlation with the blood pressure-lowering effect in dogs than the conventional method based on RIA for generated ANG I (Palmer et al. 1993).

Modifications of the Method

Pals et al. (1990) described a rat model for evaluating inhibitors of human renin using anesthetized, nephrectomized, ganglion-blocked rats. The blood pressure rise induced by sustained infusion of renin was dose dependently decreased by a renin inhibitor.

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Evaluation of Renin Inhibitors in Monkeys

Purpose and Rationale

The renin–angiotensin system as the main regulator of blood pressure can be influenced in several ways. One approach involves the inhibition of renin. Renin is an aspartyl protease that hydrolyzes angiotensinogen to release the decapeptide angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting enzyme.

Sequencing of renin and angiotensinogen from various species revealed marked species differences for both the enzyme and the substrate. Inhibitors developed for human renin show a high specificity for primate renin and show only weak inhibition of renin from subprimate species. This means that the most common laboratory animals, such as rats and dogs, are not suitable for the in vivo evaluation of renin inhibitors. The marmoset was chosen by Wood et al. (1985, 1989a) as a primate model.

Procedure

Marmosets (*Callithrix jacchus*) of both sexes weighing between 300 and 400 g are fed a pellet diet supplemented with fruit. Two days prior to the experiment, the animals are anesthetized and catheters are implanted in a femoral artery for measurement of blood pressure and in a lateral tail vein for injection or infusion of test substances. Thirty min before the experiment, the animals receive an intravenous injection of 5 mg/kg furosemide in order to stimulate renin release. During the experiment, the marmosets are sedated with diazepam (0.3 mg/kg i.p.) and kept in restraining boxes. Mean blood pressure is recorded continuously, and heart rate is measured at fixed intervals. The test compound or the standard are injected at various doses by intravenous infusion or administered orally.

Evaluation

Blood pressure is recorded after 30 min of intravenous infusion and 30 min after stopping the infusion. Comparing the changes from pretreatment values after various doses, dose–response curves can be established.

Modifications of the Method

Fischli et al. (1991) monitored arterial pressure in conscious and chronically instrumented monkeys using a telemetry system. One week before the experiment, the animals were anesthetized, and a 3 F high-fidelity pressure-tip transducer (Millar Instruments, Inc.) was inserted into the abdominal aorta through the right femoral artery. Then the catheter was tunneled subcutaneously to the back of the monkey in the interscapular region. The proximal part of the catheter was connected to a transmitter located in a jacket worn by the monkey. The blood pressure was transmitted continuously to a receiver, which transformed the signal to an analogue value of blood pressure.

Linz et al. (1994) reported on the effects of renin inhibitors in anesthetized rhesus monkeys weighing between 5 and 13 kg. The animals are sodium depleted by administration of 10 mg/kg/day furosemide-Na for 6 consecutive days. At day 7, 10 mg/kg furosemide is given i.v. 30 min before the start of the experiment. Anesthesia is induced with 20 mg/kg ketamine hydrochloride i.m. and continued with 40 mg/kg pentobarbitone-Na, slow i.v. drip. After completion of surgical procedures and after insertion of catheters under fluoroscopic control, the following hemodynamic parameters are measured: Pulse rate and systolic and diastolic blood pressures are registered with a transducer (Statham P23 ID) in one femoral artery. A catheter tip manometer (Millar Instruments, Houston, Texas, USA) is introduced into the left ventricular cavity for the determination of left ventricular pressure. Contractility is electronically deduced from left ventricular pressure with appropriate amplifiers (Hellige GmbH, Freiburg, Germany). The electrocardiogram (ECG) from conventional lead II is taken using an ECG transducer (Hellige GmbH). Heart rate is measured from QRS peaks using a biotachometer (Hellige GmbH). Cardiac output is determined using the thermodilution method. Thermodilution is integrated and converted to cardiac output readings by commercially available equipment (HMV7905, Hoyer, Bremen). To determine cardiac output, 2 ml chilled 0–5 °C isotonic glucose solution (5 %) is injected rapidly into the right ventricle by a catheter via the right jugular vein. A thermistor is placed into the aortic arch via the right carotid artery.

Hemodynamics are monitored for 30 min following i.v. injection of various doses of the potential renin inhibitor. At the end of the experiments, the ACE inhibitor ramiprilat 100 µg/kg is given i.v. to probe for an additional blood pressure-lowering effect. Blood samples for the determination of ANG II concentration, renin inhibition, and plasma drug levels are withdrawn at 10, 30, and 60 min after i.v. injection of the renin inhibitor. The volume is replaced by i.v. injections of isotonic glucose solution (5 %). After all data and blood samples have been obtained, animals are sacrificed by an overdose of pentobarbitone-Na.

For experiments after intraduodenal administration, sodium depletion and anesthesia are done as described above. A small side branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure measurements using a pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Blood samples are withdrawn via a catheter placed into the saphenous vein. A gastric fiberscope (Olympus XP 10) is introduced into the duodenum under visual control, and the renin inhibitor is administered intraduodenally through the service channel of the fiberscope in a volume of 5 ml. Blood samples are withdrawn before and at 15, 30, 45, 60, 90, and 120 min after intraduodenal administration.

Wood et al. (2005) tested an orally effective renin inhibitor (aliskiren) in marmosets. Blood pressure and heart rate were measured by telemetry in conscious animals moving freely in their home cages. Pressure transmitters (AM Unit, model TA11PA-C40, Data Sciences, USA) were implanted into the peritoneal cavity under aseptic conditions and light anesthesia. The sensor catheter was placed in the aorta below the renal artery pointing upstream.

Critical Assessment of the Method

Due to the high species specificity of renin and its substrate, angiotensinogen, renin inhibitors for treatment of hypertension have to be tested in primate models. The marmoset as well as the rhesus monkey have been proven to be suitable models.

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Penile Erection in Rabbits

Purpose and Rationale

The discovery of inhibitors of phosphodiesterase as effective drugs for patients with erectile dysfunction (Klotz et al. 2001; Porst et al. 2001) has stimulated the use of appropriate in vivo animal models. In particular, rabbits have been recommended as models for impotence research (Bischoff and Schneider 2000, 2001; Bischoff 2001; Bischoff et al. 2001; Saenz de Tejada et al. 2001) confirming earlier work in this area (Thielen et al. 1969; Sjöstrand and Klinge 1979; Naganuma et al. 1993; Lin and Lin 1996).

Procedure

Adult male Chinchilla rabbits weighing 3.5–4.5 kg are housed in individual cages for at least 1 week after arrival, at room temperature with water and food ad libitum.

For the study, an indwelling catheter filled with saline is inserted into a marginal ear vein and taped in position. The drugs are injected into the ear vein, followed by a small volume of saline. The time is noted and at appropriate times the animal gently removed from the cage and held by one research worker. The rabbit penis is not visible when it is not erect (Naganuma et al. 1993). However, when erection occurs, it is possible to examine the pudendal area and measure the length of the uncovered penile mucosa with sliding calipers.

Evaluation

Penile erection is evaluated by measuring the length to the nearest millimeter of the uncovered penile mucosa with a sliding caliper at 5, 10, 15, 30, 50, 60, 90, and 120 min after administration of the test compounds and continued hourly for up to 5 h. Mean values are calculated and results expressed as means ± SEM. The area under the curve is calculated by an integration program.

Modifications of the Method

Choi et al. (2002) compared the efficacy of vardenafil and sildenafil in facilitating penile erection in **anesthetized rabbits**. Penile erections were elicited by submaximal pelvic nerve stimulation every 5 min for 30 min. Response was assessed by continuously recording intracavernosal pressure and systemic arterial pressure.

Min et al. (2000) tested the augmentation of pelvic nerve-mediated sexual arousal in anesthetized **female rabbits** by sildenafil. The following parameters were measured before, during, and after pelvic nerve stimulation at 4, 16, and 32 Hz: (1) hemoglobin concentration and oxygen saturation in female genital (vaginal, labial, clitoral) tissues by laser oximetry, (2) clitoral blood flow by laser Doppler flowmetry, (3) vaginal luminal pressure by a balloon catheter pressure transducer, and (4) vaginal lubrication by tampon.

Carter et al. (1998) tested the effect of the selective phosphodiesterase type 5 inhibitor sildenafil on erectile dysfunction in **pentobarbital-anesthetized dogs**. Increases in intracavernosal pressure in the corpus cavernosum and penile blood flow were induced by pelvic nerve stimulation over a frequency range of 1–16 Hz. The effects of increasing doses of sildenafil on electric-stimulated intracavernosal pressure, penile blood flow, blood pressure, and heart rate were evaluated.

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Methods to Induce Experimental Hypertension

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Acute Renal Hypertension in Rats

Purpose and Rationale

Since the classical experiments of Goldblatt et al. (1934), there is clear evidence that the ischemia of the kidneys causes elevation of blood pressure by activation of the renin–angiotensin system. The principle can be used both for acute and chronic hypertension. In rats acute renal hypertension is induced by clamping the left renal artery for 4 h. After reopening of the vessel, accumulated renin is released into circulation. The protease renin catalyzes the first and rate-limiting step in the formation of angiotensin II leading to acute hypertension. The test is used to evaluate antihypertensive activities of drugs.

Procedure

Male Sprague Dawley rats weighing 300 g are used. The animals are anesthetized by intraperitoneal injection of 100 mg/kg hexobarbital sodium. A PVC-coated Dieffenbach clip is placed onto the left hilum of the kidney and fixed to the back muscles. The renal artery is occluded for 3.5–4 h.

3.5 h following the surgery, the animals are anesthetized by intraperitoneal injection of 30–40 mg/kg pentobarbital sodium. The trachea is cannulated to facilitate spontaneous respiration. To measure systolic and diastolic blood pressure, the cannula in the carotid artery is connected to a pressure transducer (Statham P 23 Db).

For administration of the test compound, a jugular vein is cannulated.

Following a stable blood pressure state, ganglionic blockade is performed with pentolinium (10 mg/kg i.v.). After obtaining stable reduced blood pressure values, the renal arterial clip is removed. This leads to a rise in blood pressure as a consequence of elevated plasma renin level. Within 15 min a stable hypertension is achieved (control = 100 %).

The test substance is then administered by intravenous injection at doses of 10 and 100 µg/kg.

Blood pressure is monitored continuously until a renewed increase to the starting level is obtained.

Ten to 12 animals are used per compound.

Evaluation

Increase in blood pressure after reopening of the renal artery and reduction in blood pressure after administration of the test drug are determined [mmHg]. Percent inhibition of hypertensive blood pressure values under drug treatment are calculated as compared to pretreatment hypertension values. Duration of the effect is determined [min]. Statistical significance is assessed by the paired *t*-test.

Modifications of the Method

A sharp and transient in systemic arterial blood pressure associated with reflex bradycardia can be elicited by injection of 5-hydroxytryptamine, cyanide, nicotine or lobeline into the coronary artery blood stream of dogs (Berthold et al. 1989). The phenomenon is named the cardiogenic hypertensive chemoreflex and 5-HT proved to be the most powerful agent for its initiation (James et al. 1975).

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Chronic Renal Hypertension in Rats

Purpose and Rationale

On the basis of the findings of Goldblatt et al. (1934) that ischemia of the kidneys induces hypertension, various modifications of the technique have been described for several animal species. One of the most effective modifications in rats is the so called 1-kidney-1-clip method.

Procedure

Male Sprague Dawley rats weighing 200–250 g are anesthetized with 50 mg/kg i.p. pentobarbital. The fur on the back is shaved and the skin disinfected. In the left lumbar area, a flank incision is made parallel to the long axis of the rat. The renal pedicel is exposed with the kidney retracted to the abdomen. The renal artery is dissected clean, and a U-shaped silver clip is slipped around it near the aorta. Using a special forceps (Schaffenburg 1959), the size of the clip is adjusted so that the internal gap ranges from 0.25 to 0.38 mm. The right kidney is removed through a flank incision after tying off the renal pedicle. The skin incisions are closed by wound clips.

Four to five weeks after clipping, blood pressure is measured, and rats with values higher than 150 mmHg are selected for the experiments. Blood pressure readings are taken on each of 3 days prior to drug treatment. Drugs are administered orally in volumes of 10 ml/kg. The rats are divided into four animals per dose, and each animal is used as his own control. Compounds are administered for 3 days, and predrug and 2-h postdrug blood pressure readings are taken.

Evaluation

Changes in systolic blood pressure are expressed in mmHg. Activity is determined by comparing treatment blood pressure values with the control blood pressure value (day 1, predrug blood pressure). Comparisons are made using the paired *t*-test for evaluation of statistical significance.

Modifications of the Method

Duan et al. (1996) induced renal hypertension in male Hartley **guinea pigs** by a two-step procedure consisting of ligation of the left caudal renal artery and right nephrectomy. Arterial blood pressure and heart rate were monitored in conscious animals. ACE inhibitors reduced blood pressure in sham-operated and in renal hypertensive guinea pigs, whereas renin inhibitors were effective only in renal hypertensive animals.

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Chronic Renal Hypertension in Dogs

Purpose and Rationale

Production of hypertension by clamping renal arteries has been first described by Goldblatt et al. (1934) in dogs. Later on, the method has been modified, e.g., as the "wrapping" technique (Abrams and Sobin 1947).

Procedure

Dogs weighing 8–12 kg are anesthetized with i.v. injection of 15 mg/kg thiopental. Anesthesia is maintained with a halothane–oxygen mixture. Under aseptic conditions, a midline abdominal incision is made. One kidney is exposed and wrapped in cellophane and then replaced. The contralateral kidney is exposed. The artery, vein, and ureter are ligated, and the kidney is removed. The abdomen is closed by sutures and clips. On the day of surgery and for 3 days following, the dogs are given antibiotics. Body temperature is measured twice daily for 4 days following surgery.

Six weeks following surgery, blood pressure is measured using a tail-cuff method. For recording, the tail-cuff is attached to a polygraph. Only animals with a systolic blood pressure higher than 150 mmHg are considered to be hypertensive and can participate in studies evaluating potential antihypertensive compounds.

For the experiment, blood pressure is recorded either by the indirect tail-cuff method or by direct measurement via an implanted arterial cannula. On day 1, readings are made every 2 h, just before, and 2 and 4 h after oral treatment with the potential antihypertensive compound. Drug administration is repeated for 5 days. On days 3 and 5, blood pressure readings are taken before and 2 and 4 h after treatment. At least three dogs are used per dose and compound.

Evaluation

The starting value is the average of the two readings before application of the drug. Each of the following readings is subtracted from this value and recorded as fall of blood pressure at the various recording times.

Modifications of the Method

Renal hypertension in rats has been achieved by many modifications of the method (Stanton 1971) such as the technique according to Grollman (1944). The kidney is exposed through a lumbar incision, the renal capsule is removed by gentle traction, and a figure-8 ligature is applied being tight enough to deform the kidney but not tight enough to cut the tissue.

Renal hypertension may be induced in the **rat** by encapsulating both kidneys with latex rubber capsules (Abrams and Sobin 1947). Molds are formed from plastic using a rat kidney as a model. The capsules are prepared by dipping the molds in liquid latex allowing them to dry in the air. Three applications of latex are applied before the capsules are toughened by placing them under warm running tap water. The kidney is exposed by lumbar incision, the renal capsule gently removed, and the capsule applied.

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Neurogenic Hypertension in Dogs

Purpose and Rationale

Vasodilator and depressor reflexes, originating in the baroreceptor areas of the carotid sinus and aortic arch, play an important part in the regulation of blood pressure. Stimulation of the afferent buffer fibers exerts an inhibitory influence on the vasomotor center, and their sectioning leads to a persistent rise in blood pressure. In this way, acute neurogenic hypertension can be induced in dogs.

Procedure

Adult dogs of either sex weighing 10–15 kg are anesthetized using 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital, and 60 mg/kg sodium pentobarbital i.v. A femoral vein and artery are cannulated using polyethylene tubing to administer compounds i.v. and record arterial pressure and heart rate, respectively. Left ventricular pressure and dp/dt are recorded via the left common carotid artery (post-deafferentation) using a Millar microtip pressure transducer. P_{max} is recorded by speeding up the chart paper. Cardiac output is determined by introducing a Swan–Ganz catheter into the right heart and pulmonary artery via a jugular vein. Five ml of cold 5 % dextrose is injected into the right atrium, and an Edwards cardiac output computer is used to calculate the cardiac output from the temperature change in the pulmonary artery. All recordings are made with a polygraph.

Both of the carotid arteries are cleared up to the bifurcation of the internal and external carotid arteries. The carotid sinus nerves are isolated, ligated, and sectioned, and a bilateral vagotomy is performed to produce neurogenic hypertension (mean arterial pressure more than 150 mmHg). The dog is allowed to equilibrate for approximately 30 min, and a bolus of the test compound is administered by intravenous injection. Heart rate, arterial pressure, left ventricular pressure, Pmax, and dp/dt are monitored for 90 min. A minimum of three dogs are used for each compound.

Evaluation

Changes of the cardiovascular parameters are expressed as percentage of the values before administration of the drug.

Modifications of the Method

Neurogenic hypertension through baroreceptor denervation has also been described in **rabbits** (Angell-James 1984) and in rats (Krieger 1984).

Critical Assessment of the Method

The neurogenic hypertension is useful for acute experiments. However, it is less useful for chronic experiments since the elevated blood pressure caused by buffer nerve section is more labile than that caused by renal ischemia.

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DOCA–Salt-Induced Hypertension in Rats

Purpose and Rationale

Mineralocorticoid-induced hypertension is thought to be due to the sodium-retaining properties of the steroid causing increases in plasma and extracellular volume. The hypertensive effect is increased by salt loading and unilateral nephrectomy in rats.

Procedure

Male Sprague Dawley rats weighing 250–300 g are anesthetized with ether. Through a flank incision, the left kidney is removed. The rats are injected twice weekly with 20 mg/kg s.c. desoxycorticosterone acetate in olive oil for 4 weeks. Drinking water is replaced with a 1 % NaCl solution. Blood pressure starts to rise after 1 week and reaches systolic values between 160 and 180 mmHg after 4 weeks.

Modifications of the Method

The regimen to induce DOCA–salt hypertension has been modified by many authors (Stanton 1971).

DOCA pellets (Peterfalvi and Jequier 1960; Passmore and Jimenez 1990) or implants in Silastic devices (Ormsbee and Ryan 1973; King and Webb 1988) were used instead of repeated injections.

DOCA–salt hypertension can also be achieved without nephrectomy (Bockman et al. 1992).

Using kininogen-deficient Brown Norway Katholiek (BN-Ka) rats, Majima et al. (1991, 1993) showed suppression of rat desoxycorticosterone–salt hypertension by the kallikrein–kinin system.

Li et al. (1996) examined small artery structure on a wire myograph and quantified endothelin-1 messenger RNA by Northern blot analysis in DOCA–salt hypertensive rats after administration of an ACE inhibitor, a calcium channel antagonist, and a nitric oxide synthase inhibitor.

Ullian (1997) described the **Wistar–Furth rat** as a model of mineralocorticoid resistance. These rats developed two-kidney, one-clip hypertension to the same degree as did Wistar rats and reacted to glucocorticoid treatment with a rapid onset of hypertension but were resistant to the development of DOCA–NaCl hypertension.

Studies in DOCA–salt hypertensive **mice** were reported by Gross et al. (1998, 1999), Honeck et al. (2000), and Peng et al. (2001).

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Fructose-Induced Hypertension in Rats

Purpose and Rationale

Increases in dietary carbohydrate intake can raise blood pressure in experimental animals. The increased intake of either sucrose or glucose was shown to enhance the development of either spontaneous hypertension or salt hypertension in rats (Hall and Hall 1966; Preuss and Preuss 1980; Young and Landsberg 1981). Hwang et al. (1987) first reported that hypertension could be induced in normal rats by feeding a high-fructose diet. Fructose feeding was also found to cause insulin resistance, hyperinsulinemia, and hypertriglyceridemia in normal rats (Zavaroni et al. 1980; Tobey et al. 1982). Dai and McNeill (1995) studied the concentration and duration dependence of fructose-induced hypertension in rats.

Procedure

Groups of 8 male Wistar rats weighing 210–250 g are used. They are housed two per cage on a 12-h light/12-h dark cycle and are allowed free access to standard laboratory diet (Purina rat chow) and drinking fluid. Drinking fluid consists either of tap water or 10 % fructose solution. Body weight, food intake, and fluid intake of each rat are measured every week during treatment. Using the tail-cuff method, systolic blood pressure and pulse rate are measured before and every week during treatment. Blood samples are collected before and every second week during treatment for determination of plasma glucose, insulin, and triglycerides.

Evaluation

Since maximum effects on the chosen parameters are achieved after 6 weeks, the duration of treatment can be limited to this time. Statistical analysis is performed using a one-way or two-way analysis of variance, followed by the Newman–Keuls test.

Modifications of the Method

Reaven et al. (1988, 1989) found an attenuation of fructose-induced hypertension by exercise training and an inhibition by somatostatin treatment.

Brands et al. (1991, 1992) found an increase of arterial pressure during chronic hyperinsulinemia in conscious rats.

Hall et al. (1995) reported the effects of 6 weeks of a high-fat diet on cardiovascular, renal, and endocrine functions in chronically instrumented conscious **dogs**. Body weight increased by approximately 16.9 kg, whereas MAP, cardiac output, and heart rate increased by 28 %, 77 %, and 68 %, respectively.

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Genetic Hypertension in Rats

Survey

Rats with inherited hypertension have been described by Smik and Hall (1958), Phelan and Smirk (1960), Laverty and Smirk (1961), and Phelan (1968) as **genetically hypertensive (GH) rats** (Simpson and Phelan 1984).

Okamoto and Aoki (1963) and Okamoto et al. (1966) reported the development of a strain of spontaneously hypertensive rats from mating one Wistar male rat with spontaneously occurring high blood pressure with a female with slightly elevated blood pressure. By inbreeding over several generations, a high incidence of hypertension with blood pressure values of 200 mmHg or more was achieved. These strains were called "spontaneously hypertensive rats (Okamoto–Aoki)" (SHR) or "Wistar–Kyoto rats" (WKY). Hypertension in these rats is clearly hereditary and genetically determined, thus comparable to primary hypertension in humans. Cardiac hypertrophy (Sen et al. 1974) and cellular ionic transport abnormalities have been observed (Yamori 1984a).

Inbred strains being **salt hypertension sensitive** and **salt hypertension resistant (RD)** have been developed by Dahl et al. (1962a, 1963), Rapp (1984), and Cicila et al. (1993). Inoko et al. (1994) reported the transition from compensatory hypertrophy to dilated, failing left ventricles in Dahl salt-sensitive rats.

Two strains of rats with inbred dissimilar sensitivity to DOCA–salt hypertension ("Sabra strain") have been separated by Ben-Ishay (1984) and Ben-Ishay et al. (1972).

Another hypertensive strain derived from Wistar rats was produced by brother–sister mating in the group of Bianchi et al. (1974, 1986) at the University of Milan called "Milan hypertensive strain" (MHS). These rats show a cell membrane defect resulting in abnormal kidney function. Salvati et al. (1990) studied the diuretic effect of bumetanide in isolated perfused kidneys of Milan hypertensive rats.

Furthermore, the "Lyon" strains of hypertensive, normotensive, and low-blood-pressure rats were developed (Dupont et al. 1973; Vincent et al. 1984; Dubay et al. 1993). These rats show a genetically determined defect in central nervous function.

Spontaneously hypertensive rats which develop failure before 18 months have been selectively bred. Several substrains of spontaneous hypertensive rats were separated by the group of Okamoto et al. (1974) including the **stroke-prone strain SHR = SHRSP**. These rats have an increased sympathetic tone and show a high incidence of hemorrhagic lesions of the brain with motor disturbances followed by death (Yamori 1984b; Feron et al. 1996).

A strain of obese spontaneously hypertensive rats has been described by Koletsky (1975), Ernsberger et al. (1993).

With new techniques of genetic engineering, **transgenic rats with hypertension** could be created. Increase of blood pressure of spontaneously hypertensive rat is determined by multiple genetic loci (Deng and Rapp 1992; Dubay et al. 1993). With new technology, not only these loci could be defined but also new models in hypertension research and models to detect antihypertensive drugs could be established (Bohlener et al. 1997; Pinto et al. 1998).

Mullins et al. (1990) reported fulminant hypertension in transgenic rats harboring the mouse Re-2 gene.

A **rat strain TGR(mREN2)27** as a monogenetic model in hypertension research was described by Peters et al. (1993), Lee et al. (1996), Langheinrich et al. (1996), and Ohta et al. (1996).

Bohlender et al. (1996) reconstructed the human renin–angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using ALZET® pumps.

Zolk et al. (1998) described the effects of quinapril, losartan, and hydralazine on cardiac hypertrophy and β-adrenergic neuroeffector mechanisms in transgenic TGR(mREN2)27 rats.

Critical Assessment of the Method

The use of spontaneously hypertensive rats to detect potential antihypertensive compounds is well established. On the basis of available data, no preference can be given to a particular strain. The most abundant experience has been gained with the Wistar–Kyoto strain. Transgenic rats with well-defined genomes are gaining more importance.

Modifications of the Method

Pijl et al. (1994) described streptozotocin-induced diabetes mellitus in spontaneously hypertensive rats as a pathophysiological model for the combined effects of hypertension and diabetes.

Rosenthal et al. (1997) used rats of the Cohen–Rosenthal diabetic hypertensive strain to examine the effects of an ACE inhibitor, an ATII antagonist, and a calcium antagonist on systolic pressure and spontaneous blood glucose levels.

Holycross et al. (1997) used hypertensive SHHF/Mcc-facp rats to study plasma renin activity during development of heart failure.

Linz et al. (1997) compared the outcome of lifelong treatment with the ACE inhibitor ramipril in young prehypertensive stroke-prone spontaneously hypertensive rats and age-matched normotensive Wistar–Kyoto rats. Lifelong ACE inhibition doubled the lifespan in hypertensive rats matching that of normotensive rats.

Studies in **genetically hypertensive mice** were reported by Rosenberg et al. (1985), Hamet et al. (1990), and Meneton et al. (2000). Ohkubo et al. (1990) generated transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes.

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Hypertension Induced by Chronic Angiotensin II Infusion

Purpose and Rationale

Angiotensin II-induced hypertension is a commonly studied model of experimental hypertension, particularly in rodents (King et al. 2007; Kuroki et al 2012 and Zimmerman et al. 2004). Ang II is administered subcutaneously for 2 weeks at a dose ranging between 100 and 200 ng kg^{-1} min^{-1} for rats (Campbell 2013). Chronic Ang II hypertensive response is characterized by a gradual rise in pressure (Abraham and Simon 1994; Brown et al. 1981; Csiky and Simon 1997; Simon et al. 1995). Simon et al. (1998) showed that Ang II-induced hypertension is dose- and time-dependent and synergistically enhanced by dietary sodium supplementation.

Ang II is administered chronically via subcutaneously implanted ALZET® osmotic minipump (DURECT, Cupertino, CA), iPRECIO® implantable pump (SMP-200, Primetech, Tokyo, Japan), or infusion pumps attached to the infusion catheter (Kuroki et al. 2014). Chronic Ang II administration by the s.c. route showed a slowly progressive increase in blood pressure. 10- to 100-fold higher doses of s.c. Ang II are required to achieve similar blood pressure elevation and increases in circulating Ang II levels as those seen following chronic i.v. (Campbell 2013).

Procedure

Male Sprague Dawley rats (400–450 g) on normal sodium (0.7 % NaCl) diet are administered Ang II (100 and 200 ng/kg/min) or vehicle (saline) for 12 weeks.

Pump Preparation: Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; A9525, Sigma-Aldrich) is dissolved in physiological saline. Acetic acid (final concentration of 0.01 N) is added to maintain the stability of Ang II. Previous studies demonstrated that this volume of acetic acid (0.01 N) has no detectable metabolic or hemodynamic effects (Simon 1992; Simon and Altman 1992). The vehicle is saline with 0.01-N acetic acid. ALZET® model 2 ML4 (28 day, DURECT Corporation, Cupertino, CA, USA) minipumps are filled with vehicle or Ang II solution using a syringe and filling tube. Flow moderator is inserted into the filled ALZET® pump until the cap or flange is flushed with the top of the pump. The filled pumps are primed in sterile saline at 37 °C overnight.

Surgical Procedure: Rats are anesthetized with isoflurane, and the back, between and slightly posterior to the scapulae, is shaved. The area is disinfected with 70 % ethanol and iodine twice. A mid-scapular incision is made and a hemostat is inserted into the incision and, by opening and closing the jaws of the hemostat, spread the subcutaneous tissue caudally to create a pocket for the pump. Filled pump is inserted into the pocket,

delivery portal first. This minimizes interaction between the compound delivered and the healing of the incision. The incision is closed with wound clips (9 mm – two clips will normally suffice). Wound clips are removed 7–10 days post procedure. Minipumps are surgically removed at weeks 4 and 8, and new ones are implanted. The rate of delivery of the 4-week minipump is 2.5 μL/h. The rats have free access to tap water and received normal (0.7 % NaCl) diet during the 12 weeks.

Tail Systolic BP Measurements: Systolic BP is measured in restrained awake rats by the tail-cuff method between 8 and 11 AM. Measurements are made on at least two occasions before the insertion of the first minipump to rule out spontaneous hypertension (SBP >130 mmHg). Rats whose SBP exceeded 130 mmHg are rejected. After insertion of the first minipump, the SBP of rats was measured weekly for 4 weeks and then every 2 weeks for the rest of the experiment. The BP load that rats were exposed to during the experiments was calculated as the area under the SBP curve. The area was calculated by subdividing the BP curve of each rat into weekly or biweekly trapezoids, by computing the area of each trapezoid, and, finally, by adding up the calculated areas. The rats were weighed to the nearest 1 g on the day of the final experiments.

Blood Collection and Separation of Plasma: At the end of the experimental procedure, whole blood (0.35 ml) is collected from a jugular venous catheter over a period of 30–60 s into a syringe containing 15 μl of an inhibitor cocktail: EDTA (125 mM, E4884, Sigma-Aldrich), pepstatin A (20 mg/ml in methanol, 77170, Sigma-Aldrich), 1,10-phenanthroline (8 mg/ml, 131377, Sigma-Aldrich), enalaprilat (80 mg/ml in 50 % ethanol, E9658, Sigma-Aldrich), 4-amidinophenylmethanesulfonyl fluoride hydro-chloride (800 mg/ml, A6664, Sigma-Aldrich), and 2-mercaptoethanol (2 %, M6250, Sigma-Aldrich) (Ledwith et al. 1993). The inhibitor cocktail is prepared fresh before each sample collection from previously prepared stock solutions. Blood is chilled on ice immediately after collection until centrifuged at 2,000 g for 10 min. Plasma is collected and recentrifuged at 16,000 g for an additional 10 min and stored at −80 °C until assayed for ANG II.

ANG II Assay: Plasma is thawed, acidified with 1 % trifluoroacetic acid (TFA; 302031, Sigma-Aldrich), and centrifuged at 16,000 g for 10 min. It is then purified through a C18 column (Y1000 SEP-COLUMNS, Peninsula Laboratories, San Carlos, CA), washed with 1 % TFA, and eluted with a 60 % solution of acetonitrile (34998, Sigma-Aldrich) containing 1 % TFA. Samples are dried in a speed vac centrifuge (heated to 50 °C during the initial 4 h of a 5-h run cycle) and resuspended in 200-μl EIA buffer (from the EIA kit). Sample pH is adjusted to pH 6–7 by adding microliter amounts of 1-M sodium phosphate buffer and NaOH. Reconstituted samples are further diluted by 15–50 % with addition of EIA buffer before measurements of ANG II levels using a commercial ELISA kit (ANG II EIA Kit no. 589301, Cayman Chemical, Ann Arbor, MI). All measurements are performed in duplicate. Absorbance at 450 nm is measured using a plate reader. After the reporter (Ellman's reagent) was loaded to each well, absorbance is serially measured at 10-min intervals for a total of 1 h to ensure linearity of the reaction. The absorbance reading from the 30-min time point is used for final analysis.

Evaluation

Results are presented as arithmetical means ± SEM. Data are analyzed by one-way ANOVA followed by multiple comparisons (Holm–Sidak) versus control (0.4 % NaCl). Analyses are performed in SigmaPlot (version 11, Systat Software, Richmond, CA).

Modifications of the Method

Lohmeier and Hildebrandt (1998) showed that cardiac and/or arterial baroreflexes chronically inhibit renal sympathetic nerve activity during ANG II hypertension in conscious dogs.

Davern and Head (2007) evaluated the brain's regions responding to chronic elevated angiotensin II in a rabbit model of Ang II-induced hypertension.

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Hypertension Induced by Chronic NO Synthase Inhibition

Purpose and Rationale

Tonic basal release of nitric oxide (NO) by vascular endothelial cells controls blood pressure in the basal state. Chronic blockade of NO synthesis in the rat produces systemic hypertension and glomerular damage (Baylis et al. 1992). This was recommended by Ribeiro et al. (1992) as a model of hypertension. Yang et al. (1996) found an increase of vascular angiotensin II receptor expression after chronic inhibition of NO synthase in spontaneously hypertensive rats. The detrimental sequels of chronic NO synthase inhibition in rats can be inhibited by treatment with ACE inhibitors (Hropot et al. 1994; Küng et al. 1995). Hsieh et al. (2004) reported that NO inhibition by $_{L}$-NAME accelerates hypertension and induces perivascular inflammation in rats.

Hropot et al. (2003) reported that angiotensin II subtype AT_1 receptor blockade prevents hypertension and renal insufficiency induced by chronic NO synthase inhibition in rats.

Procedure

Male Wistar rats at an age of 7–8 weeks weighing 210 ± 10 g were placed at random in metabolic cages, divided in four to six groups of six to eight rats each. Group 1 (control) had free access to tap water and food. Groups 2–4 were treated with 0.02 % L-NAME water solution for 6 weeks in a daily dose of 25 mg/kg. Groups 3 and 4 received the angiotensin receptor antagonists fonsartan (10 mg/kg) or losartan (30 mg/kg) for 6 weeks daily per stomach tube. Groups 5 and 6 received fonsartan and losartan alone. At the end of the study, 24-h urine samples were collected and retrobulbar blood samples were taken in short inhalation anesthesia. Plasma values of creatine, PRA, and electrolytes were determined. For clearance evaluation, rats were anesthetized with 50 mg/kg thiopentone i.p. In order to determine glomerular filtration rate and renal plasma flow, clearances of inulin and *para*-aminohippurate were performed. After the clearance experiments, the rats were sacrificed and hearts and kidneys removed. Hearts were perfused in the isolated working heart preparation via the aorta with modified Krebs–Henseleit buffer containing solvents or drugs. After a preischemic period of 20 min, acute regional myocardial ischemia was produced by clamping the left coronary artery close to its origin for 15 min (ischemic period). Thereafter, the clip was removed, and changes during reperfusion were monitored (reperfusion period). The following cardiodynamic and cardiometabolic parameters were measured: incidence and duration of ventricular fibrillation, left ventricular pressure, contractility (dP/dt max), heart rate, and coronary flow; in the coronary effluent lactate dehydrogenase, creatine kinase, and lactate; and in the myocardial tissue lactate, glycogen, ATP, and creatine phosphate. Left ventricular pressure was measured with a Statham pressure transducer (P 23 DB) which on differentiation yielded LV dP/dt max and heart rate. Coronary flow was determined by electromagnetic flow probes in the aortic cannula. For the determination of lactate release, lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate samples were taken from the coronary effluent and analyzed spectrophotometrically.

Evaluation

Results are presented as arithmetical means ± SEM. A one-way ANOVA was calculated with SYSTAT for Windows (SYSTAT, Evanston, Ill., USA) followed by multiple pairwise comparisons according to Tukey.

Modifications of the Method

Arnal et al. (1993) measured cardiac weight of rats in hypertension induced by NO synthase blockade.

Linz et al. (1999) reviewed the interactions between ACE, kinins, and NO.

Sampaio et al. (2002) reported that hypertension plus diabetes mimics the cardiomyopathy induced by NO inhibition in rats.

Rossi et al. (2003) found that chronic inhibition of NO synthase induces hypertension and cardiomyocyte, mitochondrial, and myocardial remodeling in the absence of hypertrophy.

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Pulmonary Hypertension Induced by Monocrotaline

Purpose and Rationale

The pyrrolizidine alkaloid monocrotaline, derived from *Crotalaria spectabilis*, is hepatotoxic and pneumotoxic in the rat. A single injection of monocrotaline leads to progressive pulmonary hypertension resulting in right ventricular hypertrophy and cardiac failure (Gillespie et al. 1986, 1988; Todorovich-Hunter et al. 1988). Pathologic changes and hemodynamic changes associated with monocrotaline administration include blebbing of the lung, degeneration and fragmentation of endothelial cells, perivascular edema, extravasation of red blood cells, and muscularization of the pulmonary arteries and arterioles (Valdiva et al. 1967; Lalich et al. 1977; Huxtable et al. 1978; Hislop and Reid 1979; Meyrick and Reid 1979; Meyrick et al. 1980; Ghodsi and Will 1981; Hilliker et al. 1982; Sugita et al. 1983; Hilliker and Roth 1985; Stenmark et al. 1985; Altiere et al. 1986; Molteni et al. 1986; Lai et al. 1996). Rats given monocrotaline develop severe right ventricular hypertrophy often accompanied by ascites and pleural effusions (Ceconi et al. 1989).

Amelioration by angiotensin-converting enzyme inhibitors and by penicillamine has been demonstrated (Molteni et al. 1985, 1986).

Procedure

Treatment of male Sprague Dawley rats weighing 200–225 g with the test drug (angiotensin-converting enzyme inhibitor or vehicle) is started 1 week prior to a single subcutaneous injection of 100 mg/kg monocrotaline up to sacrifice 4, 7, or 14 days later by pentobarbital anesthesia and exsanguination. Heart and lungs are excised from thoracic cavity. After removing atria from the heart, the right ventricle is separated from the left ventricle plus septum which are blotted and weighed separately. The left lung is blotted, weighed, minced, and reweighed after drying at room temperature for 14 days. Three pulmonary artery segments, main pulmonary artery, right extrapulmonary artery, and intrapulmonary artery from the right lower lobe, are isolated for study of vascular responsiveness. Cylindrical segments of each vessel are suspended between stainless steel hooks in 10-ml isolated tissue baths containing modified Krebs–Henseleit buffer aerated with 95%O_2/5% CO_2 at 37 °C. At the end of each experiment,

vessel segments are blotted and weighed and their dimensions measured. Cross-sectional area of each artery is determined from tissue weight and diameter.

Arteries are equilibrated for 1 h at 1 g of passive applied load and then are made to contract to KCl (6×10^{-2} M). After washout, the procedure is repeated with applied loads increased by 1-g increments. Responses are normalized to the maximum active force development generated by an artery in each experiment, and the data are plotted as a function of applied force. Changes in isometric force are monitored through force displacement transducers (Grass FT03) and recorded on a polygraph.

Responsiveness to contractile and relaxant agonists is assessed in pulmonary arteries from saline- and monocrotaline-treated rats both in verum- and placebo-treated groups. Cumulative concentration–response curves to hypertonic KCl, angiotensin II, and norepinephrine are generated sequentially in vessels at resting tone. Arteries are then contracted submaximally with norepinephrine, and cumulative concentration–response curves to the vasorelaxants isoproterenol and acetylcholine are determined.

Evaluation

Contractions are expressed as active tension development, force generated per cross-sectional area, and relaxations are normalized to precontraction tone. Both contractile and relaxation responses are plotted as a function of the negative logarithm of agonist concentration. Differences in mean responses are compared by a *t*-test for grouped data.

Modifications of the Method

Molteni et al. (1986) treated rats continuously with monocrotaline in the drinking water at a concentration of 2.4 mg/kg/day for a period of 6 weeks. Test rats received an ACE inhibitor during this time in the drinking water and controls the vehicle only. At the end of the experiment, hearts and lungs were weighed and examined by light and electron microscopy.

Madden et al. (1995) determined L-arginine-related responses to pressure and vasoactive agents in monocrotaline-treated rat pulmonary arteries.

Ono et al. (1995) studied the effects of prostaglandin E_1 (PGE_1) on pulmonary hypertension and lung vascular remodeling in the rat monocrotaline model of human pulmonary hypertension.

Yamauchi et al. (1996) studied the effects of an orally active endothelin antagonist on monocrotaline-induced pulmonary hypertension in rats.

Gout et al. (1999) evaluated the effects of adrenomedullin in isolated vascular rings from rats treated with monocrotaline (60 mg/kg s.c.) causing pulmonary hypertension and ventricular hypertrophy within 3–4 weeks.

Kanno et al. (2001) studied the effect of an angiotensin-converting enzyme inhibitor on pulmonary arterial hypertension and endothelial nitric oxide synthase expression in monocrotaline-treated rats. For evaluation of right ventricular hypertrophy as a result of pulmonary arterial hypertension, multislice spin-echo MRI images were acquired at 8–12 time points in a cardiac cycle with respiratory and ECG gating (Kanno et al. 2000) at 2, 3, 4, and 5 weeks after monocrotaline treatment.

Kang et al. (2003) reported that a phosphodiesterase-5 inhibitor attenuated monocrotaline-induced pulmonary hypertension in rats.

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Portal Hypertension in Rats

Purpose and Rationale

Portal hypertension is associated with hyperdynamic splanchnic circulation and reduced vascular resistance (Vorobioff et al. 1983). Tanoue et al. (1991) developed a method for inducing portal hypertension and esophageal varices in rats – partial ligation of the portal vein after devascularization of the circumference of the left renal vein and complete ligation of the portal vein on the fifth day thereafter. Tsugawa et al. (2000) used this model to study the role of nitric oxide and endothelin 1 in rat portal hypertension.

Procedure

Male Sprague Dawley rats were anesthetized with 50 mg/kg Nembutal intraperitoneally. The portal vein was isolated and stenosis created by a single ligature of 3-0 silk placed around the portal vein and a 20-gauge blunt-tipped needle after devascularization of the left renal vein. This devascularization is indispensable in preventing the development of excess collateral vessels, which inhibit the formation of esophageal varices and portal hypertensive state. The needle was then removed from the ligature. In addition, 3-0 silk was also placed at the area of partial ligation (loose ligation), and both ends were then drawn out through the abdominal wall. Five days after the operation, the ends of the silk that had been placed in the flank were simultaneously pulled to induce complete portal vein ligation. Two weeks later, this portal hypertension model was completed.

Portal venous pressure, blood flow volume in the intra-abdominal viscera, plasma NO, and plasma endothelin 1 were measured.

Evaluation

Results were expressed as mean ± standard deviation. The Student's *t*-test was used to determine significance between portal hypertension rats and sham-operated controls.

Modifications of the Method

Portal hypertension by portal vein ligation without devascularization of the left renal vein was used by Lee et al. (1985), Braillon et al. (1986), Oren et al. (1995), Fernandez et al. (1996), Moreno et al. (1996), Connolly et al. (1999), Hilzenrat et al. (1999), Chagneau et al. (2000), Yu et al. (2000), and Sakurabayashi et al. (2002).

Dieguez et al. (2002) used a surgical technique based on the development of a triple stenosing ligation to worsen the complications inherent to the prehepatic chronic portal hypertension.

Jaffe et al. (1994) and Li et al. (1998) used injection of different-sized microspheres into the portal vein of male Wistar rats to induce portal hypertension.

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Preeclampsia in Rats

Purpose and Rationale

Preeclampsia is defined by new onset of hypertension and proteinuria after 20 weeks gestation of pregnancy. Placental vasculogenesis may play an important role in the pathophysiology of preeclampsia. In preeclampsia, the ischemic placenta has been shown to release factors, including the anti-angiogenic molecules soluble fms-like tyrosine kinase 1 (sFlt1) (Maynard et al. 2003) and soluble endoglin (sEng) (Venkatesha et al. 2006), that result in vasoconstriction and end organ damage seen in the mother (Fisher and Roberts 1999; Granger et al. 2002 and Taylor and Roberts 1999). Karumanchi and Stillman (2006) created a rat model of preeclampsia by administration of a sFLT1-expressing adenovirus. The administration of the sFLT1 by this vector resulted in a

dose-dependent hypertension, proteinuria, and glomerular endotheliosis in pregnant rats (Karumanchi and Stillman 2006).

Procedure

Adenoviruses expressing Fe or sFltl are amplified from a single plaque by serial infection of 293A cells and purified by two cycles of CsCl gradient and dialyzed. Adenovirus expressing murine Fe protein (a soluble protein) in equivalent doses is used as a control to rule out nonspecific effects of adenoviruses. The final products are titered by optical absorbance method and expressed as plaque-forming units (pfu)/mL.

Pregnant Sprague Dawley rats at days 8–9 of gestation were injected in the tail vein with an adenoviral dose that is approx. 1–2 × 109 pfu in phosphate-buffered saline (PBS), total volume of 400 μL.

Blood pressures are measured through the carotid arterial catheter at day 16 and 17 (corresponds to early third trimester). Rats are first anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally), and carotid artery is isolated and cannulated with a 3-Fr high-fidelity microtip catheter connected to a pressure transducer. Blood pressures are recorded and averaged over a 10-min period, and the mean arterial pressure is then calculated (MAP = diastolic pressure in mmHg + one-third (systolic pressure - diastolic pressure in mmHg)).

Blood from the rats in ethylenediaminetetraacetic acid (EDTA) tubes is collected and plasma obtained. Urine is also collected from the rats at the time of sacrifice by direct puncture of the urinary bladder using a 1-mL tuberculin syringe. Plasma samples are processed for the measurement of sFlt-1 protein using a commercially available ELISA for murine sFlt-1. Urinary albumin is also determined using commercial ELISA kit. To normalize the albumin excretion for varying urine outputs, urinary creatinine is measured and proteinuria is reported as a ratio of urinary albumin over creatinine.

Kidneys are obtained and sliced into three pieces for light, electron microscopy and immunofluorescence. Kidney slice processed for light microscopy is fixed in Bouin's solution for 6 h and stored in 70 % ethanol at room temperature until paraffin embedding. The slice is cut at 3 μm and stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson trichrome (MT) stains, using routine methods. Another kidney slice is embedded in optimum cutting temperature (OCT) compound and frozen in a standard type cryostat. The frozen slice is cut at 4 μm and using standard techniques immunostained for fibrin (anti-rat fibrinogen). The last kidney slice is fixed in 3 % glutaraldehyde and postfixed in 2 % osmium. The blocks are stained with 2 % uranyl acetate and embedded in Epon. One-micron sections are cut and stained with methylene blue for light microscopy. Thin sections are cut (at about 130 nm) and assessed by ultrastructural study.

Modifications of the Method

Granger et al. (2006) described an experimental model of induced chronic uteroplacental ischemia. To reduce uterine perfusion, pregnant rats undergo clipping of the aorta above the iliac bifurcation and left and right uterine arcade at the ovarian artery at day 14 of gestation.

Yallampalli and Garfield (1993) showed that constant infusion of inhibitor of nitric oxide synthesis, L-nitro-arginine methyl ester, from day 17 of gestation in rats induced signs similar to those of preeclampsia, hypertension, and fetal growth retardation.

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Coronary Drugs

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Contents

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Isolated Organs

Heart–Lung Preparation

Purpose and Rationale

The isolated heart–lung of the dog was introduced by Knowlton and Starling (1912). Since then, the dog model has been used for many physiological and pharmacological studies (Krayer 1931; Krayer and Mendez 1942; Somani and Blum 1966; Takeda et al. 1973; Ishikawa et al. 1978, 1983; Ono and O'Hara 1984; Ono et al. 1984; Caffrey et al. 1986; Hausknecht et al. 1986; Fessler et al. 1988; Seifen et al. 1987, 1988; Naka et al. 1989). More recently, the rat model has been preferred (Dietz 1984, 1987; Onwochei et al. 1987; Onwochei and Rapp 1988; Kashimoto et al. 1987, 1990, 1994, 1995; Fukuse et al. 1995).

Procedure

Wistar rats weighing 300–320 g are anesthetized with 50 mg/kg pentobarbital i.p. Tracheotomy is performed and intermittent positive pressure ventilation is instituted with air. The chest is opened and flooded with ice-cold saline and the heart arrested. Cannulae are inserted into the aorta and the superior (for measurement of central venous pressure) and inferior venae cavae. The heart–lung preparation is perfused with a solution containing rat blood cells from another rat and Krebs–Ringer bicarbonate buffer, with hematocrit and pH of 25 % and 7.4, respectively. The

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concentrations of the buffer constituents (mM) are NaCl 127, KCl 5.1, $CaCl_2$ 2.2, KH_2PO_4 1.3, $MgSO_4$ 2.6, $NaHCO_3$ 15, glucose 5.5, and heparin. The perfusate pumped from the aorta passes through a pneumatic resistance and is collected in a reservoir maintained at 37 °C and then returned to the inferior vena cava. In this model, no other organs except the heart and lung are perfused. Cardiac output is determined by the inflow as long as the heart does not fail. Mean arterial pressure is regulated by the pneumatic resistance. Heart rate is recorded by a bioelectric amplifier, and cardiac output is measured with an electromagnetic blood flow meter. Arterial pressure and right atrial pressure are measured with transducers and amplifiers. The heart is perfused initially with cardiac output of 30 ml/min and mean arterial pressure of 80 mmHg. Test drugs are administered into the perfusate 5 min after start of the experiment.

Evaluation

Hemodynamic data within groups are analyzed by two-way analysis of variance (ANOVA) with repeated measures. Recovery time is measured by the Kruskal–Wallis test. The other data are analyzed by one-way ANOVA followed by the Dunnett test for multiple comparisons.

Modifications of the Method

Using the Starling heart–lung preparation in dogs, Wollenberger (1947) studied the energy-rich phosphate supply of the failing heart.

Shigei and Hashimoto (1960) studied the mechanism of the heart failure induced by pentobarbital, quinine, fluoroacetate, and dinitrophenol in dog's heart–lung preparation and effects of sympathomimetic amines and ouabain on it.

Imai et al. (1961) used heart–lung preparations of the dog to study the cardiac actions of methoxamine with special reference to its antagonistic action to epinephrine.

Capri and Oliverio (1965) and Beaconsfield et al. (1974) used the heart–lung preparation of the **guinea pig**.

Robicsek et al. (1985) studied the metabolism and function of an autoperfused heart–lung preparation of the **dog**.

The **dog heart–lung preparation** was used:

By Seifen et al. (1988) to study the interaction of a calcium channel agonist with the effects of digoxin

By Somani and Blum (1966) to study blockade of epinephrine- and ouabain-induced cardiac arrhythmias in the dog

By Riveron et al. (1988) to investigate the energy expenditure of an autoperfusing heart–lung preparation

By Namakura et al. (1987) to study the role of pulmonary innervation in an in situ lung-perfusion preparation as a new model of neurogenic pulmonary edema

By Hausknecht et al. (1986) to investigate the effects of lung inflation on blood flow during cardiopulmonary resuscitation

By Caffrey et al. (1986) to evaluate the effect of naloxone on myocardial responses to isoproterenol

By Ono et al. (1984) to estimate the cardiodepressant potency of various beta-blocking agents

By Ishikawa et al. (1983) for a graphical analysis of drug effects in the dog heart–lung preparation – with particular reference to the pulmonary circulation and effects of norepinephrine and 5-hydroxytryptamine

By Iizuka (1983) to study the cardiac effects of acetylcholine and its congeners

By Fessler et al. (988) to investigate the mechanism of reduced LV afterload by systolic and diastolic positive pleural pressure

By Takeda et al. (1973) to study the cardiac actions of oxprenolol

Beaconsfield et al. (1974) used the heart–lung preparation of **guinea pigs** to study the cardiac effect of delta-9-tetrahydrocannabinol.

The **rabbit** autoperfusing heart–lung preparation was used by Muskett et al. (1986, 1988).

The isolated heart–lung preparation in the **cat** was described by Beaufort et al. (1993).

Kontos et al. (1987, 1988) harvested heart–lung blocks from **calves**.

Kissling et al. (2000) described a modified heart–lung preparation of the **rat** to allow the

systolic coronary flow to be distinguished from diastolic flow and to permit the effect of coronary compliance on coronary circulation to be studied.

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Isolated Heart According to Langendorff

Purpose and Rationale

More than 100 years ago, Langendorff (1895) described studies on isolated surviving mammalian hearts using mainly cats as donors. Since then, the method has been improved from the technical site and is nowadays used for studies with guinea pig, rabbit, or rat hearts. In principle, the heart is perfused in retrograde direction from the aorta either at constant pressure or at constant flow with oxygenated saline solutions. Retrograde perfusion closes the aortic valves, just as in the in situ heart during diastole. The perfusate is displaced through the coronary arteries flowing off the coronary sinus and the opened right atrium. In this original setup, the ventricles do not fill with perfusate and therefore do not perform pressure-volume work. Parameters usually measured are contractile force, coronary flow, and cardiac rhythm.

Procedure

Guinea pigs of either sex weighing 300–500 g are sacrificed by stunning. For studies of biochemical parameters in tissue and perfusate, removal of the heart during barbiturate anesthesia and artificial respiration is recommended. The heart is removed as quickly as possible and placed in a dish containing Ringer's solution at 37 °C. Associated pericardial and lung tissues are removed. The aorta is located and cut just below the point of its division. A glass or plastic cannula is introduced into the aorta and tied with two threads, and perfusion is started with oxygenated Ringer's solution or Krebs–Henseleit buffer. The heart is transferred to a double-walled Plexiglass perfusion apparatus which is kept at 37 °C by the water from a thermostat. Oxygenated Ringer's solution is perfused at a constant pressure of 40 mmHg and at a temperature of 37 °C from a reservoir. A small steel hook with a string is attached to the apex of the heart. Contractile force is measured isometrically by a force transducer with a preload of 2.5 g and recorded on a polygraph. Coronary flow is measured by a drop counter. Alternatively, flow measurements can be performed using a mechanic-electronic flow meter consisting of a vertical pipe and a magnetic valve (Hugo Sachs Electronic KG, Germany). Heart rate is measured through a chronometer coupled to the polygraph. Drugs are injected into the perfusion medium just above the aortic cannula.

Critical Assessment of the Method

A reappraisal of the Langendorff heart preparation was given by Broadley (1979) underlining the usefulness to test coronary vasodilating drugs. The value of the Langendorff method can be best assessed by demonstrating a few of its applications in physiology and pharmacology. Direct effects can be measured as well as the antagonism against various physiological and pharmacological agents.

Modifications of the Method

A survey on various modifications of the Langendorff technique and the **isolated working heart preparation** has been given by Ross (1972).

Neely et al. (1967) inserted a second cannula into a pulmonary vein or the left atrium. Perfusate from a reservoir flows via this cannula through the mitral valve into the left ventricle. During the systole of the heart, the left ventricle repumps the perfusate through the aorta into the reservoir. The perfusate flowing through the coronary arteries and dripping off from the outside of the heart is collected in a vessel below the heart and recirculated into the reservoir with a roller pump.

Flynn et al. (1978) underlined the difference of this working heart preparation to the original Langendorff method and reported the effects of histamine and noradrenaline on peak left ventricular systolic pressure, contractility, sinus rate, coronary flow, aortic flow, total cardiac output, and external pressure-volume work. Therefore, this method is reported separately.

Ishiu et al. (1996) measured simultaneously Ca^{2+}-dependent indo-1 fluorescence and left ventricular pressure on a beat-to-beat basis in Langendorff guinea pig hearts and investigated the changes in Ca^{+2} transient and left ventricular function during positive inotropic stimulation and myocardial ischemia.

Hukovic and Muscholl (1962) described the preparation of the isolated **rabbit** heart with intact sympathetic nervous supply from the right stellate ganglion.

Hendrikx et al. (1994) used the isolated perfused rabbit heart to test the effects of an Na^+/H^+ exchange inhibitor on postischemic function, resynthesis of high-energy phosphate, and reduction of Ca^{2+} overload.

Michio et al. (1985) modified the Langendorff method in rabbits to a working heart preparation by cannulating the left atrium. At a pressure of 20 cm H_2O in the left atrium, the heart pumped the solution against a hydrostatic pressure of 100 cm H_2O. Aortic flow, systolic aortic pressure, coronary flow, and heart rate were measured.

The influence of an ACE-inhibitor on heart rate, lactate in the coronary effluate, and GTP level in the myocardium after 60 min hypothermic cardiac arrest was studied in working heart preparation of rabbits by Zegner et al. (1996).

Gottlieb and Magnus (1904) introduced the so-called balloon method. A small balloon fixed to the tip of a catheter is filled with water and inserted into the left ventricle via one of the pulmonary veins, the left atrium and the mitral valve. The balloon size has to fit the volume of the left ventricle, and therefore, its size depends on the animal species and body weight. The catheter can be fixed by tying the pulmonary vein stems. Via a three-way valve, the balloon can be extended to a given preload. The beating heart now exerts a rhythmic force to the balloon and thus to the membrane of a pressure transducer. The advantages of this method are that force development and preload can be stated reproducibly in pressure units [mmHg], left ventricular contraction curves can be used for further calculations, and continuous heart rate recordings can be carried out without any problems when using a rate meter.

Sakai et al. (1983) reported a similar method adapted to **mice**.

Bardenheuer and Schrader (1983) described a method whereby the balloon is inserted into the left ventricle as described above. However, isovolumetric pressure in the left ventricle is not measured. Instead, the fluid in the balloon is pumped through the cannula into a closed extracorporeal circulation. The fluid is forced into one direction by two recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat, the fluid volume expelled from the balloon corresponding to the stroke volume of the heart can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated **rat** hearts (Linz et al. 1986, 1990):

- LVP (left ventricular pressure) with Statham pressure transducer P 23DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.
- Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation

$$MVO_2 = CF \times (P_a = P_V) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mmHg), P_v is the oxygen partial pressure of the venous effluent perfusate [mmHg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37 °C (Zander and Euler 1976).

For the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities in

the perfusate, samples are taken from the coronary effluent.

After the experiments, hearts are rapidly frozen in liquid nitrogen and stored at –80 °C. Of the left ventricle, 500 mg are taken, put into 5 ml ice-cold $HClO_4$, and disrupted with an ULTRA-TURRAX (Junke and Kunkel, Ika-Werk, Type TP). Glycogen is hydrolyzed with amyloglucosidase (pH 4.8) and determined as glucose. Furthermore, ATP and creatine phosphate are measured.

Avkiran and Curtis (1991) constructed a dual lumen aortic cannula which permits independent perfusion of left and right coronary beds in isolated rat hearts without necessitating the cannulation of individual arteries.

Igic (1996) described a modification of the isolated perfused working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted into the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses coronary vessels and the rest is expelled. Because the inner cannula can be easily retracted into the outer cannula, which is placed in the aorta, the preparation provides an opportunity to use the same heart as a "working" or "nonworking" model for investigating functions of the heart.

By labeling glucose, lactate, or fatty acids in the perfusate with ^{3}H or ^{14}C, Barr and Lopaschuk (1997) directly measured energy metabolism in the isolated rat heart.

Krzeminski et al. (1991) described a new concept of the isolated heart preparation with online computerized data evaluation. Left ventricular pressure was recorded by means of a balloon catheter, while special suction electrodes obtained the high-amplitude, noise-free electrogram recordings. The coronary effluent partial pressure of oxygen was continuously monitored, which enabled the calculation of myocardial oxygen consumption (MVO_2). The effluent partial pressure of carbon dioxide and pH value were also measured simultaneously. A computerized system of data acquisition, calculation, storage, and end report was described.

Döring (1990) described continuous simultaneous **ultrasonic recording** of two cardiac diameters in an isolated perfused guinea pig heart. For the measurement of the left ventricular transversal diameter, the ultrasonic transmitter was positioned at the epicardium at the largest cardiac diameter. The corresponding ultrasonic receiver was inserted through the right atrium into the right ventricle to approximately the same height as the transmitter. In the right ventricle, which is empty in the isolated perfused Langendorff heart, it was automatically positioned opposite to the transmitter. Additional transducers were placed both at the heart's base and apex for assessment of the ventricular longitudinal diameter.

Several authors used the **isolated perfused mouse heart**.

Bittner et al. (1996) described a work-performing heart preparation for myocardial performance analysis in murine hearts using a modified Langendorff apparatus.

Sumaray and Yellon (1998a, b) constructed a specially designed Langendorff apparatus that allows perfusion of the isolated **mouse** heart. These authors reported that ischemic preconditioning reduces infarct size following global ischemia in the murine myocardium.

Brooks and Apstein (1996) measured left ventricular systolic and diastolic pressures in the isovolumically contracting (balloon in the left ventricle) mouse hearts.

Sutherland et al. (2003) reviewed characteristics and cautions in the use of the isolated perfused heart of mice.

Wang et al. (2001) studied the relationship between ischemic time and ischemia–reperfusion injury in isolated Langendorff-perfused mouse hearts.

Tejero-Taldo et al. (2002) reported that α-adrenergic receptor stimulation produces late preconditioning through inducible nitric oxide synthase in mouse heart.

Ross et al. (2003) found that the α_{1B}-adrenergic receptor decreases the inotropic response in the mouse Langendorff heart model.

Bratkovsky et al. (2004) measured coronary flow reserve in isolated hearts from mice.

Plumier et al. (1995) generated **transgenic mice** expressing the human heart heat shock protein 70. Upon reperfusion of the hearts after 30 min of ischemia in the Langendorff preparation, transgenic hearts versus non-transgenic hearts showed significantly improved recovery of contractile force.

Hannan et al. (2000) compared ENOS knockout and wild-type mouse hearts which were perfused in a Langendorff apparatus with Krebs bicarbonate buffer and subjected to 20 min of global normothermic ischemia followed by 30 min of reperfusion. Myocardial function was measured using a ventricular balloon to determine time to onset of contraction, left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and heart rate-pressure product (RPP).

Sheikh et al. (2001) generated transgenic mice overexpressing fibroblast growth factor (FGF)-2 protein in the heart. An isolated mouse heart model of ischemia–reperfusion injury was used to assess the potential of endogenous FGF-2 for cardioprotection.

Mouren et al. (2010) showed that endothelium-dependent and endothelium-independent coronary flow responses are increased in Krebs–Henseleit mixed with red blood cell-perfused heart as compared with Krebs–Henseleit-perfused heart. Krebs–Henseleit/red blood cell-perfused hearts are more sensitive for studying the pharmacological response of coronary vasculature to vasoactive drugs. They also showed that the Krebs–Henseleit/red blood cell-perfused heart was able to achieve adequate O_2 supply even when myocardial metabolic demands are markedly increased.

Applications

Positive Inotropic Effects

While negative inotropic substances can be tested in a heart beating with normal force, the evaluation of a positive inotropic compound usually requires that cardiac force is first reduced. Acute experimental heart failure can be induced by an overdose of barbiturates, such as sodium thiopental, or calcium antagonists. This kind of cardiac failure can be reversed by β-sympathomimetic drugs, cardiac glycosides, or increased Ca^{+2} concentration. In this way, the potential β-sympathomimetic activity of a new drug can be measured using isoproterenol as standard. After thiopental-Na treatment, left ventricular pressure (LVP) and dp/dt_{max} decrease considerably, whereas coronary flow is slightly enhanced. β-Sympathomimetic drugs restore LVP and dp/dt_{max} and keep coronary blood flow elevated.

Cardiac glycosides increase LVP and dp/dt_{max} and leave coronary flow unchanged.

Negative Inotropic Effects

The effects of a β-sympathomimetic drug such as isoproterenol at doses of 0.05–0.2 µg increasing contractile force as well as heart frequency are registered. After injection of a β-blocker, the effects of isoproterenol are attenuated. The effects of a potential β-blocking agent can be tested comparing the isoproterenol inhibition versus a standard such as propranolol (0.1 mg).

Coronary Vessel Dilating Effect

The Langendorff heart has been extensively used for assessing the coronary dilating activity of drugs (Broadley 1979). Rothaul and Broadley (1982) demonstrated the release of coronary vasodilator mediators from guinea pig isolated hearts by a technique employing donor and recipient hearts in series.

Calcium Antagonism

In order to demonstrate the effect of calcium antagonists, 1–5 mg $BaCl_2$ is injected which induces a pronounced spasm of the coronary arteries, thereby reducing the coronary flow. Five min later, the test drug is injected. Active compounds have a relaxing effect on coronary arteries indicated by an increase of coronary flow. After this effect has weaned, $BaCl_2$ is injected again and the test drug or a standard drug, e.g., nifedipine, is tested. The increase of coronary flow is expressed as percentage of flow during $BaCl_2$ spasm and compared with the effect of the standard. Using

various doses, dose–response curves can be established.

Effect on Potassium Outflow Induced by Cardiac Glycosides

Lindner and Hajdu (1968) described a method using the Langendorff heart in which contractile force, coronary flow, and the potassium content in the coronary outflow were determined by flame photometry. Increase in potassium outflow correlates well with the positive inotropic effect.

Gradual Determination of Hypoxic Damage

Lindner and Grötsch (1973) measured the enzymes creatine phosphokinase (CPK), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (α-HBDH), and glutamic oxalacetic transaminase (GOT) in the effluent of a guinea pig heart preparation under varying degrees of hypoxia. Potassium content and oxygen tension in the inflowing and outflowing solution were determined. The heart rate, the amplitude of contraction, and the rate of coronary vessel perfusion were recorded additionally.

Metabolic Studies with Nuclear Magnetic Resonance

Using ^{31}P, studies on metabolism of nucleotides and phosphorylated intermediates of carbohydrates in isolated hearts have been performed (Garlick et al. 1977; Jacobus et al. 1977; Hollis et al. 1978; Matthews and Radda 1984).

Arrhythmogenic, Antiarrhythmic, and Antifibrillatory Effects

The Langendorff heart preparation is also used to test the influence of compounds on cardiac rhythm. For recording monophasic action potentials, suction electrodes are applied on the heart. Ventricular fibrillation can be induced by simultaneous injection of digitoxin (12.5–25.0 µg) and aconitine (12.5–25.0 µg) into the perfusion fluid (Lindner 1963). Cardiac glycosides shorten the refractory period, decrease the conduction velocity and increase heterotopic stimulus generation. Aconitine increases markedly heterotopic stimulus generation. Both compounds together induce invariably ventricular fibrillation. Antiarrhythmic compounds can be tested in this way. Fibrillation is inhibited, at least partially, by 20 µg prenylamine, 10–20 µg quinidine, or 20 µg ajmaline.

Takeo et al. (1992) described protective effects of antiarrhythmic agents on oxygen-deficiency-induced contractile dysfunction of isolated perfused hearts. Hypoxia in isolated rabbit hearts was induced by perfusing the heart for 20 min with Krebs–Henseleit buffer saturated with a gas mixture of 95 % N_2 and 5 % CO_2 containing 11 mM mannitol. After hypoxic perfusion, the heart was reoxygenated for 45 min with oxygenated buffer containing glucose.

Dhein et al. (1989) studied the pathway and time course of the epicardial electrical activation process by means of a computer-assisted epicardial potential mapping, using a matrix of 256 unipolar AgCl electrodes (1 mm spatial and 0.25 ms temporal resolution) in isolated rabbit hearts perfused according to the Langendorff technique. From the activation times of the surrounding electrodes, the direction and velocity of activation for each electrode were calculated, thereby allowing construction of an epicardial vector field. The method was used for the assessment of arrhythmogenic and antiarrhythmic drug activity.

Electrical Stimulation and Antifibrillatory Effect

Ventricular fibrillation can be induced in the Langendorff preparation by reducing the glucose content of the perfusion medium to 0.25 g/1,000 ml and the KCl content to 0.12 g/1,000 ml (Burn and Goodford 1957; Burn and Hukovic 1960; Lindner 1963). After a perfusion period of 20 min, 10 µg epinephrine is injected into the perfusion cannula. Immediately afterwards, the heart is stimulated with a current of 40 Hz and 5 mA for 2 min. This procedure is repeated every 10 min. Standard conditions are achieved when the fibrillation continues without further electrical stimulation. Hearts treated in this way serve as controls. Other hearts stimulated in the same way are treated with continuous infusion of the test drug or the standard via the perfusion medium. Differences in the incidence of fibrillations are calculated using the χ^2 test.

Electrophysiological Evaluation of Cardiovascular Agents

Balderston et al. (1991) modified the Langendorff technique in rabbit hearts in order to perform electrophysiologic studies. His bundle electrograms were measured with a plunge electrode and allowed atrioventricular nodal physiology to be evaluated directly. Atrial conduction and refractoriness, atrioventricular node conduction and refractoriness, His–Purkinje conduction, and ventricular conduction and refractoriness could be accurately measured. The effects of verapamil and flecainide were described.

EDRF Release from the Coronary Vascular Bed

Lamontagne et al. (1992) isolated platelets from the blood of healthy human donors and injected platelet boluses into the perfusion line of the Langendorff preparation of a rabbit heart. In the effluent, cyclic GMP was determined as an index for EDRF release.

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Coronary Artery Ligation in Isolated Working Rat Heart

Purpose and Rationale

In working heart preparations of rats, ischemia can be induced by clamping the left coronary artery close to its origin. After removal of the clip, changes in the reperfusion period can be observed. Prevention of these symptoms can be an indicator of the efficacy of coronary drugs.

Procedure

The preparation used is a modification of an isolated working heart preparation originally used for guinea pig hearts (Bardenheuer and Schrader 1983). Wistar rats of either sex weighing 280–300 g are sacrificed by decapitation. The hearts are removed and dissected free from the epicard and surrounding connective tissue. A cannula is introduced into the aorta from where the coronary vessels are perfused with the non-recirculated perfusion medium according to the Langendorff technique. In the left ventricle, a

balloon closely fitting the ventricular cavity is placed and connected to an artificial systemic circulation. The fluid in the balloon is pumped through a cannula into the closed extracorporeal circulation being forced into one direction by two recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986a). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat, the fluid volume pressed from the balloon, corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated rat hearts (Linz et al. 1986b):

LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.

Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation

$$MVO_2 = CF \times (P_a - P_V) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], Pa is the oxygen partial pressure of arterial perfusate (650 mmHg), P_v is the oxygen partial pressure of the venous effluent perfusate [mmHg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37 °C (Zander and Euler 1976).

Coronary Artery Ligation

For coronary artery occlusion experiment (Scholz et al. 1992, 1993), the isolated working hearts are perfused for a period of 20 min (pre-ischemic period) with modified Krebs–Henseleit buffer at a constant pressure of 65 mmHg. Thereafter, acute myocardial ischemia is produced by clamping the left coronary artery close to its origin for 15 min (ischemic period). The clip is then reopened, and changes during reperfusion are monitored for 30 min (reperfusion period). After coronary artery ligation and reperfusion, the hearts develop ventricular fibrillation.

From the coronary effluent, samples are taken for lactate, lactate dehydrogenase (LDH), and creatine kinase (CK) determinations. After the experiment, glycogen, lactate, ATP, and creatine phosphate in myocardial tissue are measured.

The test drugs are given into the perfusion medium either before occlusion or 5 min before reperfusion. For ex vivo studies, the rats are treated orally with the test drug 1 h before sacrifice and preparation of the isolated working heart.

Evaluation

The incidence and duration of ventricular fibrillation after treatment with coronary drugs is compared with controls. Left ventricular pressure, LV dP/dt max, and coronary flow are reduced after coronary constriction by angiotensin II, whereas enzyme activities in the effluent are increased and the myocardial content of glycogen, ATP, and creatine phosphate are decreased. Cardiac protective drugs have the opposite effects. The values of each parameter are statistically compared with controls.

Modifications of the Method

Vogel and Lucchesi (1980) described an isolated, blood-perfused, **feline** heart preparation for evaluating pharmacological interventions during myocardial ischemia. Ventricular function was measured with a fluid-filled latex balloon within the left ventricle.

Vleeming et al. (1989) ligated the left coronary artery in rats after thoracotomy in ether anesthesia. Forty-eight hours after the operation, the hearts were prepared for retrograde constant pressure

perfusion, according to the Langendorff technique.

Igic (1996) presented a new method for the isolated working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted in the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses the coronary vessels and the rest is expelled. Because the inner cannula can easily be retracted into the outer cannula, which is placed in the aorta, this preparation provides an opportunity to use the same heart as a "working" or "nonworking" model for investigating functions of the heart.

Pepe and McLennan (1993) described a maintained afterload model of ischemia in erythrocyte-perfused isolated working hearts of rats.

Further characterization of the pathophysiological reactions or the isolated working heart was performed by Linz et al. (1999). The external heart power (EHP) [mJ/min/g] was calculated using the formula

$$\begin{aligned} EHP_{\mathrm{LV}} &= \text{pressure} - \text{volume} + \text{acceleration work} \\ &= [SV(MAP - LAP)] \\ &\quad + [1/2SV \times d \times (SV/\pi r^2 e^2)]HRg^{-1}_{\mathrm{LVwwt}} \end{aligned}$$

where *SV* indicates stroke volume; *MAP*, mean aortic pressure; *LAP*, mean left arterial pressure; *d*, specific weight perfusate (1.004 g/cm^3); *r*, inner radius of aortic cannula; *e*, ejection time; *HR*, heart rate; LV, left ventricle; and LVwwt, left ventricular wet weight.

The function of the left ventricle was altered by changing the aortic pressure (afterload) at constant left atrial filling load (preload). By adjusting the Starling resistance, the aortic outflow could be switched during 1 min from the fixed baseline afterload to a preset higher afterload producing step-wise rises in mean arterial pressure.

Lee et al. (1988) studied the effects of acute global ischemia on cytosolic calcium transients in perfused isolated **rabbit** hearts with the fluorescent calcium indicator indo 1. Indo 1-loaded hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm from the epicardial surface of the left ventricle. The F_{400}/F_{550} ratio was calculated by an analog circuit, which allowed cancelation of optical motion artifact. The resulting calcium transients were registered simultaneously with the ventricular pressure and demonstrated a rapid upstroke and slow decay similar to those recorded in isolated ventricular myocytes. Global ischemia rapidly suppressed contraction, but it produced a concurrent increase in the systolic and diastolic levels of calcium transients, together with an increase in the duration of the peak.

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Isolated Working Heart Model in Infarcted Rat Heart

Purpose and Rationale

The model of chronic heart failure in spontaneously hypertensive rats described by Itter et al. (2004a ▶ Methods to Induce Cardiac Hypertrophy and Insufficiency) has been used by the same group to study the isolated working heart in rats after chronic infarction (Itter et al. 2004b).

Procedure

Animals and Methods

WKY/NHsd and SHR/NHsd rats at an age of 4 months were randomized into two groups – sham and myocardial infarction (MI). The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the left ventricle (LV). Chronic heart failure (CHF) was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function, and geometric properties were determined by the "working heart" mode and in vivo determinations by MRI and heart weight.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg i.p.). The left ventrolateral thorax was shaved and prepared to create a

disinfected surgical access area. When stable anesthesia was achieved, the animals were placed on a small animal operation table, intubated, and ventilated with room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstetten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. Reflexes were evaluated before surgery. The operation took 5 min. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Nordersredt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point, the lungs were hyperinflated by increasing the positive end-expiratory pressure, and the chest was closed. The rats were placed on a heating pad and covered with a layer of unbleached tissue paper. The rats were extubated following return of reflexes. They were continuously monitored until they started moving in their cages. To avoid ventricular arrhythmias, lidocaine (2 mg/kg i.m.) was given before surgery. To prevent acute lung edema, the rats received furosemide (Lasix, 2 mg/kg bodyweight twice daily for 3 days) via the drinking water. To avoid pain and distress, the rats received metamizole treatment (Novalgin, 0.1 mg/kg body weight i.m.) once, directly after the recovery period.

Before killing the animals 8 weeks after MI, noninvasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter, the animals were anesthetized with pentobarbital (180 mg/kg i.p. pentobarbital) and subsequently heparinized (heparin natrium 500 I.U./100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE (polyethylene) tube inserted into the trachea and ventilated with room air. A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. The heart was immersed in physiological buffer chilled to 4 °C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to a perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95 % O_2/5 % CO_2) non-circulating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4$, 1.64; $NaHCO_3$, 24.88; KH_2PO_4, 1.18; glucose, 5.55; and Na pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue in the thymus or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous pO_2 measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria.

When a tight seal with no leaks had been established and after a 15 min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters, the afterload pressure was enhanced in a cumulative manner from an additional 20–140 mmHg. Thereafter, the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5–30 mmHg.

Flow and pressure signals for computation were obtained from the PLUGSYS-measuring system (Hugo Sachs Elektronik, March-Hugstetten, Germany). Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstetten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight and the left and right ventricular weights were determined. The left ventricle was

then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices. The areas of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the left ventricular mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well.

Evaluation

The data are given as mean ± SEM. Statistics were performed using the SAS system statistics package (SAS Institute, Cary, N.C., USA) with a sequential rejection *t*-test according to Holm (1979).

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Relaxation of Bovine Coronary Artery

Purpose and Rationale

Eicosanoids can regulate the tonus of coronary arteries. Prostacyclin induces relaxation, whereas thromboxane A_2 causes contraction. Spiral strips from bovine coronary artery can be used for assaying relaxation activity of test compounds (Dusting et al. 1977).

Procedure

Freshly slaughtered beef hearts are immersed in cold oxygenated Krebs solution and immediately transported in a thermos flask to the laboratory. The left descending coronary artery and several of its primary branches are cut into spiral strips (about 20 mm long and 2–3 mm wide). The specimens can be stored up to 48 h at 4 °C. The artery strips are suspended in a 4 ml organ bath under an initial tension of 2 g and immersed in a Krebs' bicarbonate solution at 37 °C being gassed with oxygen containing 5 % CO_2 throughout the experiment. The Krebs solution contains a mixture of antagonists to inhibit any actions from endogenous acetylcholine, 5-hydroxytryptamine, histamine, or catecholamines (hyoscine hydrobromide 10^{-7} g/ml, methysergide maleate 2×10^{-7} g/ml, mepyramine maleate 10^{-7} g/ml, propranolol hydrochloride 2×10^{-6} g/ml). The strips are superfused with a solution of the test compounds in concentrations of 0.01, 0.1, and 1.0 µg/ml at a rate of 10–20 ml/min with oxygenated Krebs solution containing the mixture of antagonists. Isometric contractions are recorded with Grass force-displacement transducers (type FT 03C) on a Grass polygraph. The strips are superfused with Krebs' solution 3 h prior to the experiment. Standard compounds are 100 ng/ml PGE_2 inducing contraction and 100 ng/ml PGI_2 inducing pronounced relaxation.

Evaluation

The relaxation induced by the test compound is expressed as percentage of maximal response to 100 ng/ml PGI_2.

Modifications of the Method

Campell and Paul (1993) measured the effects of diltiazem on isometric force generation, $[Ca^{2+}]_i$, and energy metabolism in the isolated **porcine** coronary artery.

Li et al. (1997) determined the ability of analogs of human α-calcitonin gene-related peptide to relax isolated porcine coronary arteries precontracted with 20 mM KCl.

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In Vivo Methods

Isoproterenol-Induced Myocardial Necrosis in Rats

Purpose and Rationale

Cardiac necrosis can be produced by injection of natural and synthetic sympathomimetics in high doses. Infarct-like myocardial lesions in the rat by isoproterenol have been described by Rona et al. (1959). These lesions can be totally or partially prevented by several drugs such as sympatholytics or calcium antagonists.

Procedure

Groups of 10 male Wistar rats weighing 150–200 g are pretreated with the test drug or the standard either s.c. or orally for 1 week. Then, they receive 5.25 and 8.5 mg/kg isoproterenol s.c. on two consecutive days. Symptoms and mortality in each group are recorded and compared with those of rats given isoproterenol alone. Forty-eight hours after the first isoproterenol administration, the rats are sacrificed and autopsied. The hearts are removed and weighed, and frontal sections are embedded for histological examination.

Evaluation

Microscopic examination allows the following grading:

Grade 0: no change
Grade 1: focal interstitial response
Grade 2: focal lesions in many sections, consisting of mottled staining and fragmentation of muscle fibers
Grade 3: confluent retrogressive lesions with hyaline necrosis and fragmentation of muscle fibers and sequestrating mucoid edema
Grade 4: massive infarct with occasionally acute aneurysm and mural thrombi

For each group, the main grade is calculated with the standard deviation to reveal significant differences.

Critical Assessment of the Method

The test has been used by many authors for evaluation of coronary active drugs, such as calcium antagonists and other cardioprotective drugs like nitroglycerin and molsidomine (Vértesi et al. 1991; Classen et al. 1993).

Modifications of the Method

Yang et al. (1996) reported a protective effect of human adrenomedullin$^{13–52}$, a C-terminal fragment of adrenomedullin$^{10–52}$ on the myocardial injury produced by subcutaneous injection of isoproterenol into rats.

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Myocardial Infarction After Coronary Ligation in Rodents

Purpose and Rationale

Ligation of the left coronary artery in rats as described by Selye et al. (1960) induces an acute reduction in pump function and a dilatation of left ventricular chamber. The method has been used to evaluate beneficial effects of drugs after acute (Chiariello et al. 1980; Flaim and Zelis 1981; Bernauer 1985) or chronic (Innes and Weisman 1981; Pfeffer et al. 1985; Linz et al. 1996) treatment.

Procedure

Male Sprague–Dawley rats weighing 200–300 g are anesthetized with diethyl ether. The chest is opened by a left thoracotomy, and a thread is inserted near the middle of the lateral margin of the cutaneous wound and carried through a tunnel of the left pectoral muscle around the cranial half of the incision. The heart is gently exteriorized by pressure on the abdomen. A ligature is placed around the left coronary artery, near its origin, and is tightened. Within seconds, the heart is repositioned in the thoracic cavity, and the ends of the musculocutaneous thread are tightened to close the chest wall and enable the animal to breathe spontaneously.

The speed of the procedure renders mechanical respiration unnecessary.

To evaluate drug effects, the rats are treated 5 min after and 24 h after occlusion by subcutaneous injection (standard 5 mg/kg propranolol).

Two days after surgery, the rats are anesthetized with 60 mg/kg i.p. pentobarbital, and the right carotid artery is cannulated with a polyethylene catheter connected to a pressure transducer. The fluid-filled catheter is then advanced into the left ventricle through the aortic valve for measurement of left ventricular systolic and end-diastolic pressure.

After hemodynamic measurements, the heart is arrested by injecting 2 ml of 2.5 M potassium chloride. The chest is opened, and the hearts are isolated and rinsed with 300 mM KCl to maintain a complete diastole. A double-lumen catheter is advanced into the left ventricle through the ascending aorta, the right and left atria are tied off with a ligature, and the right ventricle is opened. The left ventricular chamber is filled with a cryostatic freeze medium through the smaller of the two catheter lumens and connected to a hydrostatic pressure reservoir maintained at a level corresponding to the end-diastolic pressure measured in vivo. The outlet (larger lumen) is then raised to the same level as the inlet to allow fluid in the two lumens to equilibrate. The heart is rapidly frozen with hexane and dry ice.

The hearts are serially cut with a cryostat into 40 μm thick transverse sections perpendicularly to the longitudinal axis from apex to base. At a fixed distance, eight sections are obtained from each heart and collected on gelatin-coated glass slides. Sections are air-dried and incubated at 25 °C for 30 min with 490 μM nitroblue tetrazolium and 50 mM succinic acid in 0.2 M phosphate buffer (pH 7.6), rinsed in cold distilled water, dehydrated in 95 % ethyl alcohol, cleared in xylene, and mounted with a synthetic resin medium. Viable tissue appears dark blue, contrasting with the unstained necrotic tissue.

Evaluation

The infarct size can be determined by planimetry and expressed as percentage of left ventricular area, and thickness can be expressed as percentage of noninfarcted ventricular wall thickness (MacLean et al. 1978; Chiariello et al. 1980; Roberts et al. 1983). An automatic method for morphometric analysis with image acquisition and computer processing was described by Porzio et al. (1995).

Critical Assessment of the Method

Myocardial infarction following coronary artery ligation in Sprague–Dawley rats is a widely used rat model of heart failure. If the left coronary artery is not completely ligated, heart failure may occur as a consequence of chronic myocardial ischemia (Kajstura et al. 1994).

Modifications of the Method

Johns and Olson (1954) described the coronary artery patterns for mouse, rat, hamster, and guinea pig.

Kaufman et al. (1959) and Fishbein et al. (1978, 1980) used various histochemical methods for the identification and quantification of border zones during the evolution of myocardial infarction.

Sakai et al. (1981) described an **experimental model of angina pectoris in the intact anesthetized rat**. In anesthetized rats, the tip of a special carotid cannula was placed closely to the right and left coronary ostium. Single intra-aortic injections of methacholine or acetylcholine (in the presence of physostigmine) developed a reproducible elevation of the ST segment and the T wave of the electrocardiogram. Coronary drugs were tested to prevent these changes.

Ytrehus et al. (1994) analyzed the effects of anesthesia, perfusate, risk zone, and method of infarct sizing in rat and rabbit heart infarction.

Leprán et al. (1981) placed a loose ligature of atraumatic silk around the left anterior descending coronary artery under ether anesthesia in **rats**. Ten days later, acute myocardial infarction was produced by tightening the ligature.

Kouchi et al. (2000) found an increase in $G_{i\alpha}$ protein accompanying progression of postinfarction remodeling in hypertensive cardiomyopathy in **rats**. G protein α subunits were studied with immunoblotting techniques (Böhm et al. 1990). The polyclonal antiserum MB1 was raised in rabbits against the carboxyl-terminal decapeptide of retinal transduction (KENLKDCGLF) coupled to keyhole limpet hemocyanin. The MB1 recognized $G_{i\alpha 1}$ and $G_{i\alpha 2}$ but not $G_{0\alpha}$ and $G_{i\alpha 3}$ (Böhm et al. 1994). The membrane fractions were electrophoresed in SDS-polyacrylamide gels and were transferred to nitrocellulose filters. The filters were incubated with

the first antibodies for $G_{i\alpha}$ (MB1) or $G_{s\alpha}$ (RM/1) and then with the second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Amersham). Immunoreactive signals were detected by means of the ECL kit (Amersham).

Liu et al. (1997) found that ligation of the left descending coronary artery in Lewis inbred **rats** produces an uniformly large infarct with low mortality. The model may be superior to the usual model in Sprague–Dawley rats with a marked variability in infarct size and cardiac dysfunction.

Coronary artery ligation induces left ventricular remodeling with cardiomyocyte apoptosis and myocardial fibrosis indicated by morphological studies and by collagen accumulation, which can be prevented by drug treatment (Yang et al. 1992; Belichard et al. 1994; Nguyen et al. 1998; Sia et al. 2002; Bäcklund et al. 2004).

The naturally occurring peptide ***N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP)** is an inhibitor of pluripotent hematopoietic stem cell proliferation and is normally present in human plasma and circulating mononuclear cells. It is cleaved to an inactive form by the NH_2-terminal catalytic domain of ACE (Azizi et al. 1996). Acute angiotensin-converting enzyme inhibition increases the plasma level of *N*-acetyl-seryl-aspartyl-lysyl-proline (Azizi et al. 1996). By morphological studies and collagen determinations, Rasoul et al. (2004) found an antifibrotic effect of Ac-SDKP and angiotensin-converting enzyme inhibition in hypertension in rats. Similarly, Yang et al. (2004) found that Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction.

Moreover, Azizi et al. (1997) and Le Meur et al. (1998) discussed whether the plasma Ac-SDKP level is a reliable marker of chronic angiotensin-converting enzyme inhibition in hypertensive patients. An Ac-SDKP EIA Kit is available from Cayman, Ann Arbor, Mich., USA.

Chen et al. (2004) found inhibition and reversal of myocardial infarction-induced hypertrophy and heart failure by NHE-1 inhibition.

Johns and Olson (1954) described a method of experimental **myocardial infarction by coronary occlusion in small animals**, such as **mouse**, **hamster**, **rat**, and **guinea pig**.

Scholz et al. (1995) described a dose-dependent reduction of myocardial infarct size in **rabbits** by a selective sodium–hydrogen exchange subtype 1 inhibitor.

Gomoll and Lekich (1990) tested the **ferret** for a myocardial ischemia/salvage model. Varying combinations of duration of left anterior descending coronary occlusion and reperfusion were evaluated.

Kim et al. (2014) compare the image properties of PET scans obtained using a recently developed 18F-labeled phosphonium cations (Kim et al. 2012) with those images obtained using the gold standard PET myocardial tracer [13N]NH3 in rat myocardial infarction models. [18F]-Labeled (6-Fluorohexyl)triphenylphosphonium cation showed to be a useful replacement for in cardiac PET/CT applications.

Coronary Artery Ligation in Mice

Michael et al. (1995, 1999) and Gould et al. (2001) described the surgical procedure to induce myocardial ischemia in **mice** by ligation of the left anterior descending branch of the left coronary artery.

Infarct and Reperfusion Model

Male C57BL/6 mice 12–16 weeks of age (22.5–30.5 g body weight) were used. Anesthesia was produced by an intraperitoneal injection of pentobarbital sodium (4 mg/ml; 10 μl/g body weight). Mice were placed in a supine position with paws taped to the operating table. With direct visualization of the trachea, an endotracheal tube was inserted and connected to a Harvard rodent volume-cycled ventilator cycling at 100/min with volume sufficient to adequately expand the lungs but not overexpand. The inflow valve was supplied with 100 % oxygen.

For studies of the myocardial response to permanent occlusion, ligation of the anterior descending branch of the left coronary artery was achieved by tying an 8–0 silk suture around the artery. The suture was passed under the artery at a position ~1 mm from the tip of the normally positioned left auricle.

For studies of the effect of reperfusion after coronary artery occlusion, the ligature was tied

at the same location on the coronary artery used for the permanent occlusion. However, to allow subsequent reestablishment of blood flow, occlusion was produced by placing a 1 mm length of polyethylene (PE) tubing (OD = 0.61 mm) on the artery and fixing it in place with the ligature. The artery was then compressed by tightening the ligature, producing myocardial blanching and electrocardiographic (ECG) S-T segment elevation as observed in permanent ligations. After occlusion for the desired time, blood flow was restored by removing the ligature and PE tubing. The chest wall was then closed by a 6–0 Ticron suture with one layer through the chest wall and muscle and a second layer through the skin and subcutaneous layer.

After surgical closing of the chest, the endotracheal tube was removed, warmth was provided by a heat lamp, and 100 % oxygen was provided via a nasal cone. The animal was given 0.1 mg/kg butorphanol tartrate as an analgesic, and it became sternally recumbent within 1 h. After surviving the experimental infarct, the mice recovered, and this allowed postoperative physiological measurement. Sham-operated mice underwent an identical procedure with placement of the ligature but did not undergo coronary artery occlusion.

Modifications of the Method

Guo et al. (1998) demonstrated the effects of an early and a late phase of ischemic preconditioning in mice. The results demonstrated that, in the mouse, a robust infarct-sparing effect occurred during both the early and the late phases of ischemic preconditioning, although the early phase was more powerful.

Guo et al. (2005) found that late preconditioning induced by NO donors, adenosine A_1 receptor agonists, and δ_1-opioid receptor agonists is mediated by inducible NO synthase.

Lutgens et al. (1999) reported cardiac structural and functional changes after chronic myocardial infarction in the mouse.

Scherrer-Crosbie et al. (1999) described echocardiographic determination of risk area in a murine model of myocardial ischemia. Myocardial contrast echocardiography was performed before and after coronary artery ligation in anesthetized mice by intravenous injection of contrast microbubbles and transthoracic echo imaging. Time–video intensity curves were obtained for the anterior, lateral, and septal myocardial walls. After myocardial ischemia, myocardial contrast echocardiography defects were compared with the area of no perfusion measured by Evans blue staining.

Jones and Lefer (2001) described cardioprotective actions of acute HMG-CoA reductase inhibition in the setting of myocardial infarction.

Janssens et al. (2004) reported that cardiomyocyte-specific overexpression of NO synthase 3 (NOS3) improves left ventricular (LV) performance and reduces compensatory hypertrophy after myocardial infarction. The effect of cardiomyocyte-restricted overexpression of one NO synthase isoform, NOS3, on LV remodeling after myocardial infarction in mice was tested. LV structure and function before and after permanent left anterior descending (LAD) coronary artery ligation were compared in transgenic mice with cardiomyocyte-restricted NOS3 overexpression (NOS3-TG) and their wild-type littermates (WT). Before myocardial infarction, systemic hemodynamic measurements, echocardiographic assessment of LV fractional shortening (FS), heart weight, and myocyte width (as assessed histologically) did not differ in NOS3-TG and WT mice. The inotropic response to graded doses of isoproterenol was significantly reduced in NOS3-TG mice. One week after LAD ligation, the infarcted fraction of the LV did not differ in WT and NOS3-TG mice. Four weeks after myocardial infarction, however, end-systolic LV internal diameter (LVID) was greater, and FS and maximum and minimum rates of LV pressure development were less in WT than in NOS3-TG mice. LV weight/body weight ratio was greater in WT than in NOS3-TG mice.

LaPointe et al. (2004) found that inhibition of cyclooxygenase-2 (COX-2) improves cardiac function after myocardial infarction in the mouse. Myocardial infarction was produced by ligation of the LAD coronary artery in mice. Two days later, mice were treated with a selective COX-2 inhibitor or vehicle in drinking water

for 2 weeks. After the treatment period, mice were subjected to two-dimensional M-mode echocardiography to determine cardiac function. Hearts were then analyzed for the determination of infarct size, interstitial collagen content, brain natriuretic peptide (BNP) mRNA, myocyte cross-sectional area, and immunohistochemical staining for transforming growth factor (TGF)β and COX-2.

Shibuya et al. (2005) reported that *N*-acetyl-seryl-aspartyl-lysine-proline prevents renal insufficiency and matrix expansion in diabetic d*b*/d*b* **mice**.

Weinberg et al. (2005) found in coronary ligation experiments in mice that rosuvastatin reduces experimental left ventricular infarct size after ischemia–reperfusion injury but not total coronary occlusion.

Yang et al. (2005) found that the infarct-sparing effect of A_{2A}-adenosine receptor activation is due primarily to its action on lymphocytes. Chimeric mice were created by bone marrow transplantation from A_{2A}AR-knockout or green fluorescent protein (GFP) donor mice to irradiated congenic C57BL/6 (B6) recipients. In the GFP chimeras, we were unable to detect GFP-producing cells in the vascular endothelium, indicating that bone marrow-derived cells were not recruited to endothelium at appreciable levels after bone marrow transplantation and/or acute myocardial infarction. Injection of 5 or 10 μg/kg of a potent and selective agonist of A_{2A} adenosine receptor had no effect on hemodynamic parameters but reduced infarct size in B6 mice after 45 min of LAD artery occlusion followed by 24 h of reperfusion.

Kanno et al. (2003) found **connexin**43 to be a determinant of myocardial infarct size following coronary occlusion in mice.

Regulation of myocardial connexins during hypertrophic remodeling was reviewed by Teunissen et al. (2004).

Kuhlmann et al. (2006) reported that granulocyte colony-stimulating factor (G-CSF), alone or in combination with stem cell factor (SCF), can improve hemodynamic cardiac function after myocardial infarction in mice and reduces inducible arrhythmias in the infarcted heart potentially via increased connexin43 expression and arteriogenesis.

Gargiulo et al. (2012) discusses the applications of hybrid cardiac positron emission tomography/X-ray computed tomography (PET/CT) systems technology for imaging of mouse models of myocardial infarction.

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Occlusion of Coronary Artery in Anesthetized Dogs and Pigs

Purpose and Rationale

The size of infarcts is studied after proximal occlusion of the left anterior descending coronary artery in open chest dogs. Compounds potentially reducing infarct size are tested. To delineate the postmortem area at risk, coronary arteriograms are made after injection of a $BaSO_4$-gelatin mass into the left coronary ostium. The infarct's area is visualized with nitroblue tetrazolium chloride in myocardial sections.

Procedure

Dogs of either sex weighing approximately 30 kg are used. The animals are anesthetized by intravenous injection of pentobarbital sodium (bolus of 35 mg/kg followed by continuous infusion of 4 mg/kg/h). The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator.

Arterial blood gases are checked, and the ventilation rate and/or oxygen flow rate is adjusted to achieve physiological blood gas values (P_{O2}: 100–140 mmHg, P_{CO2}: 32–40 mmHg, and pH7.47). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously from lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral vein is connected to a pressure transducer (Statham P 23DB). For the determination of left ventricular pressure (LVP), a Millar microtip catheter (PC350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted.

Experimental Procedure

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened, and the left anterior descending coronary artery (LAD) is exposed. After reaching steady-state conditions for the hemodynamic parameters (approx. 45 min), the LAD is ligated just below the first diagonal branch for 360 min. No attempt is made to suppress arrhythmic activity after the ligation.

The test substance or the vehicle (controls) is administered by intravenous bolus injection and/or continuous infusion. The schedule of administration may vary. Hemodynamic parameters are registered continuously during the whole experiment. At the end of the experiment, the animals are sacrificed with an overdose of pentobarbital sodium and the heart is dissected.

Preparation to Determine Area at Risk

Coronary arteriograms are made according to Schaper et al. (1979) to delineate the anatomic postmortem area at risk. A purse-string suture is placed around the left coronary ostium in the sinus of Valsalva; a cannula is then placed in the ostium and the purse-string suture is tightened. Micronized $BaSO_4$ suspended in 12 % gelatin solution (37 °C) is injected under increasing pressure (2 min at 100 mmHg, 2 min at 150 mmHg, and 2 min at 200 mmHg). The heart is placed in crushed ice to gel the injectate. The right ventricle is removed, and the left ventricle plus septum is cut into transverse sections (approx. 1 cm thick) from the apex to the level of the occlusion (near the base). From each slice, angiograms are made with an X-ray tube at 40 kV to assess the postmortem area at risk (by defect opacity: reduction of $BaSO_4$-filled vessels in infarct tissue).

Preparation to Determine Infarct Size

The slices are then incubated in p-nitroblue tetrazolium solution (0.25 g/L in Sörensen' phosphate buffer, pH7.4, containing 100 mM D, L-maleate) in order to visualize the infarct tissue (blue-/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of the infarct area.

Left ventricle and infarct area and area at risk are measured by planimetry from projections of all slices with the exclusion of the apex and of the slice containing the ligature.

Evaluation

Mortality and the different hemodynamic parameters are determined. Changes of parameters in drug-treated animals are compared to vehicle controls. The different characteristics are evaluated separately. Mean values ± SEM of infarct area and of area at risk are calculated. Statistical analyses consist of regression and correlation analyses and of the Student's t-test. Results are considered significant at $p < 0.05$.

Modifications of the Method

Nachlas and Shnitka (1963) described the macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity in dogs by staining the cardiac tissue with nitro-BT [2,2′-di-p-nitrophenyl-5,5′ diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride] yielding a dark blue formazan in viable muscle but not in necrotic muscle fibers.

Chiariello et al. (1976) compared the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction in dogs.

Black et al. (1995) studied the cardioprotective effects of heparin or *N*-acetylheparin in an in vivo dog model of myocardial and ischemic reperfusion injury. The hearts were removed after 90 min of coronary occlusion and a 6 h reperfusion period. Area at risk was determined by the absence of Evans blue dye after perfusion of the aorta in a retrograde fashion and infarct zone by the absence of formazan pigment within the area at risk after perfusion of the circumflex coronary artery with triphenyltetrazolium chloride.

Reimer et al. (1985) tested the effect of drugs to protect ischemic myocardium in unconscious and conscious dogs. In the conscious model, dogs of either sex weighing 10–25 kg were anesthetized with thiamylal sodium (30–40 mg/kg i.v.) and underwent thoracotomy through the 4th intercostal space. Heparin-filled polyvinyl chloride catheters were positioned in the aortic root, the left atrium via the left atrial appendage, and a systemic vein. A mechanical adjustable snare-type occluder was placed around the proximal left circumflex coronary artery above or below the first marginal branch, so that temporary occlusion resulted in cyanosis of at least 75 % of the inferior wall. The catheters and snare were either exteriorized or positioned in a subcutaneous pocket at the back of the neck. Penicillin, 1,000,000 units, and streptomycin, 1.0 g, were given i.m. for the first 4 postoperative days, and at least 7 days were allowed for recovery from surgery.

Dogs were fasted overnight prior to the study. After exteriorization and flushing of the catheters, 30–40 min were allowed for the animals to adjust to laboratory conditions. Morphine sulfate, 0.25 mg/kg, i.m., was given 30 min before occlusion, and an additional 0.25 mg/kg, i.v., was given 20 min later. Heart rate and aortic and left atrial pressures were monitored continuously. Permanent coronary occlusion was produced by a sudden one-stage tightening of the snare occluder. Drugs were administered by continuous i.v. infusion over 6 h. Hemodynamic measurements were taken 5 min before occlusion and 10, 25, 105, 180, and 360 min after occlusion.

Raberger et al. (1986) described a model of **transient myocardial dysfunction in conscious dogs**. Mongrel dogs, trained to run on a treadmill, were chronically instrumented with a miniature pressure transducer in the left ventricle, and a hydraulic occluder was placed around the circumflex branch of the left coronary artery. Two pairs of piecoelectrical crystals for sonomicrometry were implanted subendocardially to measure regional myocardial functions. Comparable episodes of regional dysfunction of the left coronary artery area during treadmill runs were found after partial left coronary artery stenosis induced by external filling of the occluder.

Hartman and Warltier (1990) described a model of **multivessel coronary artery disease** using conscious, chronically instrumented dogs. A hydraulic occluder was implanted around the left anterior descending coronary artery (LAD) and an ameroid constrictor around the left circumflex coronary artery (LCCA). Pairs of piecoelectric crystals were implanted within the subendocardium of the LAD and LCCA perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion. Bolus injections of adenosine were administered daily via the left atrium to evaluate LAD and LCCA coronary reserve. After stenosis by the ameroid constrictor, radioactive microspheres were administered to compare regional perfusion within normal myocardium to flow in myocardium supplied by the occluded or stenotic coronary arteries.

Holmborn et al. (1993) compared triphenyltetrazolium chloride staining versus detection of fibronectin in experimental myocardial infarction in **pigs**.

Klein et al. (1995) used intact **pigs** and found myocardial protection by Na^+/H^+ exchange inhibition in ischemic–reperfused hearts.

Klein et al. (1997) measured the time delay of cell death by Na^+/H^+ exchange inhibition in regionally ischemic–reperfused **porcine** hearts.

Garcia-Dorado et al. (1997) determined the effect of Na^+/H^+ exchange blockade in ischemic rigor contracture and reperfusion-induced

hypercontracture in *pigs* submitted to 55 min of coronary occlusion and 5 h reperfusion. Myocardial segment length analysis with ultrasonic microcrystals was used to detect ischemic rigor (reduction in passive segment length change) and hypercontracture (reduction in end-diastolic length).

Symons et al. (1998) tested the attenuation of regional dysfunction in response to 25 cycles of ischemia (2 min) and reperfusion (8 min) of the left circumflex coronary artery in **conscious swine** after administration of a Na^+/H^+ exchange inhibitor. The animals were instrumented to measure arterial blood pressure, regional myocardial blood flow (colored microspheres), systolic wall thickening in the normally perfused left anterior descending and left circumflex coronary artery regions (sonomicrometry), left circumflex coronary artery blood flow velocity (Doppler), and reversibility to occlude the left circumflex coronary artery (hydraulic occluder).

Etoh et al. (2001) studied myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in **pigs**.

McCall et al. (2012) described a model of myocardial infarction using Yorkshire and Göttingen swines. Myocardial infarction was created with a closed-chest coronary artery occlusion–reperfusion and encompasses the anteroapical, lateral, and septal walls of the left ventricle.

Lukács et al. (2012) reviewed the small and large animal models of experimental myocardial infarction regarding the differences among species, methods, reproducibility, and interpretation.

Lukács et al. (2013) evaluated the electromechanical mapping diagnostic in parallel with cardiac magnetic resonance imaging tool in porcine myocardial infarction models (balloon occlusion in the left anterior descending coronary artery or coil deployment in the LAD or circumflex artery).

Li et al. (2013) showed that prophylactic use of amiodarone plus lidocaine reduces arrhythmia and mortality after acute myocardial infarction in sheep model without significant negative effect on hemodynamics. This improved cost and acceptance of a large myocardial infarction animal models. Spata et al. (2013) reported an acute myocardial infarction model in sheep created by catheter injection of autologous aggregated platelets into the mid-left anterior descending coronary artery.

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Acute Ischemia by Injection of Microspheres in Dogs

Purpose and Rationale

Severe left ventricular failure is induced by repeated injections of 50 μm plastic microspheres into the left main coronary artery of anesthetized dogs. Hemodynamic measurements are performed under these conditions, testing drugs which potentially improve cardiac performance. The test can be used to evaluate the influence of drugs on myocardial performance during acute ischemic left ventricular failure in dogs.

Procedure

Dogs of either sex weighing approximately 30 kg are anesthetized by an intravenous bolus injection of 35–40 mg/kg pentobarbital sodium continued by an infusion of 4 mg/kg/h. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator and controlled by measuring end-expiratory CO_2 concentration as well as blood gases. Two peripheral veins are

cannulated for the administration of narcotic (brachial vein) and test compounds (saphenous vein). The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of the right femoral vein is connected to a pressure transducer (Statham P 23DB). For the determination of left ventricular pressure (LVP), a Millar microtip catheter (Gould PC 350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured on a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate (HR) is counted. To measure right ventricular pressure, a Millar microtip catheter is inserted via the right femoral vein. Systolic, diastolic, and mean pulmonary artery pressure (PAP), mean pulmonary capillary pressure, and cardiac output are measured by a thermodilution technique using a cardiac index computer (Gould SP 1435) and a balloon tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened, and the left circumflex coronary artery (LCX) is exposed. To measure coronary blood flow, an electromagnetic flow probe (Hellige Recomed) is placed on the proximal part of the LCX.

Polystyrol microspheres (3 M Company, St. Paul, Minnesota, USA) with a diameter of 52.5 ± 2.24 μm are diluted with dextran 70, 60 mg/ml and saline at a concentration of 1 mg microspheres/ml (1 mg = approx. 12,000 beads). For administration of microspheres, an angiogram catheter (Judkins-Schmidt Femoral-Torque, William Cook, Europe Aps.BP 7) is inserted into the left ostium via the left femoral artery.

Induction of Failure

The microspheres are injected through the angiogram catheter into the left ostium initially as 10 ml and later as 5 ml boluses about 5 min apart. The microsphere injections produce stepwise elevations of LVEDP. Embolization is terminated when LVEDP has increased to 16–18 mmHg and/or PAPm has increased to 20 mmHg and/or heart rate has reached 200 beats/min. The embolization is completed in about 70 min and by injection of an average dose of 3–5 mg/kg microspheres. Hemodynamic variables are allowed to stabilize after coronary embolization for at least 30 min.

Experimental Course

The test substance or the vehicle (controls) is then administered by intravenous bolus injection or continuous infusion or by intraduodenal application.

Recordings are obtained:

- Before embolization.
- After embolization.
- Before administration of test compound.
- 5, 30, 45, 60, 90, 120 and, eventually, 150 and 180 min following administration of test drug. At the end of the experiment, the animal is sacrificed by an overdose of pentobarbital sodium.

Evaluation

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formula:

Stroke volume [ml/s], $SV = \frac{\text{cardiac output}}{\text{heart rate}}$

Tension index [mmHgs], $IT = \frac{BPs \times \text{heart rate}}{1,000}$

Coronary vascular resistance [mmHg min/ml], $CVR = \frac{BPm \times RAPm}{CBF}$

Total peripheral resistance [dyns/cm^5], $TPR = \frac{BPm \times RAPm}{\text{cardiac output}} \times 79.9$

Pulmonary artery resistance [dyns/cm^5], $PAR = \frac{PAPm - PCPm}{\text{cardiac output}} \times 79.9$

Right ventricle work [kg m/min], $RVW = (PAPm - RAPm) \times \text{cardiac output} \times 0.0136$

Left ventricle work [kg m/min], $LVW = (BPm - LVEDP) \times \text{cardiac output} \times 0.0136$

Left ventricular myocardial oxygen consumption

$$[\text{ml } O_2/\text{min}/100\text{ g}],\ MVO_2 = K_1(BPs \times HR) + K_2 \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} + 1.43$$

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25 \times 10^{-4}$
BPs = systolic blood pressure [mmHg]
BPd = diastolic blood pressure [mmHg]
BPm = mean blood pressure [mmHg]
CBF = coronary blood flow in left circumflex coronary artery [ml/min]
$RAPm$ = mean right atrial pressure [mmHg]
$PAPm$ = mean blood pressure of the A. pulmonalis [mmHg]
$PCPm$ = mean pulmonary capillary pressure
HR = heart rate [beats/min]
SV = stroke volume [ml]
BW = body weight [kg]

Changes of parameters in drug-treated animals are compared to vehicle controls; statistical significance of the differences is calculated with the Student's *t*-test.

The mean embolization times, doses of microspheres, and number of microsphere applications are evaluated.

Modifications of the Method

Gorodetskaya et al. (1990) described a simple method to produce acute heart failure by coronary vessel embolization with microspheres in rats.

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Influence on Myocardial Preconditioning

Purpose and Rationale

Damage to the mammalian heart produced by prolonged ischemia and reperfusion can be reduced by "preconditioning" the myocardium via a brief cycle of ischemia and reperfusion prior to the protracted ischemic event. Ischemic preconditioning has been shown to decrease infarct size and increase recovery of postischemic ventricular function (Murry et al. 1986) and to reduce leakage of cellular marker proteins indicative for cardiac myocyte death (Volovsek et al. 1992). In addition, preliminary preconditioning also attenuates cardiac arrhythmia associated with subsequent occlusion and reperfusion (Vegh et al. 1990).

The mechanistic basis of this phenomenon is under discussion (Parratt 1994; Parratt and Vegh 1994). Adenosine receptor involvement in myocardial protection after ischemic preconditioning in rabbits has been shown by Baxter et al. (1994). Adenosine (A_1 receptor) antagonists have been demonstrated to block the protection produced by preconditioning (Liu et al. 1991), and short-term administration of adenosine was shown to simulate the protective effects of ischemic preconditioning (Toombs et al. 1993). These observations together suggest that adenosine is generated by the short preconditioning ischemia. Other recent pharmacological studies (Gross and Auchampach 1992; Yao and Gross 1994) indicate the involvement of the ATP-sensitive potassium channel. Recent investigations indicate that an increase of NO production after ACE inhibitors

may be a part of the protective mechanism (Linz et al. 1992, 1994). Moreover, the involvement of prostanoids and bradykinin in the preconditioning process has been discussed (Wiemer et al. 1991). Gho et al. (1994) found a limitation of myocardial infarct size in the rat by transient renal ischemia, supporting the hypothesis that the mechanism leading to cardiac protection by ischemic preconditioning may not only reside in the heart itself.

Procedure

New Zealand rabbits of either sex weighing 2.5–3.5 kg are initially anesthetized with an intramuscular injection of ketamine (50 mg/ml)/xylazine (10 mg/ml) solution at a dose of 0.6 ml per kg body weight. A tracheotomy is performed to facilitate artificial respiration. The left external jugular vein is cannulated to permit a constant infusion (0.15–0.25 ml/min) of xylazine (2 mg/ml in heparinized saline) to assist in maintaining anesthesia and fluid volume. Anesthesia is also maintained by i.m. injections (0.4–0.6 ml) of ketamine (80 mg/ml) and xylazine (5 mg/ml) solution. After the xylazine infusion is started, animals are respired with room air at a tidal volume of 10 ml/kg and a frequency of 30 inflations per min (Harvard Apparatus, USA). Thereafter, ventilation is adjusted or inspiratory room air is supplemented (5 % CO_2/95 % O_2) to maintain arterial blood chemistry within the following ranges: pH 7.35–7.45, P_{CO2} 25–45 mmHg, and P_{O2} 90–135 mmHg. The right femoral artery and vein are isolated and catheterized for measurement of arterial pressure and administration of drugs, respectively.

A thoracotomy is performed in the fourth intercostal space, and the lungs are retracted to expose the heart. The pericardium is cut to expose the left ventricle, and a solid-state pressure transducer catheter (e. g., MicroTip 3F, Millar Instruments, Houston, USA) is inserted through an apical incision and secured to enable measurement of pulsatile left ventricular pressure. The maximal rate of increase in left ventricular pressure (LVd*P*/d*t* max) is determined by electronic differentiation of the left ventricular pressure wave form. A segment of 4–0 prolene suture is looped loosely around a marginal branch of the left main coronary artery to facilitate coronary occlusion during the experiment. Needle electrodes are inserted subcutaneously in a lead II configuration to enable recording of an ECG in order to determine heart rate and help confirm the occurrence of ischemia (ST segment elevation) and reperfusion of the myocardium distal to the coronary occlusion. Continuous recording of pulsatile pressure, ECG, heart rate, and LVd*P*/d*t* is simultaneously displayed on a polygraph (e.g., Gould chart recorder, Gould Inc., Valley View, USA) and digitized in real time by a personal computer. Hemodynamic data are condensed for summary and later statistical analysis.

Ischemic preconditioning is induced by tightening the prolene loop around the coronary artery for 5 min and then loosening to reperfuse the affected myocardium for 10 min prior to a subsequent 30 min occlusion. After surgical preparation, and prior to 30 min of occlusion, rabbits are randomly selected to receive ischemic preconditioning, no preconditioning, or ischemic preconditioning plus treatment with test drugs. After 30 min of occlusion, the ligature is released and followed by 120 min of reperfusion. Occlusion is verified by epicardial cyanosis distal to the suture, which is usually accompanied by alterations in hemodynamics and ECG. Reperfusion is validated by return of original color. Systemic hemodynamics are summarized for each experimental period. The experiment is terminated after 120 min of reperfusion, and the heart is excised for determinations of infarct size and area at risk.

Immediately before the animal is sacrificed, the marginal branch of the left coronary artery is reoccluded, and India ink is rapidly injected by syringe with an 18 g needle into the left ventricular chamber to demarcate blackened normal myocardium from unstained area at risk. After the rabbits are sacrificed, the heart is removed and sectioned in a breadloaf fashion from apex to base perpendicular to the long axis. The right ventricle is removed from each slice leaving only the left ventricle and septum. After each slice is weighed, the portions are washed and incubated in a phosphate buffered saline solution

of triphenyl tetrazolium chloride (1 g/ml, Sigma) for 10–15 min. Salvaged myocardium in the area at risk stains brick red, whereas infarcted tissue remains unaltered in color. Slices are then placed between sheets of Plexiglas and the areas (normal, risk, infarct) of each slice are traced on a sheet of clear acetate. Traces are then digitized and analyzed using computerized planimetry to compare the relative composition of each slice with respect to normal tissue, area at risk, and infarcted myocardium. Planimetry is performed with a computerized analysis system, e.g., Quantimet 570C image analysis system (Leica, Deerfield, USA).

Surface areas of normal tissue, area at risk, and infarcted myocardium on both sides of each slide are averaged for the individual slide. The contribution of each slide to the total infarcted and area at risk (%) and area at risk as a percentage of total left ventricular mass for the entire left ventricle is prorated by the weight of each slice (Garcia-Dorado et al. 1987). By adding the adjusted contributions from each slice to infarcted tissue, area at risk, and left ventricular mass, a three-dimensional mathematical representation of the total myocardial infarct size and risk zone can be calculated for each rabbit and a mean tabulated for each treatment group for statistical comparison.

Evaluation

All data are presented as mean ± SD. Systemic hemodynamic data are analyzed by ANOVA using Statistica/W software. Means are considered significantly different at $p < 0.05$.

Modifications of the Method

Li et al. (1990) found in dog experiments that preconditioning with one brief ischemic interval is as effective as preconditioning with multiple ischemic periods.

In contrast, Vegh et al. (1990) found in other dog experiments that two brief preconditioning periods of coronary occlusion, with an adequate period of reperfusion between, reduce the severity of arrhythmias.

Yang et al. (1996) found a second window of protection after ischemic preconditioning in conscious rabbits which minimizes both infarction and arrhythmias.

Late preconditioning against myocardial stunning in conscious pigs together with an increase of heat stress protein (HSP) 70 was described Sun et al. (1995).

Szilvássy et al. (1994) described the anti-ischemic effect induced by ventricular overdrive pacing as a conscious rabbit model of preconditioning. Rabbits were equipped with right ventricular electrode catheters for pacing and intracavital recording and polyethylene cannulae in the left ventricle and right carotid artery to measure intraventricular pressure and blood pressure. One week after surgery in conscious animals, ventricular overdrive pacing at 500 beats/min over 2, 5, or 10 min resulted in an intracavital S-T segment elevation, shortening of ventricular effective refractory period, decrease in maximum rate of pressure development and blood pressure, and increase in left ventricular end-diastolic pressure proportional to the duration of stimulus. A 5 min preconditioning ventricular overdrive pacing applied 5 or 30 min before a 10 min ventricular overdrive pacing markedly attenuated ischemic changes, whereas a 2 min ventricular overdrive pacing had no effect.

The ventricular overdrive pacing-induced preconditioning effect was lost in atherosclerotic rabbits (Szilvássy et al. 1995); however, delayed cardiac protection could be induced in these animals (Szekeres et al. 1997).

Kharbanda et al. (2002) used an experimental model of myocardial infarction in pigs to demonstrate transient ischemia of the hind limb using a tourniquet to reduce myocardial IR injury. Others (Shimizu et al. 2009) have shown that plasma dialysate from preconditioned animals provides potent cardioprotection of naïve hearts in Langendorff preparation.

Merlocco et al. (2014) described a novel method for inducing cardioprotection by transcutaneous electrical nerve stimulation (TENS). Rabbits were subjected to lower limb TENS, and cardioprotection was evaluated in a Langendorff preparation. TENS reduced infarct size and improved functional recovery during reperfusion.

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MRI Studies of Cardiac Function

Purpose and Rationale

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin–lattice relaxation time (T_1) and the spin–spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1 mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water, and because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as gray matter or lesions, have higher T_1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected (0.1–0.3 mmol kg^{-1}) and, in areas of increased blood–brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1-weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

MRI has emerged as a highly accurate and quantitative tool for the evaluation of cardiac function (Peshock et al. 1996).

Al-Shafei et al. (2002a, b) performed MRI analysis of cardiac cycle events in diabetic rats and tested the effect of angiotensin-converting enzyme inhibition.

Procedure

Diabetes was induced in Wistar rats at an age of 7, 10, and 13 weeks. The rats were anesthetized using 1–2 % halothane in oxygen, and their blood glucose levels were checked. They were then given a single intraperitoneal injection of streptozotocin 65 mg/kg body weight. The control rats received sham injections of the citrated buffer when they were 7 weeks old. One diabetic group was treated with 2 g/l captopril in the drinking water.

For **MR imaging**, rats were anesthetized using 1–2 % halothane in oxygen, weighed, and their systolic blood pressures measured noninvasively using a rat tail blood pressure monitor both before and after imaging sessions to confirm physiological stability. Electrocardiographic (ECG) monitoring used shielded subcutaneous electrodes and a Tektronix 2225 oscilloscope. The cine imaging protocols were performed with the anesthetized animal placed in a specially designed home-built half-sine-spaced birdcage radiofrequency (RF) probe unit contained within a cylindrical plastic holder fitted within a gradient set of internal diameter 11 cm. The RF probe unit was made up of a half-sine-spaced birdcage RF probe of internal diameter of 4.5 cm with open ends, an RF shield consisting of a cylinder of copper gauze surrounding and sliding over the birdcage, a tuning capacitor, and a coaxial cable to carry the RF (Ballon et al. 1990). The assembly included ECG leads, attachment plugs for the ECG leads, and a unit to anchor anesthetic delivery tubes near the nose of the animal. All experiments used a 2T Oxford Instrument (UK) superconducting magnet with a horizontal internal bore of 31 cm. A gated cine protocol synchronized line acquisition to set times following alternate electrocardiographicR waves. This acquisition was then repeated at the same slice position at 12 equally incremented times through the cardiac cycle. This sequence in turn was repeated for each of the 128 lines to generate each 128 × 128 image, which itself was acquired twice for signal averaging. The preceding procedure was in turn repeated 12 times to obtain signal-averaged images for every one of the 12 contiguous transverse slices examined. Each imaging session therefore required (128 × 12 × 2 × 2) times the cardiac cycle duration. The effective repeat time (TR) was approximately 13 ms. The short echo time (TE) of 4.3 ms reduced motion artifacts and ensured good contrast between blood and myocardium.

Evaluation

The image data were transferred from the MRI console using in-house hardware and software to remote UNIX workstations for quantitative analysis using in-house software based on CaMReS libraries (CaMReS, Dr N. J. Herrod, Herchel Smith Laboratory for Medicinal Chemistry, University of Cambridge).

Modifications of the Method

Itter et al. (2004) used noninvasive MRI techniques in a model of chronic heart failure in spontaneously hypertensive rats.

Bryant et al. (1998) and Franco et al. (1999) described MRI and invasive evaluation of development of heart failure in transgenic **mice** with myocardial expression of tumor necrosis factor-α.

Wiesmann et al. (2002) reported analysis of right ventricular function in healthy mice and a murine model of heart failure by in vivo MRI.

Kraitchman et al. (2003) described quantitative ischemia detection during cardiac magnetic resonance stress testing by use of fast harmonic phase MTI (FastHARP) in dogs.

Reddy et al. (2004) discussed the feasibility of a porcine model of healed myocardial infarction by integration of cardiac MRI with three-

dimensional electroanatomic mapping to guide left ventricular catheter manipulation.

Pelzer et al. (2005) reported that the estrogen receptor-α agonist 16α-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. Improved left ventricular function upon 16α-LE2 treatment was also observed in cardiac MRI studies.

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MRI Studies After Heart and Lung Transplantation

Purpose and Rationale

Acute cardiac allograft rejection continues to be the cause of graft loss and contributes to the morbidity and mortality after cardiac transplantation. Endomyocardial biopsy is used routinely for cardiac transplant rejection surveillance. A sensitive and noninvasive method for detecting rejection is desirable. Kanno et al. (2001) developed a rat model of heterotopic heart and lung transplantation for MRI experiments. Allotransplantations were performed with syngeneic transplantations serving as controls. MR images were obtained with a gradient echo method.

Procedure

Animals

All rats used in the experiments were male, 2–3 months of age, and weighed 220–250 g each. Animals were housed individually and provided with food and water ad libitum. Inbred Brown Norway (BN; $RT1^n$) and DA ($RT1^a$) rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind).

Heart and Lung Transplantation

Under anesthesia with injection of 35 mg/kg body weight of sodium pentobarbital IP, 500 U/kg body weight of heparin was injected. In the syngeneic group, an en bloc donor heart and lung were taken from a BN rat and transplanted to another BN rat. In the allogeneic group, a graft from a DA rat was transplanted to a BN rat. This group was divided into two groups: one group was treated with 3 mg/kg per day cyclosporine (CsA), and the other group was not given CsA. Graft survival was monitored every day by palpating contraction of the transplanted heart.

Operative procedures have been described by Kanno et al. (2000). In brief, after the chest wall of the donor rat was opened, the left lung was ligated and excised. The azygos vein with the left superior and right superior venae cavae was ligated and divided. The descending thoracic aorta was transected, and 10 ml of cold University of Wisconsin solution (UW solution, Dupont Pharma) was infused into the inferior vena cava until the fluid draining from the aorta was clear, followed by ligation and division of the inferior vena cava. The ascending aorta was dissected and transected at the portion between the left common carotid artery and the left subclavian artery, followed by ligation and division of the right brachiocephalic artery and the left common carotid artery. After removal of the heart and lung from the donor, the right lung was washed three times through the bronchus with UW solution containing penicillin G. The grafts were then placed into cold UW solution for ≈5 min until transplantation. Next, the left inguinal portion of the recipient rat was opened and dissected to make enough space for the transplanted organs. The left lower part of the abdominal wall was opened in a transverse fashion from the left femoral vessels to the midline. The abdominal organs were retracted to the right, and both the aorta and the inferior vena cava just beyond the bifurcation were dissected. The vessels were clamped, and an appropriate opening of the aorta was made to receive the aorta of the graft in an end-to-side fashion. Rhythmic heartbeats commenced spontaneously as the heart and the lung regained circulation after removal of the clamp. After hemostasis of the surgical field, the abdominal wall was sutured, with care taken not to kink or obstruct the aorta of the graft.

MRI Experiments

MRI measurements were carried out on a 4.7-T/40 cm Bruker AVANCE DRX MR instrument equipped with 15 cm, 10 gauss/cm shielded gradients. In vivo MR images of transplanted heart–lung were obtained over a period of 24 h after infusion of dextran-coated ultrasmall superparamagnetic iron oxide (USPIO) particles. The imaging sequence consisted of a gradient echo sequence, triggered to ECG and ventilator (60 strokes/min, 10 ml/kg), with TR/TE 500/10 ms, flip angle equal to Ernst angle, slice thickness 1 mm, field of view 6.0 cm, data matrix size 256 × 130 (zero filled to 256 × 256), and scan time 5 min. ECG leads were placed on both of the hind limbs of the rat with the transplant to pick up the heartbeat from the transplanted heart more effectively. The change of MRI signal intensity was measured in whole ventricular wall in each transplanted heart. The MR signal intensity of the heart was normalized to that of the leg muscle, because USPIO particles are not readily taken up by muscular tissue, according to Gellissen et al. (1999).

Dextran-coated USPIO particles were synthesized according to the method of Palmacci and Josephson (1993) with slight modifications (Dodd et al. 1999). The MR relaxivities R_1 (spin–lattice relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) and R_2 (spin–spin relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) measured at 4.7 T were 3.8×10^4 and 9.1×10^4 (mol/l)/s, respectively. For in vivo studies, dextran-coated USPIO particles were dialyzed against PBS solution and diluted to a concentration of 18 μmol Fe/ml, and 0.8 ml of the suspension (i.e., ≈3 mg Fe/kg body weight) was injected intravenously for each study.

At 6 days after transplantation, dextran-coated USPIO particles were injected intravenously as mentioned above, and the animals were subjected to MRI. Then, 24 h later, these animals were again placed inside the magnet and scanned. The regions of interest were defined manually with Bruker software. MR signal intensity in the entire ventricular wall in the plane was measured. After injection of USPIO particles at postoperative day (POD) 6, animals with allotransplants were given CsA for 4 (POD 7–10) or 7 (POD 7–13) days and reinjected with USPIO particles on POD 14.

Pathological Analysis and Immunohistochemistry

After an MR experiment was completed, the transplanted hearts were extirpated, fixed in 3.7 % formaldehyde, and embedded in paraffin for 5 5 µm mu;m sections. Hematoxylin–eosin staining and Perl's Prussian blue staining were performed in the Transplantation Pathology Laboratory of the University of Pittsburgh Medical Center. Histological analysis for pathological grading of heart rejection, which is based on the criteria established by the International Society for Heart and Lung Transplantation, was also performed by this laboratory in a blinded manner. Monoclonal antirat macrophage antibody (ED1, Serotec) was used as a primary antibody for macrophages. Immunohistochemistry was carried out with the ABC staining system (Santa Cruz Biotechnology) according to the manufacturer's protocol.

Evaluation

The results are presented as mean ± SD. The results were analyzed by ANOVA with StatView software (SAS Institute). A value of $P < 0.05$ was considered to be statistically significant.

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Ex Vivo Methods

Plastic Casts from Coronary Vasculature Bed

Purpose and Rationale

Prolonged administration of coronary drugs has been shown to increase the number and size of interarterial collaterals of dogs and pigs after coronary occlusion (Vineberg et al. 1962; Meesmann and Bachmann 1966). An increased rate of development of collateral arteries was observed after physical exercise in dogs (Schaper et al. 1965), as well as after chronic administration of coronary dilating drugs (Lumb and Hardy 1963). An even more effective stimulus for collateral development is an acute or gradual occlusion of one or several major coronary branches. Filling the arterial coronary bed with a plastic provides the possibility to make the collaterals visible and to quantify them (Schmidt and Schmier 1966; Kadatz 1969).

Procedure

Dogs weighing 10–15 kg are anesthetized with pentobarbital sodium 30 mg/kg i.v. They are respirated artificially and the thorax is opened. After opening of the pericard, ameroid cuffs are placed around major coronary branches. Gradual

swelling of the plastic material occludes the lumen within 3–4 weeks. The dogs are treated daily with the test drug or placebo. After 1 week recovery period, they are submitted to exercise on a treadmill ergometer. After 6 weeks treatment, the animals are sacrificed, the heart removed, and the coronary bed flushed with saline. The liquid plastic Araldite is used to fill the whole coronary tree from the bulbus aortae. The aortic valves are glued together in order to prevent filling of the left ventricle. Red colored Araldite is used to fill the arterial tree. The venous part of the coronary vasculature can be filled with blue colored Araldite from the venous sinus. The uniformity of the filling pressure, the filling time, and the viscosity of the material are important. Polymerization is complete after several hours. Then, the tissue is digested with 35 % potassium hydroxide. The method gives stable preparations which can be preserved for a long time.

Evaluation

Plastic casts from drug-treated animals are compared with casts from dogs submitted to the same procedure without drug treatment.

Critical Assessment of the Method

The procedure allows impressive demonstration of the formation of arterial collaterals. The results of postmortem Araldite impletion agree with the functional results of experimental coronary occlusion.

Modifications of the Method

Boor and Reynolds (1977) described a simple planimetric method for the determination of left ventricular mass and necrotic myocardial mass in postmortem hearts.

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Calcium Uptake Inhibition Activity

Michael Gralinski, Liomar A. A. Neves, and Olga Tiniakova

Contents

General Considerations

Cellular calcium flux is regulated by receptor-operated and voltage-dependent channels, which are sensitive to inhibition by calcium entry blockers. The term calcium antagonist was introduced by (Fleckenstein 1964; Fleckenstein et al. 1967) when two drugs, prenylamine and verapamil, originally found as coronary dilators in the Langendorff experiment, were shown to mimic the cardiac effects of simple Ca^{2+} withdrawal, diminishing Ca^{2+}-dependent high-energy phosphate utilization, contractile force, and oxygen requirement of the beating heart without impairing the Na^{+}-dependent action potential parameters. These effects were clearly distinguishable from β-receptor blockade and could promptly be neutralized by elevated Ca^{2+}, β-adrenergic catecholamines, or cardiac glycosides, measures that restore the Ca^{2+} supply to the contractile system. In the following years, many Ca^{2+} antagonists were introduced to therapy. Specific Ca^{2+} antagonists interfere with the uptake of Ca^{2+} into the myocardium and prevent myocardial necrotization arising from deleterious intracellular Ca^{2+} overload. They act basically as specific inhibitors of the slow transsarcolemnal Ca^{2+} influx but do not or only slightly affect the fast Na^{+} current that initiates normal myocardial excitation.

Calcium channels and the sites of action of drugs modifying channel function have been classified (Bean 1989; Porzig 1990; Tsien and Tsien 1990; Spedding and Paoletti 1992).

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Nomenclature and structure–function relationships of voltage-gated calcium channels were reviewed by Catterall et al. (2005).

Kochegarow (2003) reviewed the therapeutic application of modulators of voltage-gated calcium channels.

Four main types of voltage-dependent calcium channels are described:

1. L type (for long lasting)
2. T type (for transient)
3. N type (for neuronal)
4. P type (for Purkinje cells)

They differ not only by their function (Dolphin 1991) and localization in tissues and cells but also by their sensitivity to pharmacological agents (Ferrante and Triggle 1990; Dascal 1990; Kitamura et al. 1997) and by their specificity to radioligands.

The widely distributed **L-type channels** exist in isoforms (L1, L2, L3, L4) and consist of several subunits, known as α_1, α_2, β, γ, and δ. They are sensitive to dihydropyridines, phenylalkylamines, or benzothiazepines, but insensitive to ω-conotoxin and ω-agatoxin. The segments required for antagonist binding have been analyzed (Peterson et al. 1996; Schuster et al. 1996; Mitterdorfer et al. 1996; Hockerman et al. 1997; Striessnig et al. 1998; Catterall 1998).

Berjukow et al. (2000) discussed the molecular mechanism of calcium channel block by isradipine.

Striessnig et al. (2004) described the role of L-type Ca^{2+} channels in Ca^{2+} channelopathies.

The **T-type channels** are located mainly in the cardiac sinoatrial node and have different electrophysiological characteristics from L-type channels (Massie 1997; Perez-Reyes et al. 1998).

Reviews of the molecular physiology of low-voltage-activated T-type calcium channels were given by Perez-Reyes (2003, 2006).

N- and P-type calcium channel blockers occur in neuronal cells and are involved in neurotransmitter release (Olivera et al. 1987; Bertolino and Llinás 1992; Mintz et al. 1992; Woppmann et al. 1994; Diversé-Pierluissi et al. 1995; Miljanich and Ramachandran 1995; Fisher and Bourque 1996; Ikeda 1996; Ertel et al. 1997; Sinnegger et al. 1997).

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In Vitro Methods

^{3}H-Nitrendipine Binding in Vitro

Purpose and Rationale

Radiolabeled dihydropyridine calcium channel antagonists such as ^{3}H-nitrendipine (Salter and Grover 1987; Campiani et al. 1996) are selective ligands for a drug receptor site associated with the voltage-dependent calcium channel. A constant concentration of the radioligand ^{3}H-nitrendipine (0.3–0.4 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine

cerebral cortices. If the test drug exhibits any affinity to calcium channels, it is able to compete with the radioligand for channel binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug.

Procedure

Materials and solutions

Preparation buffer	Tris–HCl pH 7.4	50 mM
Incubation buffer	Tris–HCl Genapol	50 mM
	pH 7.4	0.001 %
Radioligand	^{3}H-nitrendipine specific activity 2.59–3.22 TBq/mmol (70–87 Ci/mmol) (NEN)	
For inhibition of ^{3}H-nitrendipine binding in nonspecific binding experiments	Nifedipine (Sigma)	

Two freshly slaughtered bovine brains are obtained from the slaughterhouse and placed in ice-cold preparation buffer. In the laboratory, approx. 5 g wet weight of the two frontal cerebral cortices is separated from the brains.

Membrane Preparation

The tissue is homogenized (glass Teflon potter) in ice-cold preparation buffer, corresponding to 1 g cerebral wet weight/50 ml buffer, and centrifuged at 48,000*g* (4 °C) for 10 min. The resulting pellets are resuspended in approx. 270 ml preparation buffer, and the homogenate is centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebral cortex wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at −77 °C. The protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 48,000*g* (4 °C) for 10 min. The resulting pellets are resuspended in a volume of ice-cold incubation buffer, yielding a membrane suspension with a protein content of 0.6–0.8 mg/ml. After homogenization (glass Teflon potter), the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

As 1,4-dihydropyridines tend to bind to plastic material, all dilution steps are done in glass tubes.

For each concentration, samples are prepared in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

Total binding:

- 50 µl ^{3}H-nitrendipine (12 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl incubation buffer

Nonspecific binding:

- 50 µl ^{3}H-nitrendipine (4 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl nifedipine (5×10^{-7} M)

Competition Experiments

- 50 µl ^{3}H-nitrendipine (1 constant concentration, 3–4 $\times 10^{-10}$ M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (0.6–0.8 mg protein/ml). The samples are incubated for 60 min in a bath shaker at 25 °C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free radioactivity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding
- Nonspecific binding
- Specific binding = total binding - nonspecific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-nitrendipine versus non-labeled drug by a computer-supported analysis of the binding data.

$$\frac{K_D{}^3\mathrm{H} \times \mathrm{IC}_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

where

IC_{50} = concentration of the test drug, which displaces 50 % of specifically bound ^{3}H-nitrendipine in the competition experiment

$[^3H]$ = concentration of ^{3}H-nitrendipine in the competition experiment

$K_D{}^3H$ = dissociation constant of ^{3}H-nitrendipine, determined from the saturation experiment

The K_i value of the test drug is the concentration at which 50 % of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data:

- Nifedipine $K_i = 2 - 4 \times 10^{-9}$ mol/l

Modifications of the Method

[^{3}H](+)-PN200–100 (isradipine) has been used by many authors as the labeled ligand for binding experiments (Grassegger et al. 1989; Nokin et al. 1990; Striessnig et al. 1991; Yaney et al. 1991; Miwa et al. 1992; Ichida et al. 1993; Kalasz et al. 1993; Ikeda et al. 1994; Rutledge and Triggle 1995; Shimasue et al. 1996; He et al. 1997; Natale et al. 1999; Matthes et al. 2000; Peri et al. 2000).

Yaney et al. (1991) performed binding experiments with [^{3}H](+)-PN200–110 to membranes of RINm5F cells.

The RINm5F pancreatic β-cells were grown on plastic culture flasks in medium RPMI 1640 supplemented with 10 % fetal bovine serum, 100 µg/ml of streptomycin, and 100 U/ml penicillin. The cells were kept in humidified incubators at 37 °C in 5 % CO_2-95 % air. Maintenance flasks were subcultured every 4–5 days at ~80 % confluency. For binding experiments of [^{3}H](+)-PN200–110 to membranes, cells were removed from the flasks by incubation for 15–20 min in 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] and 1 mM EGTA (pH 7.4) at 4 °C. The osmotically ruptured cells were homogenized with a Brinkmann Polytron followed by centrifugation at 20,000*g* for 15 min. The pellet was resuspended and washed twice. The final pellet was resuspended in 1 mM $CaCl^2$ and 20 mM MOPS (pH 7.4) at a protein concentration of 0.2–0.4 mg/ml. Membranes were incubated at room temperature with (+)-[^{3}H]PN200–110 in a final volume of 500 µl (Weiland and Oswald 1985). Bound and free radioligands were separated by vacuum filtration through a glass fiber filter (no. 32, Schleicher and Schüell) followed by three washes of the filter with 3 ml incubation buffer. Nonspecific binding was determined in the presence of 1 µM nitrendipine. Radioactivity retained by the filters was determined by liquid scintillation counting. The concentration of free radioligand was calculated by scintillation counting of aliquots of incubation mixtures under nonspecific conditions before and after centrifugation.

Several other calcium entry blockers, such as nimodipine, diltiazem, verapamil, and desmethoxyverapamil, have been labeled and used for binding studies in order to elucidate the calcium channel recognition sites and may be used for further classification of calcium antagonists (Ferry and Glossmann 1982; Glossmann et al. 1983; Goll et al. 1984; Lee et al. 1984; Glossmann et al. 1985a, b; Schoemaker and Langer 1985; Ruth et al. 1985; Reynolds et al. 1986).

Vaghy et al. (1987) identified a 1,4-dihydropyridine- and phenylalkylamine-binding polypeptide in calcium channel preparations.

Naito et al. (1989) described photoaffinity labeling of the purified skeletal muscle calcium antagonist receptor by [^{3}H]azidobutyryl diltiazem.

Watanabe et al. (1993) reported that azidobutyryl clentiazem labels the benzothiazepine binding sites in the α_1 subunit of the skeletal muscle calcium channel. Tissue heterogeneity of calcium channel antagonist binding sites has been demonstrated by Gould et al. (1983).

Photoaffinity labeling of the cardiac calcium channel with 1,4-dihydropyridine(−)-[^{3}H] azidopine was described by Ferry et al (1987).

Knaus et al. (1992) described a unique fluorescent phenylalkylamine probe for L-type Ca^{2+} channels.

Binding sites for ω-conotoxin appear to be primarily associated with the N type of voltage-dependent calcium channels (Feigenbaum et al. 1988; Wagner et al. 1988).

Cohen et al. (1992) recommended the peptide ω-agatoxin IIIA as a valuable pharmacological tool being the only known ligand that blocks L-type calcium channels with high affinity at all voltages and causes, unlike the 1,4-dihydropyridines, no block of T-type calcium channels.

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Calcium Antagonism in Isolated Organs

Calcium Antagonism on Action Potential of Isolated Guinea Pig Papillary Muscle

Purpose and Rationale

Intracellular action potential in the guinea pig papillary muscle is recorded. Partial depolarization is achieved by potassium-enriched Ringer solution and by addition of isoproterenol. Resting potential is increased to 40 mV resulting in inactivation of the fast sodium channel. Under these conditions, upstroke velocity is an indicator for calcium flux through the membrane, which is decreased by calcium blockers.

Procedure

Guinea pigs of either sex (Pirbright White strain) weighing 300–400 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Initially, normal Ringer solution oxygenated with carbogen ($95\%O_2/5\%CO_2$) at a temperature of 36 °C is used. A standard microelectrode technique is applied to measure the action potential via a glass microelectrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at intervals of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold. The intracellular action potential is amplified; differentiated for registration of upstroke velocity (Hugo-Sachs microelectrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000); and recorded (Gould 2400 recorder).

After an incubation period of 30 min, the Ringer solution is changed to the following composition containing 5 times more potassium and 10 % less sodium:

- NaCl 8.1 g/l
- KCl 1.0 g/l
- $CaCl_2$ 0.2 g/l
- $NaHCO_3$ 0.1 g/l
- Glucose 5.0 g/l

For further depolarization, isoproterenol (1.0 mg per 100 ml) is added. By this measure, resting potential is increased to about 40 mV, resulting in inactivation of the fast inward sodium channel. The resulting slow rising action potential is sensitive to calcium antagonistic drugs (Kohlhardt and Fleckenstein 1977).

The test compound is added at a concentration of 1 μg/ml. Effective compounds are tested at lower concentrations and compared with the standard (nifedipine at concentrations of 0.01 and 0.1 μg/ml).

Evaluation

The decrease of upstroke velocity is tested at various concentrations of the test compound and compared with the standard.

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Calcium Antagonism in the Isolated Guinea Pig Atrium

Purpose and Rationale

k-Strophanthine (and other cardiac glycosides) inhibits the membrane-bound Na^{+}-/K^{+}-activated ATPase which leads to an increase in intracellular Ca^{2+} concentration. Ca^{2+} ions activate the contractile apparatus, causing a distinctive enhancement of contractions. The procedure can be used to evaluate a compound's calcium channel blocking activity by measuring its ability to decrease atrial contractions induced by k-strophanthine.

Procedure

Apparatus

HSE-stimulator 1 (Hugo-Sachs Elektronik, D-79232 March-Hugstetten, Germany)

Stimulation data:

- Frequency 1.5 Hz
- Duration 3 ms
- Voltage 3–8 V

Experiment

Guinea pigs of either sex weighing 200–500 g are sacrificed with a blow to the nape of the neck and exsanguinated. The left atrium is removed, placed in an organ bath, and attached to an isotonic strain gauge, its base being wired to an electrode of the stimulator. The Ringer solution is aerated with carbogen and kept at 36 °C. The atrium is continuously stimulated via stimulator 1, the voltage being slowly increased up to the threshold level. Contractions are recorded on a polygraph. Prior to drug administration, two prevalues are obtained by adding 2 μg/ml k-strophanthine-α (Cymarin) to the organ bath and measuring the increase in contractile force. Following a 15 min washout and recovery period, the test drug is added to the bath followed by administration of k-strophanthine-α 10 min later. The change in contractile force is always measured 10 min after the addition of k-strophanthine-α.

Standard compounds:

- Verapamil hydrochloride
- Nifedipine

Evaluation

The percent inhibition of k-strophanthine-α-induced contraction is determined.

Modifications of the Method

Calcium antagonists can also be evaluated in the Langendorff heart preparation (Lindner and Ruppert 1982).

Leboeuf et al. (1992) reported the protective effect of bepridil and flunarizine against veratrine-induced contracture in rat atria concluding from the results in this model that these agents may be more effective as L-type calcium ion channel blockers in protecting against calcium overload during ischemia and reperfusion injury.

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Calcium Antagonism in the Isolated Rabbit Aorta

Purpose and Rationale

Contraction of aorta rings is induced by adding potassium chloride or norepinephrine to the organ bath containing slightly modified Krebs bicarbonate buffer. Test drugs with calcium channel blocking activity have a relaxing effect.

Procedure

Rabbits of either sex weighing 3–4 kg are sacrificed with an overdose of pentobarbital sodium. The chest cavity is opened, and the descending thoracic aorta (from the level of the aortic arch to the level of the diaphragm) is rapidly removed and placed in a beaker of oxygenated Krebs bicarbonate buffer at 37 °C.

The content of magnesium and calcium is slightly diminished in the Krebs bicarbonate buffer resulting in the following composition:

- NaCl 118.4 mMol
- KCl 4.7 mMol
- KH_2PO_4 1.2 mMol
- $MgSO_4{}^{\circ}2H_2O$ 1.2 mMol
- $CaCl_2{}^{\circ}2H_2O$ 1.9 mMol
- $NaHCO_3$ 25.0 mMol
- Dextrose 10.0 mMol
- EDTA 0.013 mMol

The tissue is then transferred to a dish containing fresh oxygenated, warmed Krebs solution. Fat and loose connective tissue is carefully removed while keeping the tissue moist with the solution. Eight rings of 4–5 mm width are obtained and each is mounted in a 20 ml tissue bath which contains the oxygenated warmed Krebs solution. Initial tension is set at 1.0 g. The tissue is allowed to incubate over a period of 2 h, during which time the Krebs solution is changed every 15 min. Also during this time, tension is maintained at 1.0 g. Just prior to the end of the 2 h equilibration period, the Krebs solution is changed again and the tissue is allowed to stabilize at 1.0 g tension. A sustained contraction is then generated by addition of either 40 mM KCl or 2.9×10^{-3} mM norepinephrine.

Twenty min after addition of the agonist, the test drug is added so that the final concentration in the bath is 1×10^{-5} M. The percent relaxation reading is taken 30 min after addition of the test drug. If at least 30 % relaxation occurs, an accumulative concentration–relaxation curve is established. There is a 30 min period of time between the addition of each concentration of test compound.

Evaluation

Active tension is calculated for the tissue at the time point just prior to the addition of the test compound and also at the point 30 min after the addition of each concentration of test compound. Active tension is defined as the difference between the generated tension and the baseline tension. The percent relaxation from the predrug, precontracted level is calculated for each concentration of test compound. A number of 5 experiments constitute a dose range. An ID_{50} is calculated by linear regression analysis.

Modifications of the Method

Hof and Vuorela (1983) compared three methods for assessing calcium antagonism on rabbit aorta smooth muscle.

Matsuo et al. (1989) reported a simple and specific screening method for Ca entry blockers. In the presence of various Ca channel blockers, 1×10^{-4}M Ca^{2+} causes relaxation of rat uterine smooth muscle that has been tonically contracted with oxytocin in calcium-free medium after prolonged preincubation with 3 mM EGTA.

Micheli et al. (1990) used spirally cut preparations of rat aorta and rings of rabbit ear artery to test calcium entry blocker activity.

Rüegg et al. (1985) described a smooth muscle cell line originating from fetal rat aorta to be suitable for the study of voltage-sensitive calcium channels. Calcium channel antagonists inhibited both the basal and the potassium chloride-stimulated $^{45}Ca^{2+}$ uptake.

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Calcium Antagonism in the Isolated Guinea Pig Pulmonary Artery

Purpose and Rationale

Contraction of the pulmonary artery is induced by changing the normal Tyrode solution in the organ bath against a potassium-enriched solution. This contraction can be inhibited by calcium blockers.

Procedure

The following solutions are used:

	Normal Tyrode solution [mMol]	Potassium-enriched Tyrode solution [mMol]
NaCl	135.0	89.0
KCl	3.7	50.0
$MgSO_4$	0.81	0.81
NaH_2PO_4	0.41	0.41
$NaHCO_3$	11.0	11.0
$CaCl_2$	2.25	2.25
Glucose	5.6	5.6

Guinea pigs (Pirbright White strain) of either sex weighing 400–500 g are sacrificed by stunning. The pulmonary artery is removed and cut spirally at an angle of 45°. The resulting strip is cut to lengths of 2 cm, and one piece is suspended in oxygenated normal Tyrode solution in an organ bath at 37 °C with a preload of 1 g. Contractions

are registered with an isotonic strain transducer and recorded on a polygraph.

After 1 h equilibrium time, normal solution is exchanged with potassium-enriched Tyrode solution. The artery strip reacts with a contraction which achieves after 10 min 90–95 % of its maximum. After an additional 10 min, exchange to normal Tyrode solution is performed. Ten minute later, again a contraction is induced by potassium-enriched solution. When the height of the contraction has reached a constant level, the test substance is added and again potassium-induced contraction recorded. The height of the contraction is expressed as percent of initial potassium-induced contraction.

After lavage, the procedure is repeated with a higher dose or the standard.

Evaluation

For calculation of a regression line, the decrease of contraction versus control after various doses is measured in mm. The percentage of inhibition after various doses is taken for calculation of an ED_{50}.

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In Vivo Methods

Evaluation of Calcium Blockers in the Pithed Rat

Purpose and Rationale

Using the cardioaccelerator response in pithed rats, calcium entry blockers can be distinguished from other agents which have modes of action not involving direct blockade of calcium entry (Clapham 1988).

Procedure

Male Sprague–Dawley rats (250–350 g) are anesthetized with methohexitone sodium (50 mg/kg i.p.). Following cannulation of the trachea, the rats are pithed through one orbit with a stainless steel rod and immediately artificially respired with room air (78 strokes/min, 1 ml/100 g body weight) via a Palmer small animal respiration pump. A jugular vein is cannulated for administration of drugs. Arterial blood pressure is recorded from a carotid artery using a pressure transducer. Heart rate is derived from the phasic arterial pressure signal with a phase lock loop ratemeter (BRL Instrument Services). Both parameters are displayed on a recorder. The animals are kept warm by an incandescent lamp positioned about 25 cm above them. The pithing rod is withdrawn so that the tip lays in the thoracic portion of the spinal cord. All rats then receive (+)tubocurarine (1.5 mg/kg i.v.) and are bilaterally vagotomized.

The cardioaccelerator response is obtained by continuous electrical stimulation of the thoracic spinal cord with square wave pulses of 0.5 ms duration, at supramaximal voltage at a frequency of 0.5 Hz using the pithing rod as a stimulating electrode. An indifferent electrode is inserted subcutaneously in the femoral region. Only rats with a resulting tachycardia of more than 100 beats/min are included into the experiments.

When the cardioaccelerator response has stabilized for about 3–5 min, cumulative intravenous doses of the drug or corresponding vehicle are administered. Succeeding doses are

given when the response to the previous dose has stabilized.

Calcium antagonists and β-blockers inhibit dose-dependent tachycardia elicited by electrical stimulation of the spinal cord, whereas lignocaine and nicorandil are not effective.

Doses of β-blockers or calcium antagonists, which reduce the tachycardia to 50 %, are tested again. Three min after administration of the drug, calcium gluconate (1 mg/min) or water (0.1 ml/min) is infused using a Harvard apparatus compact infusion pump. The effects of calcium entry blockers, but not of β-adrenoreceptor blockers, are antagonized.

Evaluation

The level of tachycardia immediately prior to drug administration is taken as 100 %, and responses to drugs are expressed as a percentage of this predose tachycardia. If an inhibitory effect >50 % is seen, then an ID_{50} (with 95 % confidence limits) is interpolated from linear regression analysis. Significance of differences between the groups receiving calcium gluconate and their parallel vehicle controls is calculated by Student's *t*-test.

Critical Assessment of the Method

Differentiation between the effects of β-blockers and calcium antagonists can be achieved in a relatively simple in vivo model.

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Anti-Arrhythmic Activity

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General Considerations

Guidelines for the study of arrhythmias in man and animals regarding the experimental design as well as the classification, quantification, and analysis were given as the Lambeth Conventions by Walker et al. (1988).

Classification

Antiarrhythmic drugs have been classified into various groups and subgroups (Vaughan Williams 1970, 1975, 1984, 1988, 1991, 1992; Borchard et al. 1989; Frumin et al. 1989; Harumi et al. 1989; Colatsky and Follmer 1990; Podrid et al. 1990; Coromilas 1991; Nattel 1991; Rosen and Schwartz 1991; Scholz 1991; Woosley 1991; Ravens 1992; Sanguinetti 1992; Grant 1992; Nattel 1993; Scholz 1994). This classification is based on electrophysiological effects (e.g., action potential) and on interaction with membrane receptors and ion channels. The heterogeneity of classification criteria resulted in vivid discussions (The Sicilian Gambit 1991; Vaughan Williams 1991, 1992). In particular, a clinical study (CAST Investigators 1990) challenged the therapeutic value of some antiarrhythmic drugs.

Weirich and Antoni (1990, 1991) proposed a subdivision of class 1 antiarrhythmic drugs according to the saturation behavior of frequency-dependent block and its onset kinetics.

Class I antiarrhythmic drugs directly alter membrane conductance of cations, particularly those of Na^+ and K^+. They reduce upstroke velocity, V_{max}, of the cardiac action potential by blockade of the fast Na^+ channel. This leads to a depression of conduction velocity, a prolongation of the voltage- and time-dependent refractory period and an increase in the threshold of excitability in cardiac muscle. Class I antiarrhythmic drugs are subclassified according to their effect on the action potential duration.

Class IA antiarrhythmic drugs (quinidine-like substances, e.g., disopyramide, procainamide, ajmaline) lengthen the action potential duration which is reflected in the ECG as lengthening of

the QT interval. This effect is added to that on fast sodium channel resulting in delayed recovery from inactivation.

Class IB antiarrhythmic drugs (lidocaine-like drugs, e.g., mexiletine, phenytoin, tocainide), in contrast, shorten the action potential duration.

Class IC antiarrhythmic drugs (e.g., encainide, flecainide, propafenone, indecainide) produce quinidine- and lidocaine-like effects and exert differential actions on the duration of action potential in Purkinje fibers (shortening) and ventricular muscle.

Class II antiarrhythmic drugs are β-adrenergic antagonists. They exert their antiarrhythmic effects by antagonizing the electrophysiological effects of catecholamines which are mainly mediated by an increase in slow calcium inward current.

Class III antiarrhythmic drugs (e.g., dofetilide, amiodarone, bretylium, sotalol) prolong the action potential and lead to a corresponding increase in the effective refractory period. The action is mainly due to a block of outward repolarizing currents. However, activation of sodium and calcium inward currents that prolong the plateau of the action potential may also be involved.

Class IV antiarrhythmic drugs (e.g., verapamil, diltiazem) are slow calcium channel blockers suppressing the slow calcium inward current and calcium-dependent slow action potentials.

Experimentally Induced Arrhythmias

Winslow (1984) reviewed the methods for the detection and assessment of antiarrhythmic activity.

Szekeres (1979) suggested a rational screening program for the selection of effective antiarrhythmic drugs.

Arrhythmia models in the rat were reviewed by Cheung et al. (1993).

Arrhythmogenic stimuli can be divided into three groups: chemical, electrical, and mechanical (Szekeres and Papp 1975; Wilson 1984).

Chemically Induced Arrhythmias

A large number of chemical agents alone or in combination are capable of inducing arrhythmias. Administration of anesthetics like chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus, such as intravenous adrenaline, or cardiac glycosides (usually ouabain), aconitine, and veratrum alkaloids, causes arrhythmias. The sensitivity to these arrhythmogenic substances differs among various species.

Electrically Induced Arrhythmias

The possibilities to produce arrhythmias by electrical stimulation of the heart and the difficulties for evaluation of antiarrhythmic drugs by this approach have been discussed by Szekeres (1971). Serial electrical stimulation results in flutter and fibrillation, and it is possible to reproduce some of the main types of arrhythmias of clinical importance. The flutter threshold or the ventricular multiple response threshold may be determined in anesthetized dogs before or after the administration of the test drug.

Mechanically Induced Arrhythmias

Arrhythmias can be induced directly by ischemia or by reperfusion. After ischemia either by infarction or by coronary ligation, several phases of arrhythmias are found. The two-stage coronary artery ligation technique described by Harris (1950) focuses on late arrhythmias.

Curtis and Walker (1988) examined seven scores in an attempt to validate the use of arrhythmia scores in an in vivo model of conscious rats.

The influence on reperfusion arrhythmias can be tested in various species, e.g., rat, pig, dog, and cat (Bergey et al. 1982; Winslow 1984; Curtis et al. 1987; Brooks et al. 1989).

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Electrocardiography in Animals

Purpose and Rationale

Recording of the electrocardiogram is an essential tool in the evaluation of antiarrhythmic drugs (Johnston et al. 1983; Curtis and Walker 1986; Adaikan et al. 1992). Similar to the heart rate, the electrocardiogram is different between various species (Bazett 1920; Kisch 1953; Heise and Kimbel 1955; Beinfield and Lehr 1968; Budden et al. 1981; Driscoll 1981; Osborn 1981; Hayes et al. 1994). Many authors used the bipolar lead II between right foreleg and left hind leg, which is in line with the neutrally placed heart. Additionally, lead I (between the right and left foreleg) stated to lie in the axis of the horizontal heart, and lead III (between the left foreleg and left hind leg) in line with the vertical heart, may be used as well as unipolar leads (usually designed as V1–V6) and the unipolar leads designed as aVL, aVR, and aVF. Out of several species being used, the procedure for rats (Penz et al. 1992; Hayes et al. 1994) is described.

Procedure

Male Sprague-Dawley rats weighing 250–300 g are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital. The right jugular vein is cannulated for injections, while the left coronary artery is cannulated for recording blood pressure on a polygraph. The ECG is recorded using a lead type II of configuration along the anatomical axis of the heart as determined by palpation. ECGs are recorded at a standard chart speed of 100 mm/s on a polygraph and simultaneously on a storage oscilloscope. Measurements of intervals are made on the chart recorder and from the memory trace of the monitor.

Since in the rat it is difficult to detect a T wave that corresponds exactly with the T wave seen in other species (Beinfield and Lehr 1968; Driscoll

1981; Surawicz 1987), T wave calculations are made on the basis of the repolarization wave that follows the QRS complex. The following variables are measured:

- σT = time for the depolarization wave to cross the atria, *PR* interval, *QRS* interval, *QT* interval, and *RSh* (the height between the peak of R and S wave). The *RSh* magnitude is taken as a measure of the extent of S wave depression as exerted by class I sodium channel blocking antiarrhythmics.

Evaluation

Statistical analyses are based on ANOVA followed by Duncan's test for differences of means. In order to demonstrate the relationships between drug effects, standard cumulative dose-response curves are constructed.

Modifications of the Method

Osborne (1973, 1981) described a restraining device facilitating electrocardiogram recording in **conscious rats**.

Curtis and Walker (1986) and Johnston et al. (1983) studied the responses to ligation of a coronary artery and the actions of antiarrhythmics in conscious rats.

Hayes et al. (1994) studied the ECG in **guinea pigs**, rabbits, and primates.

Stark et al. (1989) described an epicardic surface and stimulation technique (SST-ECG) in Langendorff-perfused guinea pig hearts.

Epicardial His bundle recordings in the guinea pig in vivo were described by Todt and Raberger (1992).

Chronic recording from the His bundle in awake nonsedated **dogs** was reported by Karpawich et al. (1983) and by Atlee et al. (1984).

Van de Water et al. (1989) reported a formula to correct the QT interval of the electrocardiogram in dogs for changes in heart rate.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-reentry and ventricular antifibrillatory studies in anesthetized dogs. The reentry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The pacing-induced tachycardia that results from circus movements around the tricuspid ring was very persistent in duration and regular in cycle length. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

Weissenburger et al. (1991) developed an experimental model of the long QT syndrome in conscious dogs for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes."

Bauer et al. (2004) described pro- and antiarrhythmic effects of fast cardiac pacing in a canine model of acquired long QT syndrome.

Holter monitoring in conscious dogs was described by Krumpl et al. (1989a, b).

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Baboons and **monkeys** (*Macaca* sp.) were used by Adaikan et al. (1992).

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Aconitine Antagonism in Rats

Purpose and Rationale

The plant alkaloid aconitine persistently activates sodium channels. Infusion of aconitine in the anesthetized rat causes ventricular

arrhythmias. Drugs considered to have antiarrhythmic properties can be tested in aconitine-intoxicated rats.

Procedure

Male Ivanovas rats weighing 300–400 g are used. The animals are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. Five μg/kg aconitine dissolved in 0.1 N HNO_3 is administered by continuous infusion into the saphenous vein of 0.1 ml/min, and the ECG in lead II is recorded every 30 s. The test compound is injected orally or intravenously at a screening dose of 3 mg/kg 5 min before the start of the aconitine infusion. Eight to ten animals are used per compound.

Evaluation

The antiarrhythmic effect of a test compound is measured by the amount of aconitine/100 g animal (duration of infusion) which induces:

- Ventricular extrasystoles
- Ventricular tachycardia
- Ventricular fibrillation
- Death

Higher doses of aconitine in the treated group as compared to an untreated control group are an indication of antiarrhythmic activity.

Statistical significance between the groups is assessed by the Student's *t*-test.

The scores are allotted for the intensity and the duration of the effect relative to the efficacy of standard compounds.

Standard data:

- Procainamide, 5 mg/kg i.v., and lidocaine, 5 mg/kg, i.v., lead to an increase in LD_{100} by 65 % (corresponds to LD_{100} of approximately 9 μg/100 g).

Critical Assessment of the Method

Aconitine – antagonism in vivo has been proven as a valuable screening method for antiarrhythmic activity.

Modifications of the Method

Scherf (1947) studied the auricular tachycardia caused by aconitine administration in **dogs**.

Scherf et al. (1960) provoked atrial flutter and fibrillation in anesthetized dogs by application of a few crystals of aconitine or delphinine to the surface of the right atrium in the appendix area near the head of the sinus node.

McLeod and Reynold (1962) induced arrhythmia by aconitine in the isolated **rabbit** atrium.

Nwangwu et al. (1977) used aconitine as arrhythmogenic agent for screening of antiarrhythmic agents in **mice**.

Yamamoto et al. (1993) used urethane-anesthetized rats under artificial respiration with tubocurarine pretreatment. After thoracotomy and incision of the pericardium, a piece of filter paper soaked with aconitine solution was applied to the right atrium. Test drugs were applied by continuous i.v. infusion. In addition to ECG lead II, intra-atrial ECG was monitored.

Aconitine antagonism in conscious mice as screening procedure has been recommended by Dadkar and Bhattacharya (1974) and in anesthetized mice by Winslow (1980).

Nakayama et al. (1971) described the topical application of aconitine in a small cup placed on the right atrium of dogs to induce supraventricular arrhythmias.

A method using the **cat** has been developed by Winslow (1981).

Other Arrhythmogenic Agents

In addition to the aconitine model, Vaille et al. (1992) demonstrated the selectivity of a $CaCl_2$ continuous infusion screening method in rats for the evaluation of antiarrhythmic calcium antagonists.

A mouse chloroform model was recommended by Lawson (1968).

Vargaftig et al. (1969) induced ventricular fibrillation in mice by inhalation of chloroform.

Papp et al. (1967) proposed the experimental $BaCl_2$-arrhythmia as a quantitative assay of anti-arrhythmic drugs.

Al-Obaid et al. (1998) used calcium chloride-induced arrhythmias for antiarrhythmic activity evaluation in anesthetized male rats. Cardiac arrhythmias were induced by a single intravenous injection of 10 % $CaCl_2$ (50 mg/kg). The induced arrhythmias were then analyzed for magnitude of initial bradycardia, onset, incidence, and duration of the induced fibrillations. After the induction of the arrhythmia, the animal was allowed to recover completely (15–20 min), and the test compound was injected in different doses intravenously. The effect of the test compound on the basal heart rate was then examined, and the percentage change in the heart rate was calculated. Seven minutes later, the arrhythmogenic dose of $CaCl_2$ was readministered, and the effect of the treatment on the induced arrhythmia parameters was evaluated as percentage change in the measured parameters or as protection or non-protection against the induced fibrillations.

Tripathi and Thomas (1986) described a method for the production of ventricular tachycardia in the rat and guinea pig by exposing the animals to benzene vapors for 2 min followed by an intravenous adrenaline injection.

Arrhythmias could be induced by changing the medium of cultured rat heart muscle cells (Wenzel and Kloeppel 1978).

In isolated rat hearts, ventricular fibrillation was induced by isoprenaline and a catechol-O-methyltransferase inhibitor at high perfusion temperature (Sono et al. 1985).

Takei (1994) described experimental arrhythmia in guinea pigs induced by grayanotoxin I, a biologically active diterpenoid from the plant family of Ericaceae.

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Digoxin-Induced Ventricular Arrhythmias in Anesthetized Guinea Pigs

Purpose and Rationale

Overdose of cardiac glycosides, such as digoxin, induces ventricular extrasystoles, ventricular fibrillation, and finally death. The occurrence of these symptoms can be delayed by antiarrhythmic drugs.

Procedure

Male guinea pigs (Marioth strain) weighing 350–500 g are anesthetized with 35 mg/kg pentobarbital sodium intraperitoneally. Trachea, one jugular vein, and one carotid artery are catheterized. Positive pressure ventilation is applied with a respiratory pump (Rhema GmbH, Germany) at 45 breaths/min. The carotid artery is used for monitoring systemic blood pressure via a pressure transducer. Digoxin is infused into the jugular vein with a perfusion pump (ASID BONZ PP 50) at a rate of 85 µg/kg in 0.266 ml/min until cardiac arrest. The electrocardiogram (lead III) is recorded with subcutaneous steel-needle electrodes (Hellige 19).

Treated groups (n = 5–10 animals) receive the test drug either orally 1 h or intravenously 1 min prior to the infusion. The control group (n = at least five animals) receives the digoxin infusion only. The period until the onset of ventricular extrasystoles, ventricular fibrillation, and cardiac arrest is recorded. The total amount of infused digoxin (µg/kg) to induce ventricular fibrillation is calculated. Standard drugs are lidocaine (3 mg/kg i.v.) or ramipril (1 mg/kg p.o.).

Evaluation

Using Student's *t*-test, the doses of digoxin needed to induce ventricular extrasystoles, or ventricular fibrillation, or cardiac arrest, respectively,

after treatment with antiarrhythmic drugs are compared statistically with controls receiving digoxin only.

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Strophanthin- or Ouabain-Induced Arrhythmia

Purpose and Rationale

Acute intoxication with the cardiac glycoside strophanthin K induces ventricular tachycardia and multifocal ventricular arrhythmias in dogs. This can be used as a test model to evaluate the effect of potential antiarrhythmic drugs on ventricular arrhythmias.

Procedure

Male or female dogs of either sex weighing approximately 20 kg are used. The animals are anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium. Two peripheral veins are cannulated for the administration of the arrhythmia-inducing substance (*V. brachialis*) and the test compound (*V. cephalica antebrachii*). For intraduodenal administration of the test drug, the duodenum is cannulated. Electrocardiogram is registered with needle electrodes from lead II. Heart frequency is derived from R peaks of ECG. Two to three animals are used for one compound.

Strophanthin K is administered by continuous i.v. infusion at a rate of 3 μg/kg/min. Thirty to forty minutes later, signs of cardiac glycoside intoxication appear leading to ventricular tachycardia or to multifocal ventricular arrhythmias. When this state is achieved, the strophanthin infusion is terminated. When the arrhythmias are stable for 10 min, the test substance is administered intravenously in doses between 1.0 and 5.0 mg/kg or intraduodenally in doses between 10 and 30 mg/kg.

ECG II recordings are obtained at times −0.5, 1, 2, 5, and 10 min following administration of test drug.

For i.v. administration: A test compound is considered to have an antiarrhythmic effect if the extrasystoles immediately disappear. If the test compound does not show a positive effect, increasing doses are administered at 15 min intervals. If the test substance does reverse arrhythmias, the next dose is administered after the reappearance of stable arrhythmias.

For i.d. administration: A test compound is considered to have a definite antiarrhythmic effect if the extrasystoles disappear within 15 min. The test drug is considered to have "no effect" if it does not improve strophanthin intoxication within 60 min following drug administration.

Evaluation

Evaluation of the therapeutic effect of a drug is difficult and somewhat arbitrary since there is no clear-cut correlation between effectiveness of a test compound and duration of its effect, i.e., return to normal ECGs. The standard drugs ajmaline, quinidine, and lidocaine reestablish normal sinus rhythm at doses of 1 and 3 mg/kg (i.v.) and 10 mg/kg (i.d.). Arrhythmias are eliminated

for 20 min (i.v.) and for >60 min (i.d.) following drug administration.

Modifications of the Method

Ettinger et al. (1969) used arrhythmias in dogs induced by ouabain to study the effects of phentolamine in arrhythmia.

Garrett et al. (1964) studied the antiarrhythmic activity of *N*,*N*-diisopropyl-*N*′-diethylaminoethylurea hydrochloride in anesthetized dogs with arrhythmias induced by ouabain, aconitine, or acetylcholine. Furthermore, ultra-low-frequency ballistocardiograms with ECG registration were performed in dogs.

Raper and Wale (1968) studied the effects on ouabain- and adrenaline-induced arrhythmias in **cats**.

Kerr et al. (1985) studied the effects of a vasodilator drug on ouabain-induced arrhythmias in anesthetized dogs.

A modified method for the production of cardiac arrhythmias by ouabain in anesthetized cats was published by Rao et al. (1988).

Brooks et al. (1989) infused ouabain intravenously to **guinea pigs** and determined the onset of ventricular extrasystoles and of fibrillation.

Thomas and Tripathi (1986) studied the effects of α-adrenoreceptor agonists and antagonists with different affinity for α_1- and α_2-receptors on ouabain-induced arrhythmias and cardiac arrest in guinea pigs.

Krzeminski (1991) and Wascher et al. (1991) used of ouabain-induced arrhythmia in guinea pigs for the evaluation of potential antidysrhythmic agents.

Al-Obaid et al. (1998) used ouabain-induced arrhythmias in anesthetized **Wistar rats** for evaluation of cyclopenteno[b]thiophene derivatives as antiarrhythmic agents.

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Ventricular Fibrillation Electrical Threshold

Purpose and Rationale

The use of antiarrhythmic drugs in the treatment of ventricular arrhythmias aims to prevent the development of ventricular fibrillation. Several electrical stimulation techniques have been used to measure ventricular fibrillation threshold such as single pulse stimulation, train of pulses stimulation, continuous 50 Hz stimulation, and sequential pulse stimulation.

Procedure

Adult dogs weighing 8–12 kg are anesthetized with sodium pentobarbital (35 mg/kg) and ventilated with air using a Harvard respiratory pump. Systolic arterial pressure is monitored and body temperature maintained by a thermal blanket. The chest is opened by a midline sternotomy and the heart suspended in a pericardial cradle. The sinus node is crushed and a 2.0 mm diameter Ag-AgCl stimulating electrode is embedded in a Teflon disk sutured to the anterior surface of the left ventricle. The heart is then driven by 3 ms square anodal constant current pulses for 400 ms of the basic cycle and is prematurely stimulated by one 3 ms test stimulus through the driving electrode. Electrical stimulation is programmed by a digital stimulator. A recording electrode is placed on the surface of each ventricle. A silver plate is implanted under the skin in the right femoral region as indifferent electrode. Lead II of the body surface electrocardiogram is monitored. To determine ventricular fibrillation threshold (VFT), a 0.2–1.8 s train of 50 Hz pulses is delivered 100 ms after every eighteenth basic driving stimulus. The current intensity is increased from the diastolic threshold in increments of 10 μA to 1.0 mA or until ventricular fibrillation occurs. The minimal current intensity of the pulse train required to induce sustained ventricular fibrillation is defined as the VFT. When ventricular fibrillation occurs, the heart is immediately defibrillated and allowed to recover to control conditions for 15–20 min. Antiarrhythmic drugs are administered through the femoral vein.

Evaluation

Ventricular fibrillation threshold (VFT) is determined before and after administration of test drugs at given time intervals. The mean values of 10 experiments are compared using Student's *t*-test.

Modifications of the Method

Marshall et al. (1981) and Winslow (1984) suggested to determine VFT in the pentobarbital-anesthetized rat.

Wu et al. (1989) recommended a conscious dog model for reentrant atrial tachycardia.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-reentry and ventricular antifibrillatory studies in dogs. The reentry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

A chronically prepared rat model of electrically induced arrhythmias was described by Walker and Beatch (1988).

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Coronary Artery Ligation, Reperfusion Arrhythmia, and Infarct Size in Rats

Purpose and Rationale

Coronary artery ligation in anesthetized rats results in arrhythmias and myocardial infarction. Following occlusion of the left main coronary artery, very marked ventricular dysrhythmias occur. Electrocardiogram is recorded during ligation and subsequent reperfusion. The amount of infarcted tissue is measured by means of p-nitroblue tetrazolium chloride staining in myocardial sections. The model is used to test drugs with potential antiarrhythmic activities.

Procedure

Groups of 8–10 male Sprague-Dawley rats weighing 350–400 g are used. The animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. The trachea is intubated to allow artificial ventilation (Starling pump). A catheter is placed in an external jugular vein for administration of test compounds. Peripheral blood pressure is recorded from the common carotid artery using a pressure transducer and a polygraph. The chest is opened by left thoracotomy at the forth intercostal space. After opening the pericard, the heart is exteriorized by gentle pressure on the chest walls, and a thin silk thread (Ethicon 1.5 metric, 4–0) attached to an atraumatic needle is placed around the left coronary artery about 2–3 mm distal of the origin of the left coronary artery for later ligation. From that point on, the animal is ventilated with room air using a stroke volume of 1 ml/100 g body weight at a rate of 54 strokes/min. The heart is then placed back in the chest cavity. Any animal in which this procedure itself produces dysrhythmias or a sustained fall in mean arterial blood pressure to less than 70 mmHg has to be discarded from the study.

After an equilibration time of approx. 45 min, the test substance or the vehicle (control) is

administered by intravenous injection. Five minutes later, the ligature at the left coronary artery is closed either for 15 or 90 min (in case infarct size is assessed) and subsequently reperfused for 30 min. For oral application, the test compounds are dissolved or suspended in the vehicle 30 min before occlusion. Peripheral blood pressure and ECG lead II are recorded continuously during the whole experiment. Rectal temperature is maintained at 38 °C. The numbers of ventricular premature beats (VPB), ventricular tachycardia (VT), and ventricular fibrillation (VF) are counted in the occlusion and reperfusion periods and evaluated according to the guidelines of the Lambeth Convention (Walker et al. 1988).

Preparation to Determine Infarct Size

At the end of the reperfusion period, the animal is sacrificed with an overdose of pentobarbital sodium; the heart is dissected and cut into transversal sections (approx. 1 mm thick) from the apex to the base. The slices are stained with p-nitro-blue tetrazolium chloride solution (0.25 g/L p-nitro-blue tetrazolium chloride in Sörensen phosphate buffer, containing 100 mM D, L-malate) in order to visualize the infarct tissue (blue-/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of infarct area. Left ventricle and infarct area are measured by planimetry from projections of all slices with the exclusion of the apex and the slice containing the ligature.

Evaluation

The following parameters are evaluated:

- Mortality
- Hemodynamics
  - Peripheral blood pressure [mm Hg]
  - Heart rate [beats/min]
  - Pressure rate index (PRI) (BPs × HR) [mmHg × beats/1,000]
- Arrhythmias
  - Ventricular extrasystoles (= premature ventricular contractions) (PVC)
    - Percent animals with PVC
    - Number of PVC/5 or 30 min
  - Ventricular tachycardia (VT) (VT defined as any run of seven or more consecutive ventricular extrasystoles)
    - Percent animals with VT
    - Duration [s] of VT/5 or 30 min
  - Ventricular fibrillation (VF)
    - Percent animals with VF
    - Duration [s] of VF/5 or 30 min
- Infarct size (area)

The different characteristics are evaluated separately and compared with a positive control (5 mg/kg nicainoprol i.v.).

Changes of parameters in drug-treated animals are compared to vehicle control values.

Statistical significance is assessed by the Student's *t*-test.

Modifications of the Method

Leprán et al. (1983) placed a loose silk loop around the left coronary artery and passed the thread through a cylinder-shaped polyethylene tube outside the thorax. The rats were allowed to recover from primary surgery. The loose ligature was tightened 7–10 days thereafter and arrhythmias recorded by ECG tracings.

Johnston et al. (1983) described the responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics.

As reported in sections "Isolated Heart According to Langendorff" and "Coronary Artery Ligation in Isolated Working Rat Heart" in chapter "▶ Coronary Drugs," the isolated heart according to Langendorff and the isolated working rat heart preparation can be used for ligation experiments inducing arrhythmias. Lubbe et al. (1978) reported ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart as a

model for assessment of antifibrillatory action of antiarrhythmic agents.

Bernier et al. (1986) described reperfusion-induced arrhythmias in the isolated perfused rat heart. The isolated rat heart was perfused according to the Langendorff technique. A ligature was placed around the left anterior descending coronary artery close to its origin. The arterial occlusion was maintained for 10 min followed by reperfusion. Test compounds were included in the perfusion medium. With epicardial ECG electrodes, the number of premature ventricular complexes, the incidence and duration of ventricular fibrillation, and the incidence of ventricular tachycardia were recorded.

Abraham et al. (1989) tested antiarrhythmic properties of tetrodotoxin against occlusion-induced arrhythmias produced by ligation of the left anterior descending coronary artery in the rat.

MacLeod et al. (1989) tested a long-acting analogue of verapamil for its actions against arrhythmias induced by ischemia and reperfusion in conscious and anesthetized rats, as well as for effects on epicardial intracellular action potentials.

Harper et al. (1993) found that the inhibition of Na^+/H^+ exchange preserves viability, restores mechanical function, and prevents the pH paradox in reperfusion injury to rat neonatal myocytes.

Scholz et al. (1993, 1995) reported protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion in rats.

Likewise, Yasutake et al. (1994) found protection against reperfusion-induced arrhythmias in rats by intracoronary infusion of a Na^+/H^+ exchange inhibitor.

Aye et al. (1997) tested the effects of a Na^+/H^+ exchange inhibitor on reperfusion ventricular arrhythmias in rat hearts.

Ferrara et al. (1990) studied the effect of flecainide acetate on reperfusion- and barium-induced ventricular tachyarrhythmias in the isolated perfused rat heart by monitoring heart rate, coronary flow rate, left ventricular systolic pressure, dp/dt_{max}, and the voltage of the epicardial electrogram.

Heterogeneity of ventricular remodeling after acute myocardial infarction in rats has been reported by Capasso et al. (1992).

Bellemin-Baurreau et al. (1994) described an in vitro method for evaluation of antiarrhythmic and anti-ischemic agents by using programmed electrical stimulation of the isolated rabbit heart after ligation of the left ventricular branch of the coronary artery and a reperfusion period of 15 min.

The use of the rat in models for the study of arrhythmias in myocardial ischemia and infarction has been reviewed by Curtis et al. (1987).

Black and Rodger (1996) and Black (2000) reviewed the methods used to study experimental myocardial ischemic and reperfusion injury in various animal species.

Linz et al. (1997) reported that in isolated rat hearts with ischemia-reperfusion injuries, perfusion with bradykinin reduces the duration and incidence of ventricular fibrillations, improves cardiodynamics, reduces release of cytosolic enzyme, end preserves energy-rich phosphate and glycogen stores.

Mulder et al. (1998) studied the effects of chronic treatment with calcium antagonists in rats with chronic heart failure induced by coronary artery ligation.

The effect of antihypertensive agents on cardiac and vascular remodeling was discussed by Mallion et al. (1999).

Ciulla et al (2009) reviewed animal models of myocardial injury used in cardiovascular regenerative medicine.

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Ventricular Arrhythmia After Coronary Occlusion

Ventricular Fibrillation After Coronary Occlusion and Reperfusion in Anesthetized Dogs

Purpose and Rationale

Coronary artery occlusion in anesthetized dogs is accompanied by an increase in heart rate, heart contractility, left ventricular end-diastolic pressure, and blood pressure as well as by ventricular arrhythmias. During a subsequent reperfusion

period, a high percentage of control animals die from ventricular fibrillation. Drugs with potential protective effects are tested which reduce both hemodynamic and electrical changes.

Procedure

Dogs of either sex weighing 20–25 kg are used. Anesthesia is induced by intravenous injection of 30 mg/kg thiobutabarbital sodium and maintained by i.v. administration of 20 mg/kg chloralose and 250 mg/kg urethane followed by subcutaneous administration of 2 mg/kg morphine. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator (Bird Mark 7). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral artery is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured on a high-sensitivity scale; heart rate (HR) is determined from the LVP wave form. Myocardial contractility is measured as the rate of rise of LVP (dp/dt max). The sum of ST segment elevations is calculated from five values of the peripheral limbs in ECG lead II. The pressure-rate index ($PRI = BPs \times HR$) serves as a measure of oxygen consumption.

Experimental Course

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal spaces, the pericard is opened, and the left anterior descending coronary artery (LAD) is prepared. A silk suture is placed around the LAD, just below the first diagonal branch. After an equilibration period of approx. 45 min., the test substance or the vehicle (controls) is administered as an intravenous bolus. Twenty min later, the ligature at the coronary artery is closed for 90 min. During the occlusion period, the test compound or the vehicle (controls) is given by continuous infusion. After release of the coronary obstruction, the animal is monitored for a 30 min reperfusion period. All parameters are recorded during the whole experiment. At the end of the test, surviving animals are sacrificed by an overdose of pentobarbital sodium.

Evaluation

The following parameters are evaluated:

- Mortality
- Hemodynamics
- Arrhythmias
  - Ventricular extrasystoles (= premature ventricular contractions) (PVC)
  - Percent animals with PVC
  - Number of PVC/5 or 30 min
  - Ventricular tachycardia (VT) (VT defined as any sequence of seven or more consecutive ventricular extrasystoles)
  - Duration [s] of VT/5 or 30 min
  - Ventricular fibrillation (VF)
  - Percent animals with VF

The different characteristics are evaluated separately. Changes of parameters in drug-treated animals are compared to vehicle controls. Statistical significance of the differences is calculated by means of the Student's *t*-test.

Standard data:

Mortality: In a representative experiment, 10 out of 12 of control animals died from ventricular fibrillation during the 30 min reperfusion period. One out of eight molsidomine-treated animals died, and the death was also from ventricular fibrillation during the reperfusion phase. (Molsidomine was given as a continuous infusion of 0.5 mg/kg/ml/min during the occlusion period; controls received saline.)

Modifications of the Method

Varma and Melville (1963) described ventricular fibrillation induced by coronary occlusion during hypothermia in **dogs**.

Wilkerson and Downey (1978) described a technique for producing ventricular arrhythmias in dogs through coronary occlusion by an embolus (glass beads) being introduced into the coronary circulation via a rigid cannula which is inserted through the carotid artery.

Weissenburger et al. (1991) described a model in dogs suitable for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes."

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Thiemermann et al. (1989) described a rabbit model of experimental myocardial ischemia and reperfusion. Drugs were administered by intravenous infusion 5 min after the occlusion of the left anterior-lateral coronary artery and continued during the 60 min occlusion and subsequent 3 h reperfusion periods.

Hendrikx et al. (1994) reported that the Na^+-H^+ exchange inhibitor HOE 694 improves postischemic function and high-energy phosphate resynthesis and reduced Ca^{2+} overload in the isolated perfused rabbit heart.

Barrett et al. (1997) described a method of recording epicardial monophasic action potentials and ischemia-induced arrhythmias following coronary artery ligation in intact rabbits.

Naslund et al. (1992) described a closed-chest model in *pigs*. Occlusion was induced in pentobarbital-anesthetized, mechanically ventilated **pigs** by injection of a 2 mm ball into a preselected coronary artery. Reperfusion was achieved by retraction of the ball via an attached filament.

D'Alonzo et al. (1994) evaluated the effects of potassium channel openers on pacing- and ischemia-induced ventricular fibrillation in anesthetized pigs.

Sack et al. (1994) described the effects of a Na^+/H^+ antiporter inhibitor on postischemic reperfusion in pig heart.

Premaratne et al. (1995) used a **baboon** open-chest model of myocardial ischemia and reperfusion. Baboons underwent occlusion of the left anterior descending coronary artery for 2 h. Fifteen minutes after occlusion, the treated group received hyaluronidase i.v. over a 10 min period. The ischemic period was followed by 22 h of reperfusion. At the end of the reperfusion period, the hearts were excised, and the perfusion bed at risk for infarction was determined by infusion of a microvascular dye.

Acute myocardial infarction in minipigs model was utilized to evaluate cell therapy (Fan et al. 2014; Malliaras et al. 2013). MI was created by a balloon dilation catheter (TREK®, Abbott Vascular) inserted into the left anterior descending (LAD) coronary artery prior to cell implantation.

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Harris Dog Model of Ventricular Tachycardia

Purpose and Rationale

In 1950, Harris found that the mortality in dogs after coronary occlusion with a 2-stage ligation procedure was lower than with 1-stage ligation. The left descending coronary artery is partially occluded for 30 min after which time total ligation is performed. Under these conditions, arrhythmias develop within 4–7 h, reach a peak between 24 and 48 h, and abate within 3–5 days.

Procedure

Surgical Procedure

Dogs of either sex are anesthetized by intravenous injection of methohexitone sodium (10 mg/kg), an endotracheal tube is inserted, and anesthesia maintained with halothane. The heart is exposed through an incision in the fourth or fifth intercostal space. The anterior descending branch of the left coronary artery is dissected free below its second branch and ligated in two stages. Two ligatures are placed around the artery and a 21-gauge needle. The first ligature is tied round the artery and the needle, which is then removed. Thirty minutes later, the second ligature is tied tightly round the artery. The chest is closed in layers 30 min after the second ligature has been tied, and the dog is allowed to recover.

Test Procedure

Further observations are made when the dogs are conscious, e.g., 22–24 h after ligation of the coronary artery. The dogs are positioned to lie on their side and remain in this position throughout the experiment. Mean blood pressure is recorded from a catheter placed in the femoral artery. Lead II and aV_L of the electrocardiogram and blood pressure are continuously recorded for a control period of 30 min before and during drug administration. Drugs are administered either by injection or by continuous infusion via a hind leg vein.

Evaluation

The number of sinus and ectopic beats are counted for each successive 5 min period. Beats with a distinct P wave preceding a mean frontal QRS vector of normal duration are counted as sinus in origin; all others are denoted as ectopic.

Modifications of the Method

The model which resembles late arrhythmias occurring in postinfarction patients has been used with modifications by many authors (e.g., Kerr et al. 1985; Reynolds and Brown 1986; Gomoll 1987; Garthwaite et al. 1989; Krumpl et al. 1989a, b; Trolese-Mongheal et al. 1985, 1991; Spinelli et al. 1991).

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Protection against Sudden Coronary Death

Purpose and Rationale

The group of Lucchesi described an experimental dog model to test protection against sudden coronary death (Patterson et al. 1982; Uprichard et al. 1989a, b; Chi et al. 1990a, b, 1991; Kitzen et al. 1990; Black et al. 1991, 1993).

Surgical Preparation

Purpose-bred male mongrel dogs weighing 14–22 kg are anesthetized with 30 mg/kg pentobarbital i.v. The dogs are ventilated with room air through a cuffed endotracheal tube and a Harvard respirator. A cannula is inserted in the left external jugular vein. A left thoracotomy is performed between the fourth and fifth ribs, and the heart is exposed and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) is isolated at the tip of the left atrial appendage, and the left circumflex coronary artery (LCX) is isolated ~1 cm from its origin. After a 20-gauge hypodermic needle has been placed on the LAD, a ligature is tied around the artery and the needle. The needle is then removed, resulting in critical stenosis of the vessel. The LAD is perfused for 5 min in the presence of the critical stenosis. Ischemic injury of the anterior ventricular myocardium is achieved by 2 h occlusion of the LAD by a silicon rubber snare. The vessel is reperfused after 2 h in the presence of the critical stenosis. During the period of LAD reperfusion, an epicardial bipolar electrode (1 mm silver posts, 3 mm interelectrode separation) is sutured on the left atrial appendage for subsequent atrial pacing. A bipolar plunge electrode (25-gauge stainless steel, 5 mm long, 3 mm separation) is sutured on the interventricular septum, adjacent to the occlusion site and overlying the right ventricular outflow tract (RVOT). Two similar stainless steel bipolar plunge electrodes are sutured to the left ventricular (LV) wall: one at the distribution of the LAD distal to the occlusion (infarct zone, IZ) and the second in the distribution of the LCX (non-infarct zone, NZ). A 30-gauge silver-coated copper wire electrode is passed through the wall and into the lumen of the LCX and sutured to the adjacent surface of the heart. Silver disk electrodes are implanted subcutaneously for ECG monitoring. The surgical incision is closed, and the animals are allowed to recover.

Drug Treatment

The animals are treated after the recovery period during the 3 days of programmed electrical stimulation either with the test drug or with the solvent.

Electrophysiological Studies and Programmed Electrical Stimulation

Programmed electrical stimulation (PES) is performed between days 3 and 5 after induction of anterior myocardial infarction by occlusion/perfusion of the LAD. Animals are studied while conscious and unsedated. Heart rate, ECG

intervals, and other electrophysiologic parameters (for details, see original publications) are determined before PES is started. Premature ventricular stimuli are introduced in the region of the right ventricular outflow tract. The extra-stimuli are triggered from the R wave of the ECG, and the R-S_2 coupling interval is decreased from 350 ms until ventricular refractoriness occurs. At this time, double and triple ventricular extra-stimuli are introduced during sinus rhythm. Ventricular tachyarrhythmias are defined as "non-sustained," if five or more repetitive ventricular responses are initiated reproducibly, but terminated spontaneously. Ventricular tachyarrhythmias are defined as "sustained," if they persist for at least 30 s or, in the event of hemodynamic compromise, require ventricular burst pacing for their termination.

Sudden Cardiac Death

A direct anodal 15 μA current from a 9 V nickel-cadmium battery is passed through a 250 Ω resistor and applied to the electrode in the lumen of the left circumflex coronary artery. The cathode of the battery is connected to a s.c. implanted disk electrode. Lead II ECG is recorded for 30 s every 15 min on a cardiocassette recorder. After 24 h of constant anodal current or development of ventricular fibrillation, the animals are sacrificed, the hearts are excised, and the thrombus mass in the LCX is removed and weighed. The heart is sectioned transversely and incubated for 15 min at 37 °C in a 0.4 % solution of tetrazolium triphenyl chloride for identification of infarcted areas. Time of onset of ventricular ectopy and of lethal arrhythmia is provided from recordings of the cardiocassette.

Evaluation

Non-sustained and sustained tachyarrhythmias are evaluated.

Critical Assessment of the Method

Sudden coronary death is one of the leading causes of death in developed countries. These facts warrant the use of complicated models in higher animals for search of active drugs. Some compounds previously believed to be inert have been demonstrated as proarrhythmic in the model (Chi et al. 1990).

Modifications of the Method

Schwartz et al. (1984) described an experimental preparation for sudden cardiac death in dogs. The animals were chronically instrumented and studied 1 month after an anterior myocardial infarction. A balloon catheter around the circumflex coronary artery was inflated to produce acute myocardial ischemia, and the occlusion was maintained for 2 min. Several days later, the animals were subjected to a submaximal stress on a motor-driven treadmill for 12–18 min. During the last minute of exercise, the left coronary artery was occluded, the treadmill stopped, and the occlusion was maintained for a second minute.

Schwartz et al. (1988) analyzed the baroreceptor reflexes in conscious dogs with and without a myocardial infarction to get insights in the mechanisms of sudden death.

Cahn and Cervoni (1990) reviewed the use of animal models of sudden cardiac death for drug development.

Pak et al. (1997) found that canine tachycardia-induced cardiomyopathy is a useful model for studying mechanisms and therapy of sudden cardiac death in heart failure. Adamson et al. (1994) performed a longitudinal study in dogs at high and low risk for sudden death and found an unexpected interaction between β-adrenergic blockade and heart rate variability before and after myocardial infarction.

Basso et al. (2004) recommended arrhythmogenic right ventriculopathy causing sudden death in boxer dogs as an animal model of human disease.

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Ventricular Fibrillation Induced by Cardiac Ischemia During Exercise

Purpose and Rationale

Billman and his group developed methods to evaluate antiarrhythmic drugs for their activity in cardiovascular parameters in an exercise-plus-ischemia test.

Procedure

Surgical Preparation

Mongrel dogs, weighing 15.4–19.1 kg, are anesthetized and instrumented to measure left circumflex CBF, left ventricular pressure, and ventricular electrogram (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984). The animals are given Innovar-Vet (0.02 mg/kg fentanyl citrate and 1 mg/kg hydroperidol i.v.) as a preanesthetic, whereas a surgical plane of anesthesia is induced with sodium pentobarbital (10 mg/kg i.v.). A left thoracotomy is made in the fourth intercostal space, and the heart is exposed and supported by a pericardial cradle. A 20 MHz pulsed Doppler flow transducer and a hydraulic occluder are placed around the left circumflex artery. A pair of insulated silver-coated wires are sutured to the epicardial surface of both the left and right ventricles. These electrodes are used for ventricular pacing or to record a ventricular electrogram from which HR is determined using a Gould Biotachometer (Gould Instruments, Cleveland, OH). A precalibrated solid-state pressure transducer (Konigsberg Instruments, Pasadena, CA) is inserted into the left ventricle via a stab wound in the apical dimple. Finally, a two-stage occlusion of the left anterior descending coronary artery is performed approximately one third the distance from the origin to induce an anterior wall myocardial infarction. This vessel is partially occluded for 20 min and then tied off. All leads from the cardiovascular instrumentation are tunneled under the skin to exit on the back of the animal's neck. A transdermal fentanyl patch that delivers 75 μg/h for 72 h is placed on the back of the neck (secured with adhesive tape) to decrease postoperative discomfort. In addition, bupivacaine HCl, a long-acting local anesthetic, is injected to block the intercostal nerves (i.e., pain fibers) in the area of the incision. Each animal is placed on prophylactic antibiotic therapy (amoxicillin 500 mg p.o.) three times daily for 7 days. The animals are placed in an "intensive care" setting for the first 24 h and placed on antiarrhythmic therapy (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984).

Exercise-Plus-Ischemia Test

The studies begin 3–4 weeks after the production of the myocardial infarction. The animals are walked on a motor-driven treadmill and trained to lie quietly without restraint on a laboratory table during this recovery period. Susceptibility to VF is then tested. The animals run on a motor-driven treadmill while workload is increased every 3 min for a total of 18 min. The protocol begins with a 3 min warm-up period, during which the animals run at 4.8 km/h at 0 % grade. The speed is increased to 6.4 km/h, and the grade is increased every 3 min as follows: 0 %, 4 %, 8 %, 12 %, and 16 %. During the last minute of exercise, the left circumflex coronary artery is occluded, the treadmill is stopped, and the occlusion is maintained for 1 additional minute (total occlusion time, 2 min). Large metal plates (diameter, 11 cm) are placed across the animal's chest so that electrical defibrillation can be achieved with minimal delay but only after the animal is unconscious (10–20 s after VF begin). The occlusion is immediately released if VF occur.

The animals then receive one or more of the following treatments:

1. The exercise-plus-ischemia test is repeated after pretreatment with the standard drug glibenclamide (1.0 mg/kg i.v.). The drug is injected in a cephalic vein, 3 min before exercise begins.
2. The exercise-plus-ischemia test is repeated after pretreatment with the test drug.
3. Finally, a second control (saline) exercise-plus-ischemia test is performed 1 week after the last

drug test. At least 5 days are intermitted between drug treatments. Drugs are given in a random order.

Refractory Period Determination

On a subsequent day, the effective refractory period is determined using a Medtronic model 5325 programmable stimulator, both at rest and during myocardial ischemia. The heart is paced for eight beats (S_1; intrastimulus interval, 300 ms; pulse duration, 1.8 ms at twice-diastolic threshold of ~6 mA). The intrastimulus interval is progressively shortened between the last paced beat and a single extra-stimulus (S_2). The refractory period represents the shortest interval capable of generating a cardiac response and is measured using either the left or right ventricular electrodes. This procedure is completed within 30 s. Once the control values are determined, refractory period measurements are repeated after the standard drug glibenclamide (1.0 mg/kg i.v.), or the test drug. After the completion of these studies, refractory period is determined during myocardial ischemia (2 min occlusion of the left circumflex coronary artery) ~60 s after the onset of the coronary occlusion.

Reactive Hyperemia Studies

The K_{ATP} has been implicated in vascular regulation, particularly CBF (Aversano et al. 1991; Belloni and Hintze 1991; Daut et al. 1990). Therefore, the effects of standard and test drug on the response to brief interruptions in CBF are also evaluated. The left circumflex coronary is occluded three or four times for 15 s, at least 2 min (or until CBF had returned to preocclusion base line) elapse between occlusions. The occlusions are then repeated 5 min after standard and test drug. On the subsequent day, the studies are repeated with the drug that had not been given the previous day.

Evaluation

All hemodynamic data are recorded on a Gould model 2800S eight-channel recorder (Cleveland, OH) and a Teac model MR-30 FM tape recorder (Tokyo, Japan). Coronary blood flow is measured with a University of Iowa Bioengineering flowmeter model 545 C-4 (Iowa City, IA). The rate of change of left ventricular pressure [$d(LVP)$/dt] is obtained by passing the left ventricular pressure through a Gould differentiator that has a frequency response linear to >300 Hz. The data are averaged over the past 5 s of each exercise level. The coronary occlusion data are averaged over the last 5 s before and at the 60 s line point (or VF onset) after occlusion onset. The total area between the peak CBF and return to base line is measured for each 15 s occlusion, and the percent repayment is calculated. The reactive hyperemia response to each occlusion is then averaged to obtain one value for each animal. The data are then analyzed using analysis of variance for repeated measures. When the F ratio is found to exceed a critical value ($P < 0.05$), Scheffe's test is used to compare the mean values. The effects of the drug intervention on arrhythmia formation are determined using a χ^2 test with Yates' correction for continuity. All data are reported as mean ± SEM. Cardiac arrhythmias, PR interval and QT interval are evaluated at a paper speed of 100 mm/s. QT interval is corrected for HR using Bazett's method.

Critical Assessment of the Method

Tests combining coronary constriction with physical exercise may resemble most closely the situation in coronary patients.

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Experimental Atrial Fibrillation

Atrial Fibrillation by Atrial Pacing in Dogs

Purpose and Rationale

Morillo et al. (1995) published a model of sustained atrial fibrillation by chronic rapid atrial pacing in dogs. Halothane-anesthetized mongrel dogs underwent insertion of a transvenous lead at the right atrial appendage that was continuously paced at 400 beats/min for 6 weeks. Two-dimensional echocardiography was performed to assess the effects of rapid atrial pacing on atrial size. Atrial vulnerability was defined as the ability to induce sustained repetitive atrial responses during programmed electrical stimulation. Effective refractory period (ERP) was measured at two endocardial sites of the right atrium. Sustained atrial fibrillation (AV) was defined as AF $\geq$15 min. In animals with sustained AF, 10 quadripolar epicardial electrodes were surgically attached to the left and right atria. The local atrial fibrillatory cycle length (AFCL) was measured in a 20 s window. Marked biatrial enlargement was documented after 6 weeks of continuous rapid atrial pacing. An increase in atrial area of at least 40 % was necessary to induce sustained AF.

More studies using this method were performed by Gaspo et al. (1997a, b), Yue et al. (1997), Sun et al. (1998), and Nattel and Li (2000).

Shiroshita-Takeshita et al. (2004) studied the effect of drugs on atrial fibrillation promotion by atrial tachycardia remodeling on dogs.

Procedure

Mongrel dogs were anesthetized with ketamine (5.3 mg/kg i.v.), diazepam (0.25 mg/kg i.v.), and halothane (1.5 %). Unipolar leads were inserted through jugular veins into the right ventricular apex and the right atrial appendage and connected to pacemakers (Medtronic) in

subcutaneous pockets in the neck. A bipolar electrode was inserted into the right atrium for stimulation and recording during serial electrophysiological study. AV block was created by radiofrequency ablation of control ventricular response during atrial tachypacing. The right ventricular pacemaker was programmed to 80 beats/min.

After 24 h for recovery, a baseline closed-chest serial electrophysiological study was performed under ketamine/diazepam/isoflurane anesthesia, and then atrial tachypacing (400 beats/min) was initiated. The closed-chest electrophysiological study was repeated at 2, 4, and 7 days of atrial tachypacing, and a final open-chest electrophysiological study was performed on day 8 under morphine-chloralose anesthesia.

Results of atrial tachypacing in drug-treated dogs were compared with results of dogs without treatment (controls).

Study Protocol

Dogs were anesthetized and ventilated mechanically. The atrial pacemaker was deactivated, and a right atrium appendage effective refractory period was measured at basic lengths of 150, 200, 250, 300, and 360 ms with 10 basic stimuli (S_1) followed by a premature extra-stimulus (S_2) with 5 ms decrements. The longest S_1-S_2 failing to capture defined the effective refractory period. AF was induced by atrial burst pacing at 10 Hz and 4 times threshold current. To estimate mean AF duration in each dog, AF was induced 10 times if AF duration was <20 min and 5 times if AF lasted 20–30 min and then averaged. If AF lasted longer than 30 min, it was considered sustained and was terminated by DC cardioversion. A 20 min rest period was then allowed before continuing measurements.

For open-chest electrophysiological studies, dogs were anesthetized and ventilated mechanically. A femoral artery and both femoral veins were cannulated for pressure monitoring and drug administration. A median sternotomy was performed, and bipolar electrodes were hooked to the right atrial and left atrial appendages for recording and stimulation. A programmable stimulator (Digital Cardiovascular Instruments) was used to deliver twice-threshold currents. Five silicon sheets containing 240 bipolar electrodes were sutured onto the atrial surface (Fareh et al. 2001). Atrial effective refractory periods were measured as multiple basic cycle lengths in the right and left atrial appendages and at basic cycle length 300 ms in six additional sites: right and left atrium posterior wall, right and left atrium inferior wall, and right and left atrium Bachmann's bundle. Atrial fibrillation vulnerability was determined as the percentage of atrial sites at which AF could be induced by single extra-stimuli.

Evaluation

Data are presented as mean ±SEM. Multiple group comparisons were obtained by ANOVA. AF duration data were analyzed after logarithmic transformation. Bonferroni-corrected *t*-tests were used to evaluate individual-mean differences.

Modifications of the Method

Verheule et al. (2004) described a canine model of atrial fibrillation due to chronic atrial dilatation.

Courtemanche et al. (1999) and Ramirez et al. (2000) published mathematical models of fibrillation-induced electrical remodeling and of canine atrial action potentials.

Pinto and Boyden (1999) reviewed electrical remodeling in ischemia and infarction.

Cabo and Boyden (2003) performed a computational analysis of electrical remodeling of the epicardial border zone in the canine infarcted heart.

Sakabe et al. (2004) reported that enalapril prevents perpetuation of atrial fibrillation by suppressing atrial fibrosis and overexpression of connexin43 in a canine model of atrial pacing-induced left ventricular dysfunction.

Baartscheer et al. (2005) induced combined volume and pressure overload in New Zealand white **rabbits**. In a first surgical procedure, volume overload was produced by rupture of the

aortic valve until pulse pressure increased by about 100 %, and after 3 weeks, pressure overload was created by suprarenal abdominal aortic constriction of 50 %. In these animals, chronic inhibition of the Na^+/H^+ exchanger attenuated cardiac hypertrophy and prevented cellular remodeling in heart failure.

Using **cultured atrial myocytes** (HL-1 cells), Yang et al. (2005) found that rapid field stimulation (300 beats/min) causes electrical remodeling. Nishida et al. (2010) reviewed the animal models that have been used to study the pathophysiology of atrial fibrillation.

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Atrial Fibrillation in Chronically Instrumented Goats

Purpose and Rationale

Atrial fibrillation is the most common tachyarrhythmia in humans. It causes palpitations, decreased cardiac output, heart failure, and systemic thromboembolism and is associated with significant mortality. Wijffels et al. (1995) demonstrated in a chronically instrumented conscious goat model that episodes of atrial fibrillation may be self-perpetuating ("Atrial fibrillation begets atrial fibrillation") and have suggested that there may be a purely electrophysiological explanation (termed atrial electrical remodeling) for the increase of atrial fibrillation with time. This model has been extensively used to study pathophysiological mechanism and the influence of drugs (Wijffels et al. 1997, 1999, 2000; Allessie et al. 1998; Tieleman et al. 1999; Duytschaever et al. 2000, 2005; Garratt and Fynn 2000; Van der Velden et al. 2000a, b; Veloso 2001; Brendel and Peukert 2003; Shan et al. 2004; Blaauw et al. 2004a).

Blaauw et al. (2004b) studied the efficacy and atrial selectivity of a blocker of the early ultrarapid component of the delayed rectifier (I_{kur}) in remodeled atria of the goat.

Procedure

Female goats weighing 52 ± 2 kg were used. According to the method of Duytschaever et al. (2001), Teflon-felt plaques with multiple electrodes were sutured onto the free wall of each atrium, Bachmann's bundle, and the left ventricle. All leads were tunneled subcutaneously to the neck and exteriorized by four 30-pole connectors. Experiments were started 3–4 weeks after surgery. Atrial fibrillation was induced by a fibrillation pacemaker (Wijffels et al. 1995).

The atria were paced with biphasic stimuli of 2 ms duration and $4 \times$ threshold. The atrial effective refractory period was measured at the free wall of the right and left atria during regular pacing (interval, 400–200 ms). Single interpolated stimuli were applied after eight basic stimuli, starting within the refractory period. The longest interval that failed to capture the atria (2 ms increments) was taken as the atrial effective refractory period. Atrial conduction velocity was measured along Bachmann's bundle during right atrial pacing. The distance over which conduction velocity was measured ranged from 3.5 to 5 cm.

The length of the fibrillation waves was determined at the right atrial free wall by measuring the refractory period and conduction velocity during AF. The refractory period was measured by slow, fixed-rate pacing (1 Hz), resulting in a series of single, randomly coupled, premature stimuli. Local capture of AF was evidenced by radial spread of activation from the pacing site and a short delay between stimulus and response. For each coupling interval, the percentage of capture was determined. The shortest interval capturing the atrium ≥ 50 % was taken as the refractory period. Conduction velocity was determined with a mapping electrode containing 5×6 electrodes (interelectrode distance, 4 mm) from the local conduction vectors within areas of 3×3 electrodes. At least 50 AF cycles were used to determine conduction velocity.

Inducibility of AF was measured at the right and left atria by single premature stimuli applied during regular pacing (400 ms). In case a premature beat induced a rapid irregular rhythm lasting >1 s, AF was considered inducible. The AF cycle length was measured automatically by an algorithm detecting the negative intrinsic deflection of the fibrillation electrogram. A median value of 300 consecutive intervals was calculated. QT duration was measured during atrial pacing and persistent AF from either an epicardial electrogram or a precordial ECG. Because Bazett's formula cannot be applied during AF, another approach was used to correct QT duration. In each goat, the relationship between the RR interval and QT duration was determined during 20 s of AF. The RR-QT relationship after drug administration was compared with the normal RR-QT relation.

The electrophysiological effects of the test drug and a conventional class III drug were

measured before and after 48 h (1–4 days) of AF. The drugs were infused intravenously over 1 h, during which time AF cycle length was monitored. After 30 min of infusion, refractory period, conduction velocity, median RR interval, and QT duration were measured. Successful cardioversion was defined as termination of AF within $\leq$1 h of drug administration.

Evaluation

Differences between groups were evaluated by paired Student's *t*-test or by two-way repeated ANOVA with post hoc Bonferroni's *t*-test. McNemar's test was used to compare AF inducibility. Changes in corrected QT duration were calculated by the one-sample *t*-test. Differences were considered significant at $P < 0.05$. Results are presented as mean $\pm$ SEM.

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Influence on Ultrarapid Delayed Rectifier Potassium Current in Pigs

Purpose and Rationale

Blockade of cardiac potassium channels and the resulting prolongation of repolarization and refractoriness are the mode of action of class III antiarrhythmic drugs. In the human heart, the ultrarapid delayed rectifier potassium current (I_{Kur}) was identified in atrial, but not in ventricular tissue. It appears to contribute to action potential repolarization (Wang et al. 1993; Li et al. 1996) in the atrium but not the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current seems to be the Kv1.5 protein (Fedida et al. 1993; Wang et al. 1993; Feng et al. 1997, 1998).

Since prolongation of ventricular repolarization seems to be invariably associated with proarrhythmia (early after depolarizations leading to torsades de pointes arrhythmia) as shown with available potassium channel blockers (class III drugs) such as the highly selective and potent I_{Kr}-channel blocker dofetilide (Torp-Pedersen et al. 1999), blockade of a cardiac current that is exclusively present in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. Therefore, the atrial Kv1.5 channel is a highly attractive target in the search for new and safer atrial antiarrhythmic drugs (Nattel and Singh 1999).

In a series of studies, Wirth and Knobloch (2001), Knobloch et al. (2002, 2004), and Wirth et al. (2003) investigated electrophysiological and antiarrhythmic effects of I_{Kur} channel blockers on left versus right pig atrium in vivo in comparison with I_{Kr} blockers in pigs.

Procedure

Surgery

Castrated male pigs (24–30 kg) of the German Landrace were premedicated with 3 ml Rompun 2 % i.m. (xylazine HCL, 23.3 mg/ml = 3 mg/kg i.m.) and 6 ml Hostaket (ketamine HCl, 115 mg/ml = 20 mg/kg i.m.) and anesthetized with an i.v. bolus of 5 ml Narcoren (pentobarbital, 160 mg/ml = 25–30 mg/kg i.v.) followed by a continuous intravenous infusion of 12–17 mg/kg per h pentobarbital. Animals were ventilated with room air and oxygen by a respirator (ABV-Intensiv; Stephan, Gackenbach, Germany). After a left thoracotomy, the lung was retracted, the pericardium incised, and the heart suspended in a pericardial cradle. Bipolar body surface ECG was recorded using subcutaneous needle electrodes in the classical lead II or lead III arrangement.

Atrial Effective Refractory Period Measurements

Atrial effective refractory period (ERP) measurements at different basic cycle lengths (BCL 240/300/400 ms) were performed (Wirth and Knobloch 2001). Atrial responses to the pacing procedure were visualized via monophasic action potential (MAP) from the left and right atrium as will be described below. A conditioning train of ten basic stimuli (S1) at twice-diastolic pacing threshold was followed by a diastolic extrastimulus (S2, pulse duration 1 ms) starting about 30 ms above the expected ERP with a 5 ms decrement (UHS 20, universal heart stimulator; Biotronik, Berlin, Germany). The longest coupling interval unable to elicit a propagated atrial response was taken as the atrial ERP.

MAP Recording Sites and Atrial Pacing Electrodes

Left atrial ERP was measured via a MAP pacing catheter (EP Technologies, Model 1675; Boston Scientific, La Garenne-Colombes, France), which was fixed in each pig in the middle of the left atrial free wall in an approximately perpendicular position by a holding device (Yuan et al. 1994). The tip of the MAP pacing catheter was covered by a sponge. Programmed stimulation was performed by the MAP pacing catheter. Right atrial MAP for ERP measurement was taken from the endocardium of the right atrium also via a steerable MAP pacing catheter. The catheter, inserted via the V. femoralis, was used for atrial stimulation too. Its position in the right atrium was checked by palpitation and by the typical atrial MAP morphology and duration and, additionally, by short rapid atrial pacing at a BCL of 240 ms, which the ventricle was not able to follow 1:1 as indicated by frequent P waves dissociated from the QRS complex. There were no significant differences between endocardial and epicardial ERP measurements in the free walls of either atrium at baseline and after drug.

Left Atrial Vulnerability

During the ERP measurement procedure, the mere S2 extra-stimulus, which followed the ten conditioning S1 stimuli during the ERP measurement procedure, frequently triggered runs of atrial tachycardia in the left, not the right, atrium. The occurrence of tachycardias was primarily unintended, but then exploited as a parameter for the judgment of the antiarrhythmic efficacy of compounds (referred to as left atrial vulnerability). Whether or not a run of S2-triggered atrial tachyarrhythmia occurred during the ERP measurement procedure at a given BCL was noted. The occurrences of triggered tachyarrhythmias were summed up for the three BCLs tested over three time points (during a 30 min period before or after a drug). Thus, the maximal occurrence of S2 tachyarrhythmias during the control or drug period in an individual animal was nine.

Drugs

Drugs were dissolved in polyethyleneglycol (PEG) 400 (Riedel-de Haen, Seelze, Germany) and administered i.v. over 5 min in a volume of 3 ml. Vehicle was injected at least 30 min before each drug. For each drug, a separate group of pigs was used.

Evaluation

All data were presented as means ±SEM. Two-way ANOVA for repeated measures followed by Student's *t*-test was used for the calculation of statistically significant differences between left and right atrial ERP prolongations at the three basic cycle lengths and the inhibition of left atrial vulnerability. A value of $P < 0.05$ was accepted as significant. The longest ERP at each pacing rate after drug administration was taken and expressed as absolute or percent increase from vehicle control. Interatrial difference in refractoriness was calculated as the difference between the left and right atrial ERP.

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Characterization of Antiarrhythmic Activity in the Isolated Right Ventricular Guinea Pig Papillary Muscle

Purpose and Rationale

According to Vaughan-Williams (1970), antiarrhythmic drugs are divided into four different classes depending on their mode of action. Class I antiarrhythmic agents decrease the upstroke velocity of the action potential through blockade of Na^+ channels. Class II drugs block β-receptors. Class III antiarrhythmic agents prolong action potential duration, presumably through blockade of K^+ channels. Class IV antiarrhythmic agents inhibit the slow calcium influx during the plateau of the action potential through Ca^{2+} channel blockade. These electrophysiological actions also have functional manifestations, e.g., Na^+ channel blockade decreases excitability, K^+ channel blockade lengthens refractory period, and Ca^{2+} channel blockade decreases tension of cardiac muscle. A simple and accurate non-microelectrode method is necessary to identify and classify potential antiarrhythmic drugs into the classes I, III, and IV. In right ventricular guinea pig papillary muscle, developed tension (DT), excitability (EX), and effective refractory period (ERP) are measured.

Procedure

Guinea pigs of either sex weighing 200–400 g are stunned, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed and placed into a container of prewarmed, preoxygenated physiologic solution, and the pericardium, atria, and other tissues are removed. The heart is then pinned to a dissection dish, and the right ventricle is opened. The tendinous end of the papillary muscle is ligated with a silk thread, and the chordae tendineae are freed from the ventricle. The opposite end of the papillary muscle is then cut free close to the ventricular wall. The non-ligated end of the papillary muscle is clamped into a tissue holder, the end of which is a leucite block containing platinum wire field electrodes.

The preparation is transferred to a tissue bath containing 75 ml of a physiological salt solution that is gassed continuously with 95 % O_2/5 % CO_2 and maintained at a temperature of 35 °C and a pH of 7.4. The silk thread is used to connect the muscle to a Grass FT03C force transducer. An initial resting tension of 1 g is established. Muscles are field stimulated to contract isometrically. The stimulus duration is 1 ms, the frequency 1 Hz,

and the voltage twice threshold. Pulses are delivered with the use of a Grass S88 constant voltage stimulator, and developed tension is recorded with the use of a polygraph recorder. The preparation is equilibrated in this manner for 90 min with bath solution changes every 15 min. Control measurements of the force-frequency curve, stimulus strength-duration curve, and the effective refractory period are made following the 75 min bath exchange, i.e., during the last 15 min of equilibration.

The force-frequency curve is obtained by measuring developed tension over a range of stimulation frequencies (0.3, 0.5, 0.8, 1.0, and 1.2 Hz). The tissue is contracted for 90 s at each of these frequencies with a brief period of stimulation at 1.5 Hz inserted between increments. The purpose of the 1.5 Hz insert is to keep "pacing history" constant as well as to minimize progressive, nonspecific depression during the lower-frequency stimulation series. Both pre- and postdrug developed tension (at each frequency) are expressed as a percentage of the predrug developed tension at 1 Hz. The percent change in posttreatment (versus pretreatment) developed tension at 1 Hz is used to quantitate an agent's inotropic effect.

The stimulus strength-duration curves are determined by varying the stimulus duration (0.1, 0.4, 0.8, 1.0,1.5,3.0, and 3.4 ms) and finding the threshold voltage that produced a 1:1 correspondence between stimulus and response at each duration. The degree of shift in the strength-duration curve is measured by computing the area between the pre- and posttreatment curves. The boundaries for the area are determined by the first (x-axis parallel) and the last (x-axis perpendicular) durations and by lines from the origin to the second and fourth durations.

Effective refractory period (ERP) is measured at 1 Hz using twin pulse stimuli. After every 8–10 pulses, a second delayed stimulus (S_2) identical to the basic drive pulse (S_1) is introduced. This procedure is repeated, shortening the delay (S_1–S_2) by 5 ms increments. The value of the ERP is taken as the longest delay (S_1–S_2) for which there is a single response to twin pulses. The change in ERP is computed as the difference (ms) between the pre- and posttreatment ERP values.

At the conclusion of the 90 min predrug equilibration period, an aliquot of the test drug designed to achieve the desired final concentration is added to the bath. The tissue must equilibrate for 1 h in the drug solution before postdrug measurements of the force-frequency curve, stimulus strength-duration curve, and effective refractory period are obtained.

Evaluation

The changes in effective refractory period (ERP) (posttreatment minus pretreatment), the degree of shift in the strength-duration curve (geometrical area between pre- and posttreatment curves), and the percent changes in posttreatment developed tension at 1 Hz are calculated. The results of these calculations are used to classify the compound as a class I, III, or IV antiarrhythmic agent on the basis of its effect on developed tension, excitability, and effective refractory period. An upward and right shift of the strength-duration curve (decrease in excitability) is characteristic for a class I antiarrhythmic agent, such as disopyramide. Selective prolongation of effective refractory period is characteristic for class III antiarrhythmic agents, such as sotalol. Depression of developed tension and/or flattening or reversal of the force-frequency curve is characteristic for a class IV antiarrhythmic agent, such as verapamil.

Critical Assessment of the Method

The model of the electrically stimulated isolated guinea pig papillary muscle is a simple method to classify antiarrhythmic agents. Some drugs have multiple actions and, therefore, belong in more than one class. For further characterization, analysis of the action potential is necessary.

Modifications of the Method

O'Donoghue and Platia (1991) recommended the use of monophasic action potential recordings for the evaluation of antiarrhythmic drugs.

Shibuya et al. (1993) studied the effects of the local anesthetic bupivacaine on contraction and membrane potential in isolated canine right ventricular papillary muscles. From analysis of action potential, it is concluded that at low concentrations contraction is depressed mainly due to a Na^+ channel block, whereas at high concentrations also Ca^{2+} channels may be blocked.

Kodama et al. (1992), Maryuama et al. (1995) studied the effects of potential antiarrhythmics on maximum upstroke velocity and duration of action potential in isolated right papillary muscles of guinea pigs as well as the influence of these agents on single ventricular myocytes.

Borchard et al. (1982) described a method for inducing arrhythmias or asystolia by the application of 50 Hz alternating current (ac) to electrically driven isolated left atria and right papillary muscles of the guinea pig. An increase in driving frequency from 1 to 3 Hz effected a significant reduction of the threshold of ac-arrhythmia in guinea pig papillary muscle, but no change in atria. A decrease in temperature from 31 °C to 25 °C and an increase in Ca^{2+} from 1.25 to 5 mmol/l elevated the threshold for ac-arrhythmia and asystolia. Fast sodium channel inhibitors increased threshold of ac-arrhythmia in left atria and papillary muscles, whereas the slow channel inhibitor verapamil was ineffective in concentrations up to 6 µmol/l.

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Action Potential and Refractory Period in Isolated Left Ventricular Guinea Pig Papillary Muscle

Purpose and Rationale

Intracellular action potential in the left ventricular guinea pig papillary muscle is recorded after electrical stimulation. The stimulation frequency is varied in order to determine the refractory period. Resting potential, upstroke velocity, duration of action potential, threshold, refractory period, and contractile force can be measured in vitro. Compounds which affect the duration of the effective refractory period may have antiarrhythmic or proarrhythmic effects. In addition, the inotropic effect (positive or negative) of the test compound is determined.

Procedure

Guinea pigs of either sex (Marioth strain) weighing 250–300 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed and placed in a container of prewarmed, preoxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Ringer solution oxygenated with carbogen (95 % O_2/5 % CO_2) at a temperature of 36 °C is used.

A standard microelectrode technique is applied to measure the action potential via a glass microelectrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at an interval of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold.

The intracellular action potential is amplified, differentiated for registration of upstroke velocity (dV/dt) (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

The effects on fast sodium channels as well as on calcium channels can be studied. The former requires measurement of the normal action potential and the latter the slow action potential obtained at 30 mM K^+. To estimate the relative refractory periods, the second stimuli are set in decremental intervals until contraction ceases. Relative refractory period is defined as the minimum time interval of two stimuli at which each of the stimuli is answered by a contraction. The stimulation threshold is also measured.

After an equilibrium time of 30 min, the test compound is added. After 15 and 30 min, the following parameters are compared with the predrug values:

- Resting potential mV
- Upstroke velocity V/s
- Duration of action potential ms
- Stimulation threshold V
- Refractory period ms
- Contraction force mg

The organ bath is flushed thoroughly between two consecutive applications of increasing test drug doses.

Evaluation

Contractile force [mm] and relative refractory period [ms] are determined before and after drug administration. ED_{25ms} and ED_{50ms} values are determined. ED_{25ms} or ED_{50ms} is defined as the concentration of test drug in the organ bath at which the relative refractory period is reduced or prolonged by 25 or 50 ms.

Since many antiarrhythmic agents possess additionally negative inotropic effects, changes in the force of contraction are also determined.

ED_{50} values are calculated from log-probit analyses. Scores are allotted relative to the efficacy of standard compounds (lidocaine, propranolol, quinidine).

The following changes are indicators for antiarrhythmic activity:

- Increase of stimulation threshold
- Decrease of upstroke velocity
- Prolongation of action potential
- Increase of refractory period

Upstroke velocity and duration of action potential are used for **classification purposes**.

Modifications of the Method

Tande et al. (1990) studied the electromechanical effects of a class III antiarrhythmic drug on guinea pig and rat papillary muscles and atria using conventional microelectrode technique.

Shirayama et al. (1991) studied with a similar technique the electrophysiological effects of sodium channel blockers in isolated guinea pig left atria.

Dawes (1946) described a method of examining substances acting on the refractory period of cardiac muscle using isolated rabbit auricles.

The same method was recommended as first step of a screening program for quinidine-like activity by Schallek (1956).

Wellens et al. (1971) studied the decrease of maximum driving frequency of isolated guinea pig auricles after antiarrhythmic drugs and beta-blockers.

Salako et al. (1976) recorded electropotentials along the conducting system after stimulation of the proximal part of the His bundle in rabbits.

Brown (1989), Wu et al. (1989), and Gwilt et al. (1991a, b) measured in vitro transmembrane action potential in Purkinje fibers and endocardial ventricular muscles from dogs.

Voltage clamp techniques in isolated cardiac myocytes from guinea pigs have been used by Wettwer et al. (1991).

Nygren et al. (2004) described heterogeneity of action potential durations in isolated mouse left and right atria recorded using voltage-sensitive dye mapping.

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Methods to Induce Cardiac Hypertrophy and Insufficiency

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Animal models of cardiac hypertrophy and insufficiency have been reviewed by Hasenfuss (1988), Muders and Elsner (2000), Vanoli et al. (2004), Patten and Hall-Porter (2009), Dubi and Arbel (2010), Gomes et al. (2013), and Szymanski et al. (2012).

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Cardiac Hypertrophy and Insufficiency in Rats

Aortic Banding in Rats

Purpose and Rationale

Blood flow restriction of the aorta in rats induces not only hypertension but also cardiac hypertrophy within several weeks. Angiotensin-converting enzyme inhibitors, even at subantihypertensive doses, but not other antihypertensive drugs, inhibit cardiac hypertrophy (Linz et al. 1991, 1992a, 1996; Schölkens et al. 1991; Gohlke et al. 1992; Bruckschlegel et al. 1995; Ogawa et al. 1998).

Procedure

Male Sprague Dawley rats weighing 270–280 g are fasted 12 h before surgery. Anesthesia is induced by i.p. injection of 200 mg/kg hexobarbital. The abdomen is shaved, moistened with a disinfectant, and opened by a cut parallel to the linea alba. The intestine is moistened with saline and placed in a plastic cover to prevent desiccation. The aorta is prepared free from connective tissue above the left renal artery and underlaid with a silk thread. Then, a cannula no. 1 (0.9 × 40 mm) is placed longitudinally to the aorta and both aorta and cannula are tied. The cannula is removed, leaving the aortic lumen determined by the diameter of the cannula. The intestine is placed back into the abdominal cavity with the application of 5.0 mg rolitetracycline (Reverin). In sham-operated controls, no banding is performed. The skin is closed by clipping.

The animals are treated once daily over a period of 6 weeks with doses of the ACE inhibitor or other antihypertensive drugs found previously effective to lower blood pressure in rats. At the end of the experiment, blood pressure is measured under hexobarbital anesthesia (200 mg/kg i.p.) via indwelling catheters in the left carotid artery. Blood pressure measurement in conscious rats with the conventional tail-cuff method is not possible due to the large pressure difference across the ligature. Therefore, only one measurement at the end of the study is possible. The hearts are removed, rinsed in saline until free of blood, and gently blotted to dryness. Total cardiac mass is determined by weighing on an electronic balance to the nearest 0.1 mg. The atria and all adjacent tissues are trimmed off, and the weight of the left ventricle including the septum as well as the remaining cardiac tissue representing the right ventricle is determined separately. Weights are calculated per 100 g body weight.

Evaluation

The total cardiac mass and weight of left and right ventricle of treated rats are compared with operated controls and sham-operated controls.

Modifications of the Method

Uetmasu et al. (1989) described a simple method for producing graded aortic insufficiencies in rats and subsequent development of cardiac hypertrophy. Selective perforation of the right cup of the aortic valve or in combination with that of the left valve cup was performed using a plastic rod inserted from the right common carotid artery. Hypertrophy of the heart, but no hypertension or cardiac insufficiency, was observed.

Similar methods were used by Yamazaki et al. (1989) to study the alterations of cardiac adrenoceptors and calcium channels subsequent to aortic insufficiency, by Umemura et al. (1992) to study baroreflex and β-adrenoceptor function, and by Ishiye et al. (1995) to study the effects of an angiotensin II antagonist on the development of cardiac hypertrophy due to volume overload.

Hyperplastic growth response of vascular smooth muscle cells in the thoracic aorta was found following induction of acute hypertension in rats by aortic coarctation by Owens and Reidy (1985). Changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with aortic banding were studied by Feldman et al. (1993). Muders et al. (1995) produced aortic stenosis in rats by placing a silver clip (inner diameter 0.6 mm) on the ascending aorta. Schunkert et al. (1995) studied alteration of growth responses in established cardiac pressure-overload hypertrophy in rats with aortic banding.

Prevention of cardiac hypertrophy after aortic banding by ACE inhibitors probably mediated by bradykinin could be shown (Linz et al. 1989, 1992a, b, 1993, 1994; Linz and Schölkens 1992; Schölkens et al. 1991; Weinberg et al. 1994).

Weinberg et al. (1997) studied the effect of angiotensin AT1 receptor inhibition on hypertrophic remodeling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis. Molina et al. (2009) described a novel experimental model of pressure-overload hypertrophy in young Sprague Dawley rats (200–250) created by placing a small titanium clip (internal diameter – 0.6 mm) in the aorta proximal to the right brachiocephalic artery. A decrease of 25 % in FS was observed 24–28 weeks after aortic constriction. Increased expression of β-myosin heavy chain, atrial natriuretic peptide, interleukin-1, interleukin-6, and TNF-α was also reported.

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Chronic Heart Failure in Rats

Purpose and Rationale

Rat models of heart failure were reviewed by Muders and Elsner (2000). Chronic heart failure

can be induced in rats by occlusion of coronary arteries. One of the first reports was by Selye et al. (1960). More recent reports are by Pfeffer et al. (1979), Hodsman et al. (1988), Van Veldhuisen et al. (1994, 1995), Kajstura et al. (1994), Gómez et al. (1997), Liu et al. (1997a, b), and Jadavo et al. (2005).

Itter et al. (2004) described a model of chronic heart failure (CHF) in spontaneously hypertensive rats.

Procedure

Study Design

Adult male 4-month-old SHR/NHsd and WKY/NHsd rats (Harlan Sprague Dawley, Winkelmann, Germany) weighing 250–300 g were used. Cardiovascular failure was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function, and geometric properties were determined by the "working heart" mode and in vivo determinations by MRI and heart weight. Hydroxyproline/proline ratio was measured as an indicator of heart fibrosis.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg) i.p. The left ventrolateral thorax was shaved and prepared to create a disinfected surgical access area. When a stable anesthesia was achieved, the animals were placed on a small animal operation table, intubated, and ventilated with room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstetten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Norderstedt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point, the lungs were hyperinflated by increasing the positive end-expiratory pressure, and the chest was closed. The rats were placed on a heating pad. They were continuously monitored until they start moving in their cages. To avoid ventricular arrhythmias, lidocaine (2 mg/kg i.m.) was given before surgery. The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the LV. To prevent acute lung edema, the rats received furosemide 2 mg/kg twice daily for 3 days via the drinking water.

Measurements at the End of the Study

Before killing the animals 8 weeks after MI, noninvasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter the animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The right carotid artery was cannulated with a polyethylene catheter to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure, and heart rate over a stable time course of 10 min.

A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. Thereafter the heart weight, and the left and right ventricular weights were determined. For infarct size determination, the left ventricle was sectioned transversely into four slices from the apex to the base. The infarct size was determined by planimetry and expressed as a percentage of LV mass. Lung weight and further lung histology sections were evaluated. Hydroxyproline/proline ratio was determined in paraffin-embedded slices of the left ventricle.

Magnetic Resonance Imaging

The animals were monitored by MRI at day 7 and day 42 post-MI. The rats were anesthetized with a mixture of 1 % halothane and 30/70 N_2O/oxygen with a specially manufactured rat mask. The fully anesthetized rats (phase III) were placed on a cradle made of Plexiglas in a supine position. Respiration and ECG were monitored continuously. MRI experiments were performed according to Rudin et al. (1991). The images were acquired by a spin-echo sequence SE (500/20), the field of view was 50 mm, and the image resolution was 256 × 256 pixels with a dimension of 0.2 × 0.2 mm. Four adjacent transverse slices were recorded; slice thickness was 1.5 mm. Before the acquisition of data, a coronary pilot scan was measured for adequate positioning of the transverse slices. MRI data acquisition was gated to the cardiac cycle by a Physiograd SM 785 MR monitoring system (Bruker, Karlsruhe, Germany). Two sets of transverse images were acquired, one at end-systole and another at end-diastole. End-diastole was defined as the image obtained 8 ms after the onset of the R wave of the ECG, corresponding to the largest cavity area. End-systole was defined as the image with the smallest LV cavity area. The image analysis was done using Bruker software (Karlsruhe, Germany). The parameters of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), septum size, infarct size, ejection fraction (EF), left ventricular chamber diameter (r), and circumference were measured. EF was estimated in percentage terms by the subtraction of LVESV from LVEDV. After the procedure, the rats were ventilated with oxygen, the mask was replaced, and they were brought back into their cages. They were monitored until they started moving in the cage.

Blood Pressure/Heart Rate

The animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved, the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The right carotid artery was cannulated with a polyethylene catheter. The catheter was connected to a PLUGSYS measuring system (Hugo Sachs Elektronik, March-Hugstetten, Germany) to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure, and heart rate over a stable time course of 10 min.

Working Heart

For the final investigations, the heart of the anesthetized rat was rapidly removed and immersed in physiological buffer chilled to 4 °C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to a perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95 % O_2/5 % CO_2) noncirculating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4$, 1.64; $NaHCO_3$, 24.88; KH_2PO_4, 1.18; glucose, 5.55; and Na-pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue, thymus, or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous pO_2 measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria. When a tight seal with no leaks had been established and after a 15-min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters, the afterload pressure was enhanced in a cumulative manner from an additional 20–140 mmHg. Thereafter, the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5–30 mmHg. Flow and pressure signals for computation were obtained from the PLUGSYS measuring system. Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstetten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight and the left and right ventricular weights were determined. The left ventricle was then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices.

The area of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the LV mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well. According to Pfeffer et al. (1985) and Pfeffer and Pfeffer (1987), rats with infarct sizes <20 % and >40 % were excluded from the study.

Lung Histological Determination

After lung weight determination, the organ was immersed in 4 % formalin (pH 7.0–7.5; 0.1 M). The lung was cut into small pieces, dehydrated, and embedded in paraffin. Hematoxylin and eosin (HE) sections were evaluated by light microscopy.

Hydroxyproline/Proline Ratio

After embedding, the rest of the fixed left ventricular tissue was freeze-dried. Proline and hydroxyproline was then analyzed according to the method of López de León and Rojkind (1985) and the ratio of both were calculated.

Evaluation

The data are given as mean ± SEM. Statistics were performed using the SAS system statistics package (SAS Institute, Cary, N.C., USA) with a sequential rejection *t*-test.

Modifications of the Method

Jain et al. (2000) studied the effects of angiotensin II receptor blockade after coronary ligation and exercise training on treadmill in rats.

Medvedev and Gorodetskaya (1993) induced heart failure in rats by microembolization of coronary vessels with 15-μm plastic microspheres.

Katona et al. (2004) found that selective sensory denervation by capsaicin aggravates adriamycin-induced cardiomyopathy in rats.

A simple and rapid method of developing high output heart failure and cardiac hypertrophy in rats by producing **aortocaval shunts** was described by Garcia and Diebold (1990). Rats weighing 180–200 g were anesthetized with 30 mg/kg i.p. pentobarbitone. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two-thirds caudal to the renal artery and one-third cephalic to the aortic bifurcation with an 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating in the vena cava. A bulldog vascular clamp was placed across the aorta caudal to the left renal artery. Once the aorta was clamped, the needle was fully withdrawn, and a drop of cyanoacrylate glue was used to seal the aorta-punctured point. The clamp was removed 30 s later. The patency of the shunt was verified visually by swelling vena cava and the mixing of arterial and venous blood. The peritoneal cavity was closed with silk thread stitches and the skin with metallic clips. Rats with aortocaval shunts developed cardiac hypertrophy with significantly higher absolute and relative heart weights.

Other studies with aortocaval shunts in rats were published by Flaim et al. (1979) and Liu et al. (1991).

Isoyama et al. (1988) studied myocardial hypertrophy after creating aortic insufficiency in rats.

Terlink et al. (1998) studied ventricular dysfunction in rats with diffuse isoproterenol-induced myocardial necrosis.

Studies (Inoko et al. 1994; Klotz et al. 2006) have shown that Dahl-salt-sensitive rats when placed on a high-salt diet from the 6th week of age will develop concentric LV hypertrophy without chamber dilation around the 11th week and decompensate heart failure between the 15th and the 20th week. Introduction of the high-salt diet at 7 or 8 weeks of age will result in diastolic heart failure or systolic heart failure phenotypes, respectively (Doi et al. 2000).

Another rat model of systemic hypertension inducing heart failure is created by clipping one renal artery while leaving the contralateral kidney untouched. This induces systemic hypertension and LV concentric remodeling within 8 weeks (Junhong et al. 2008; Rizzi et al. 2010). Extensive LV fibrosis and diastolic dysfunction was also reported (Junhong et al. 2008).

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Cardiac Hypertrophy and Insufficiency in Mice

Cardiac Hypertrophy in Mice

Purpose and Rationale

Rockman et al. (1991, 1993) developed a model of ventricular hypertrophy in the intact mouse by use of microsurgical techniques.

Procedure

Eight-week-old adult mice weighing 18–22 g are anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine, 5 mg/kg xylazine, and 2.5 mg/kg morphine. Animals are placed under a dissecting microscope in the supine position, and a midline cervical incision is made to expose the trachea and carotid arteries. After endotracheal intubation, the cannula is connected to a volume-cycled rodent ventilator on supplemental oxygen with a tidal volume of 0.2 ml and a respiratory rate of 110 per min. Both left and right carotid arteries are cannulated with flame-stretched PE50 tubing. Catheters are connected to modified P50 Statham transducers.

The chest cavity is entered in the second intercostal space at the left upper sternal border

through a small incision, and the thymus is gently deflected out of the field of view to expose the aortic arch. After the transverse aorta is isolated between the carotid arteries, it is constricted by a 7.0 nylon suture ligature against a 27-gauge needle, the latter being promptly removed to yield a constriction of 0.4-mm diameter and provide a reproducible transverse aortic constriction of 65–75 %.

The hemodynamic effects of acute and chronic constriction are followed by monitoring the pressure gradient between the two carotid arteries in anesthetized animals. Systolic and mean arterial pressure at baseline, during total occlusion when the ligature is tied, and early (15 min) and late (7 days) after transverse aortic constriction are recorded. The increase in systolic pressure provides an adequate mechanical stimulus for the development of cardial hypertrophy.

To confirm myocardial hypertrophy, both sham-operated and aortic-constricted hearts are examined 7 days after operation. Hearts examined for *cell size* are perfused with 4 % paraformaldehyde/1 % glutaraldehyde through the apex, immersed in osmium tetroxide, dehydrated in graded alcohols, and embedded in araldite. Tissue blocks are sectioned at a thickness of 1 μm, mounted on slides, and stained with toluidine blue. Cell areas are measured by manually tracing the cell outline on an imaging system connected to a computer.

At the end of the experiment, mice were sacrificed in anesthesia, heart excised, and weighed, the atria and ventricles separately frozen in liquid nitrogen for Northern blot analysis. Total RNA is extracted by a single-step extraction with guanidinium thiocyanate. The RNA is size fractionated by agarose gel electrophoresis, transferred to nylon membranes by vacuum blotting, and hybridized with the appropriate complementary DNA probes labeled with ^{32}P by random priming to a specific activity of 0.95–1.2 $\times$ 10^6 cpm/ng.

Evaluation

Variables measured are expressed as mean $\pm$ SD. Statistical significance of differences between sham-operated and thoracic aortic-constricted animals is assessed by Student's *t*-test.

Modifications of the Method

Dom et al. (1994) studied myosin heavy chain regulation and myocytes' contractile depression after LV hypertrophy in aortic-banded mice.

Okada et al. (2004) subjected mice to transverse aortic constriction. Echocardiographic analysis demonstrated cardiac hypertrophy and failure 1 and 4 weeks after surgery. Cardiac expression of endoplasmatic reticulum chaperones was significantly increased, indicating that pressure overload by transverse aortic constriction induced prolonged endoplasmatic reticulum stress.

Stansfield et al. (2007) described a minimally invasive murine model of transverse aortic constriction debanding, in which the band is removed up to 4 weeks after constriction through the same suprasternal incision. This reversible model of pressure overload was shown as an interesting model to study the molecular mechanisms involved in LV reverse remodeling.

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Chronic Heart Failure in Mice

Purpose and Rationale

Several authors reported the development of murine models of cardiac failure (Kaplan et al. 1994; Rockman et al. 1994; Balasubramaniam et al. 2004; Suzuki et al. 2004; Walther et al. 2004; Wang et al. 2004; Liao et al. 2005).

Xu et al. (2004) studied cardioprotection in mice with heart failure by dual inhibition of angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP).

Procedure

Mice with a targeted deletion of the B_2 kinin receptor gene or C57BL/6 J mice at an age of 10–12 weeks were anesthetized with 50 mg/kg sodium pentobarbital i.p., intubated and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space; the lungs were retracted to expose the heart, and the pericardium was opened. The left anterior descending coronary artery was ligated with an 8–0 nylon suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was considered successful when the anterior wall of the left ventricle turned pale and an obvious ST segment elevation was observed. The lungs were inflated by increasing positive end-expiratory pressure and the thoracotomy site was closed. Sham-operated mice were subjected to the same procedure except that the suture around the left anterior coronary artery was not tied. Systolic blood pressure was measured in conscious mice using a noninvasive computerized tail-cuff system. Cardiac geometry and function were evaluated with a Doppler echocardiographic system. LV diastolic dimension was measured and ejection fraction was calculated from

$$\left[\frac{(\mathrm{LVAd} - \mathrm{LVAs})}{\mathrm{LVAd}}\right] \times 100,$$

where LVAd is the LV diastolic area and LVAs is the LV systolic area.

Four weeks after surgery, each strain was separated into one group treated with an ACE inhibitor, one group treated with a NEP inhibitor, one group treated with both inhibitors, and one control group. All drugs were administered in drinking water for 20 weeks.

At the end of the study, all mice were anesthetized with pentobarbital and the heart stopped at diastole by intraventricular injection of 15 % KCl. The heart, lungs, and liver were weighed to assess hypertrophy and congestion. Infarct size was determined by Gomori trichrome staining and expressed as the ration of the infarcted portion to total LV circumference.

Sections (6 μm) from each slice were double stained with fluorescein-labeled peanut agglutinin to delineate the myocyte cross-sectional area and interstitial space and rhodamine-labeled *Griffonia simplicifolia* lectin I to show the capillaries. To calculate interstitial collagen fraction, the total surface area (microscopic field), interstitial space (collagen plus capillaries), and area occupied by capillaries alone were measured by computer-assisted videodensometry.

After 20 weeks of treatment, plasma renin was measured.

Evaluation

Data were expressed as mean ± SE. Mortality rates were compared using χ^2 tests. For the echo, blood pressure, heart weight, lung weight, infarct size, plasma renin concentration, and histology data, paired or two-sample tests using nonparametric methods were used to perform all comparisons of interest.

Modifications of the Method

Scheuermann-Freestone et al. (2001) established a new mouse model of chronic volume overload

by an aortocaval shunt. Congestive heart failure was induced, which resulted in the development of myocardial hypertrophy, impaired cardiac function, and increased expression of the natriuretic peptides in the left ventricle.

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Transgenic Mice and Heart Failure

Purpose and Rationale

Several hundreds of papers on transgenic mice and heart failure are published. Only a few can be mentioned here.

Chien (1995) described cardiac muscle diseases in genetically engineered mice.

Edwards et al. (1996) described severe cardiomyopathy in transgenic mice overexpressing the skeletal muscle myogenic regulator *myf5*.

Arber et al. (1997) found that MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure.

Graham et al. (1997) described a mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoforms of the adenine nucleotide translocator.

Iwase et al. (1997) studied cardiomyopathy in transgenic mice induced by overexpression of the cardiac stimulatory G protein α-subunit.

Knollmannn et al. (2000) reported remodeling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin.

Beggah et al. (2002) described reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes.

Verheule et al. (2004) found increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-β1.

Duncan et al. (2005) found that chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy.

Hartil and Charron (2005) reviewed mouse models where transgenic technology has been utilized to alter expression of genes involved in cardiac uptake and metabolism of either lipid or carbohydrate.

Hilfiker-Kleiner et al. (2005) reported that STAT3 knockout mice harboring a cardiomyocyte-restricted deletion of STAT3 showed enhanced susceptibility to cardiac injury caused by myocardial ischemia, systemic inflammation, or drug toxicity.

Sanbe et al. (2005) studied reversal of amyloid-induced heart disease in desmin-related cardiomyopathy.

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Cardiac Insufficiency in Guinea Pigs

Purpose and Rationale

Congestive heart failure in man is characterized by cardiac hypertrophy, peripheral edema, lung and liver congestion, dyspnea, hydrothorax, and ascites. Effective treatment is achieved by cardiac glycosides. Based on techniques reported by Selye et al. (1960), a method was developed to induce congestive heart failure in guinea pigs with symptoms very close to human pathology (Vogel and Marx 1964; Vogel et al. 1965).

Procedure

Male guinea pigs weighing 250–400 g are used. The fur at the ventral thorax is shaved and the

skin disinfected. The animal is anesthetized with ether. The skin is cut with scissors on the left side at a length of 4 cm. The left musculus pectoralis is cut at the costal insertion and elevated. The forth intercostal space is opened with two blunted forceps. The heart is pressed against the opening with the left hand. The pericardium is opened with a fine forceps and pulled back to the basis of the heart. The beating heart is extruded from the thorax wound by pressure with the left hand on the right thorax wall. A ring-shaped clamp covered with a thin rubber tube is placed around the basis of the heart, keeping the heart outside of the thorax without closing off the blood circulation. A thread soaked with diluted disinfectant solution is placed as a loop around the apex of the heart and tightened so that the apical third of both ventricles is tied off. The degree of tightening of the loop is essential. Complete interruption of blood supply to the apical third resulting in necrosis has to be avoided as well as the loop's slipping off. Technical skill is necessary to place the loop around the beating heart into the correct position. After removal of the clamp, the heart is placed back, the incision between the fourth and fifth costal rib closed, and the musculus pectoralis placed over the wound. Intrathoracal air forming a pneumothorax is removed by pressure on both sides of the thorax. After application of an antibiotic emulsion, the skin wound is closed. The surgical procedure has to be finished within a short period of time.

The animals develop symptoms of severe congestive heart failure with a death rate of 80 % within 14 days. Lung weight and relative heart weight are significantly increased. Exudate in the thorax cavity and ascites amount between 3.5 and 7.5 ml with extreme values of 17.5 ml. Lung edema and liver congestion are found histologically. Peripheral edema and preterminal dyspnea and tachypnea are observed. When treated with various doses (0.1–100 μg/kg) of cardiac glycosides s.c. or i.m. over a period of 14 days, the symptoms of cardiac insufficiency, e.g., volumes of transudate as well as death rate, are dose-dependent diminished.

Evaluation

From survival rate, ED_{50} values of cardiac glycosides can be calculated which are in the same dosage range as therapeutic doses in man.

Critical Assessment of the Method

The experimental model in guinea pigs reflects very closely the symptoms of cardiac insufficiency in man, e.g., lung congestion, hydrothorax, liver congestion, ascites, peripheral edema, and cardiac hypertrophy. The therapeutic potency of cardiac glycosides can be evaluated with this method. Additional factors being known to enhance the symptoms of congestive heart failure in man, like salt load and diphtheria toxin, further increase mortality and hydropic symptoms. The method can be used for special purposes; however, it needs considerable training and technical skill.

Modifications of the Method

Siri et al. (1989, 1991) produced left ventricular hypertrophy in the guinea pig by gradually increasing ventricular afterload. A mildly constricting band was placed around the ascending aorta of very young guinea pigs (225–275 g). With growth to 500–1,000 g, left ventricular systolic pressure increased and ventricular hypertrophy developed. Only some of the animals developed dyspnea and severe ventricular dysfunction.

Kiss et al. (1995) studied the effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure in guinea pigs. The descending aorta was banded for 4 and 8 weeks in adult guinea pigs.

Tweedle et al. (1995) assessed subrenal banding of the abdominal aorta as a method of inducing cardiac hypertrophy in the guinea pig.

Pfeffer et al. (1987) induced myocardial infarction in **rats** by ligation of the left coronary artery and found hemodynamic benefits and prolonged survival with long-term captopril therapy.

Acute ischemic left ventricular failure can be induced in anesthetized **dogs** by repeated injections of plastic microspheres into the left coronary artery (see chapter "▶ Coronary Drugs", section "Acute Ischemia by Injection of Microspheres in Dogs").

Huang et al. (1997) created congestive heart failure in **sheep** by selective sequential intracoronary injection of 90 μm microspheres under 1.5 % isoflurane injection.

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Cardiomyopathic Syrian Hamster

Purpose and Rationale

Cardiomyopathy in Syrian hamsters has been described by Bajusz et al. (1966), Bajusz and Lossnitzer (1968), Bajusz (1969), Bajusz et al. (1969a, b), Homburger and Bajusz (1970), and Gertz (1972). The disease originates from an autosomal, recessively transmissible disorder, which leads to degenerative lesions in all striated muscles and in particular in the myocardium. Histopathological changes consist of myocytolytic necrosis followed by fibrosis and calcification. The evolution of the cardiomyopathic disease can be characterized by five distinct phases: a prenecrotic stage, in which no pathology is evident, a time of active myocytolysis and cellular necrosis, a phase of fibrosis and calcium deposition, an overlapping period of reactive hypertrophy of the remaining viable myocytes, and a final stage of depressed myocardial performance and failure.

Procedure

The model of cardiomyopathy in Syrian hamsters has been used by several authors. One has to note that several strains of cardiomyopathic hamsters have been used: strain Bio 53:58 by Capasso et al. (1989, 1990) and by Chemla et al. (1992, 1993), strain BIO 14.6 by Tapp et al. (1989) and

by Sen et al. (1990), strain CHF 146CM by van Meel et al. (1989) and by Haleen et al. (1991), strain BIO82.62 by ver Donck et al. (1991), strain J-2-N by Kato et al. (1992), and strain CHF 147 by Desjardins et al. (1989) and Hanton et al. (1993).

Various experimental protocols have been described. Most authors use survival rate and heart weight as end point (e.g., van Meel et al. 1989; ver Donck et al. 1991; Hanton et al. 1993). Generally, the experiments are started with animals at an age of 120–200 days.

Capasso et al. (1989, 1990) studied the mechanical and electrical properties of cardiomyopathic hearts of Syrian hamsters using isolated left ventricular posterior papillary muscles.

Tapp et al. (1989) tested stress-induced mortality in cardiomyopathic hamsters by five consecutive daily 2-h periods of supine immobilizations at 4 °C.

Sen et al. (1990) tested the inotropic and calcium kinetic effects of calcium channel agonists and antagonists in primary cultures of isolated cardiac myocytes.

Haleen et al. (1991) tested the effects of an angiotensin-converting-enzyme inhibitor not only on survival but also on left ventricular failure in the isolated Langendorff heart by measurement of left ventricular end-diastolic pressure, dP/dt_{max}, and mean coronary flow.

Dixon et al. (1997) tested the effect of an AT_1 receptor antagonist on cardiac collagen remodeling in the cardiomyopathic Syrian hamster.

In addition to the effects on left ventricular papillary muscles strips, Chemla et al. (1992) tested the effects on diaphragm contractility in the cardiomyopathic Syrian hamster.

Whitmer et al. (1988) and Kuo et al. (1992) tested sarcolemmal and sarcoplasmatic reticulum calcium transport in the cardiomyopathic Syrian hamster.

Nigro et al. (1997) identified the Syrian hamster cardiomyopathy gene.

Tanguay et al. (1997) tested the coronary and cardiac sensitivity to a vasoselective benzothiazepine-like calcium antagonist in isolated, perfused failing hearts of Syrian hamsters.

Bilate et al. (2003) recommended the Syrian hamster as a model for the dilated cardiomyopathy of Chagas disease. Female hamsters were infected via the intraperitoneal route with *Trypanosoma cruzi* Y strain blood trypomastigotes. Survival was monitored, echocardiography was performed after 4 and 12 months, and histopathological examinations were carried out at the end of the study period.

Critical Assessment of the Method

Positive effects of various drugs have been found in the cardiomyopathic hamster, such as cardiac glycosides, inotropic compounds, beta-blockers, calcium antagonists, and ACE inhibitors. The specificity of the effects has to be challenged.

Modifications of the Method

The **tight skin (TSK) mouse** is a genetic model of pulmonary emphysema connected with right ventricular hypertrophy (Martorana et al. 1990; Gardi et al. 1994).

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Cardiac Failure in Rabbits

Purpose and Rationale

Rabbit models of heart failure were reviewed by Muders and Elsner (2000).

Rapid pacing was used by Masaki et al. (1994), Porsa et al. (1994), Eble et al. (1998), Li et al. (2003), and Rose et al. (2005); coronary artery ligation by Pennock et al. (1997), Currie and Smith (1999), Romanic et al. (2001), and Miller et al. (2004); combined pressure and volume overload by Ezzaher et al. (1991), Mohammadi et al. (1997), Dekker et al. (1998), and Baartscheer et al. (2003a, b); aortic insufficiency and aortic constriction by Bouanani et al. (1991) and Pogwizd et al. (1999); regurgitation after damage of the mitral valve by Gunawardena et al. (1999); and regurgitation after aortic valve destruction by Magid et al. (1988, 1994), Yoshikawa et al. (1993), King et al. (1997), Liu et al. (1998), and Luchner et al. (2001) used a rabbit model of progressive left ventricular dysfunction to investigate differential expression of cardiac atrial natriuretic peptide and brain natriuretic peptide. Ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system.

Procedure

Male rabbits (chinchilla bastard) underwent implantation of a programmable cardiac pacemaker (Medtronic Minix 8340, Minneapolis, Mn., USA). Under anesthesia (ketamine 60 mg/kg xylazine 5 mg/kg i.m.), the right internal jugular vein was dissected and cannulated with a single-lumen central venous catheter (Braun, Germany). The catheter was then advanced into the right ventricle under pressure guidance. A transvenous screw-in pacemaker lead (Medtronic) was advanced through the catheter into the ventricular apex and implanted endocardially. The pacemaker was implanted subcutaneously into the right abdominal wall, and the pacemaker lead was connected subcutaneously with the pacemaker. Rapid ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system. All rabbits were allowed to recover for at least 10 days after surgery before the pacemaker was started for the induction of heart failure. Proper pacemaker function was checked intraoperatively, at the time of programming, and subsequently all 10 days.

Rabbits (CHF group) underwent pacing with a stepwise increase of stimulation frequencies over 30 days. During the first 10 days, animals were paced at 330 beats/min (bpm). This protocol results in ELVD, as defined by significant LV systolic dysfunction with cardiac enlargement and decreased perfusion pressure, but no clinical signs of heart failure. The pacing rate was then increased to 360 bpm for 10 days and 380 bpm for another 10 days, and ELVD evolved to CHF with further cardiac enlargement and further decreased perfusion pressure together with clinical signs of fluid retention (ascites). At baseline (control), after being paced at 330 bpm for 10 days (ELVD) and at the end of the protocol (CHF), conscious arterial pressure was measured invasively via the medial ear artery and a 2-D-guided M-mode echocardiogram was obtained. At the end of the pacing protocol, rabbits were killed by i.v. euthanasia and tissue was rapidly harvested. Hearts were trimmed on ice, snap frozen in liquid nitrogen, and stored at −80 °C until further processing.

Echocardiography

A long- and short-axis echocardiogram (HP Sonos 5500, 12 MHz probe) was performed under light sedation (5 mg midazolam i.m.) in a supine position from the left parasternal window. LV end-diastolic (LVEDd) and end-systolic (LVESd) dimensions and diastolic and systolic thickness of the left ventricular anterior wall (AEDth and AESth) and posterior wall (PEDth and PESth) as well as left atrial diameter (LAd) were determined from three repeated 2-D-guided M-mode tracings using the ASE convention. From those measurements, fractional shortening (FS) was calculated as

$$\mathrm{FS} = \frac{(\mathrm{LVEDd} - \mathrm{LVESd})}{\mathrm{LVEDd}}.$$

Analytical Methods

For analysis of cardiac natriuretic peptide expression, mRNA was extracted from all atrial and left ventricular samples utilizing a commercial kit (Fasttrack, Invitrogen).

As a probe for brain natriuretic peptide (BNP), a 750-bp *Eco*R1/*Hind*III DNA restriction fragment containing the gene for rabbit BNP was used.

Evaluation

Results of the quantitative studies were expressed as mean ± SEM. Comparisons between the control, ELVD, and CHF groups were performed by analysis of variance (ANOVA) followed by Fisher's least significant difference test. Comparison between the atrial and LV tissues as well as between atrial natriuretic peptide (ANP) and BNP was performed by paired Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Modifications of the Method

Arnolda et al. (1985) studied adriamycin cardiomyopathy in the rabbit.

Klimtova et al. (2002) performed a comparative study of chronic toxic effects of daunorubicin and doxorubicin in rabbits.

Alexander et al. (1993) studied electrographic changes following coronavirus-induced myocarditis and dilated cardiomyopathy in rabbits.

Sanbe et al. (2005) described a transgenic model for human troponin I-based hypertrophic cardiomyopathy in the rabbit.

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Cardiac Failure in Dogs

Purpose and Rationale

Several methods are described, to induce congestive heart failure in dogs, such as rapid ventricular pacing (Armstrong et al. 1986; Freeman et al. 1987; Wilson et al. 1987; Komamura et al. 1992, 1993; Perreault et al. 1992; Travill et al. 1992; Cheng et al. 1993; Redfield et al. 1993; Cory et al. 1994; Kiuchi et al. 1994; Ohno et al. 1994; Vatner et al. 1994; Wang et al. 1994; Williams et al. 1994; Eaton et al. 1995; Spinale et al. 1995; Wolff et al. 1995; Zile et al. 1995; Ravens et al. 1996; Shinbane et al. 1997; O'Rourke et al. 1999; Winslow et al. 1999).

Luchner et al. (1996) assessed circulating, renal, cardiac, and vascular angiotensin II in a canine model of rapid ventricular pacing-induced heart failure that evolves from early left ventricular dysfunction to overt congestive heart failure.

Procedure

Male mongrel dogs underwent implantation of a programmable cardiac pacemaker (Medtronic). Under pentobarbital sodium anesthesia and artificial respiration, the heart was exposed via a small left lateral thoracotomy and pericardiotomy, and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker was implanted subcutaneously into the left chest wall and connected to the pacemaker lead. The dogs were allowed to recover for at least 10 days after surgery before the pacemaker was started. During the first 10 days, dogs were paced at

180 beats/min (bpm), resulting in early left ventricular dysfunction as defined by significant systolic dysfunction with decreased cardiac output, cardiac enlargement, and increased filling pressures but maintained systemic perfusion pressure and renal sodium excretion and no clinical signs of heart failure. The pacing rate was then increased weekly to 200, 210, 220, and 240 bpm, and early left ventricular dysfunction evolved to overt congestive heart failure with avid sodium retention and clinical signs of congestion. At baseline (control), after dogs had been paced at 180 bpm for 10 days and at the end of the protocol (overt CHF), urine was collected for measurement of sodium excretion; conscious mean arterial pressure was measured via a port catheter; a 2-D-guided M-mode echogram was obtained; and arterial blood was drawn. Cardiac filling pressures and cardiac output were measured by the thermodilution method at baseline and at the end of the protocol. Arterial blood was collected in EDTA tubes for measurement of ANP, BNP, cGMP, PRA, aldosterone, and Ang II. After euthanasia, hearts were rapidly trimmed and left ventricles weighted for calculation of the index LV weight to body weight.

Evaluation

Results were expressed as mean ± SE. Comparison between the control, early LV dysfunction, and overt CHF were performed by ANOVA followed by Fisher's least significant difference test.

Modifications of the Method

Kleaveland et al. (1988) and Nagatsu et al. (1994) used the technique of experimental **mitral regurgitation** in dogs to induce left ventricular dysfunction. A 30-cm, 7-F sheath was introduced across the aortic valve through the carotid artery. A urologic calculus retrieval forceps was advanced through the sheath to the mitral valve apparatus and was used to sever chordae tendineae. When pulmonary capillary wedge pressure rose to 20 mmHg and forward stroke volume was reduced to 50 % of its baseline, a ventriculogram was performed to confirm angiographically that severe mitral regurgitation had been created.

Dell'Italia et al. (1995) and Su et al. (1999) induced mitral regurgitation by percutaneous chordal rupture in dogs.

Kinney et al. (1991) published a method to induce acute, reversible tricuspid insufficiency in anesthetized dogs. A wire spiral is advanced through the atrioventricular canal from the right atrium. The spiral causes regurgitation by preventing complete apposition of the valve leaflets while permitting retrograde flow to occur through the spiral lumen. The degree of regurgitation can be controlled by the use of spirals of different size. Creation of tricuspid insufficiency was demonstrated by onset of right atrial pressure V waves, a ballooning of the right atrium during ventricular systole, palpation of an atrial thrill, or color Doppler echocardiography. The model is reversible and allows repeated trials of various grades of regurgitation.

Carlyle and Cohn (1983) described a non-chirurgical model of chronic left ventricular dysfunction. The method is accomplished by repetitive DC shock with a guidewire introduced percutaneously and positioned in the left ventricle along the intraventricular septum and an external paddle at the left ventricular apex.

McDonald et al. (1992) produced localized left ventricular necrosis without obstruction of the coronary blood flow in dogs by transmyocardial direct-current shock.

Sabbah et al. (1991, 1993, 1994) and Gengo et al. (1992) produced chronic heart failure in dogs by multiple sequential intracoronary **embolizations with microspheres**. The dogs underwent three to nine intracoronary embolizations with polystyrene latex microspheres (70–102 μm in diameter) performed 1–3 weeks apart. Embolizations were discontinued when left ventricular ejection fraction was less than 35 %. Vanoli et al. (2004) used multiple coronary microembolizations in dogs, whereby three to nine embolizations were performed 1 week apart. The first three embolizations consisted of 2 ml of microsphere suspension injected

subselectively into either the left anterior descending or left circumflex coronary artery in an alternating fashion. Subsequent embolizations consisted of 3–6 ml of microspheres divided equally between the left anterior descending or left circumflex coronary artery until LV ejection fraction was <35 %.

Magovern et al. (1992) described a canine model of left ventricular dysfunction caused by five weekly intracoronary infusions of **adriamycin**.

Koide et al. (1997) described premorbid determinants of left ventricular dysfunction in a model of gradually induced pressure overload in dogs. Mongrel dogs were studied through 8 weeks of gradually imposed ascending aortic constriction with the use of a **novel banding technique**. During banding, an initial gradient of 30 mmHg was created. Before banding, at 2, 4, and 6 weeks after banding, hemodynamics and left ventricular mechanics were examined at cardiac catheterization; then the pressure overload was increased by tightening the band.

Valentine et al. (1988) and Devaux et al. (1993) described **X-linked muscular dystrophy in dogs** with cardiac insufficiency similar to Duchenne muscular dystrophy in men and recommended this as an animal model for cardiac insufficiency.

Bilateral renal wrapping model in adult male dogs (20–26 kg) has been described previously (Page 1939; Hart et al. 2001; Maniu et al. 2002); in this model, the kidneys were wrapped with silk without constriction of renal vessels. Increased in systolic blood pressure and LV mass index was observed at 5 weeks post renal wrapping. At 12 weeks post renal wrap, an increase in LVEDP was observed. LV end-diastolic volume, ejection fraction, stroke volume, and cardiac output were not changed in this model. No changes in circulating angiotensin II, endothelin, catecholamines, and plasma renin activity were also noted. A modification of this method has been published by Hayashida et al. (1997, 1998); in these studies only the left kidney was wrapped. The dogs developed hypertension and diastolic dysfunction with increased LV weight/body weight ratio and LVEDP and without significant changes in fractional shortening or LV diameters an increased Ang II levels.

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Cardiac Failure in Pigs

Purpose and Rationale

Cardiac failure was studied in pigs using several experimental procedures.

Chow et al. (1990) recommended rapid ventricular pacing in pigs as an experimental model of congestive heart failure.

Farrar et al. (1993) studied pacing-induced dilated cardiomyopathy in pigs. Congestive heart failure was produced by rapid ventricular pacing at 230 bpm for 1 week.

Spinale et al. (1990a, b, 1991, 1992) examined the consequences of chronic supraventricular tachycardia on various parameters of ventricular dysfunction and subendocardial changes in pigs.

Carroll et al. (1995) investigated gene expression in a swine model of ventricular hypertrophy during pressure overload.

Multani et al. (2001) studied long-term angiotensin-converting enzyme and angiotensin I receptor inhibition in pacing-induced heart failure in pigs. Heart failure was induced by rapid atrial pacing (240 bpm for 3 weeks).

Kassab et al. (1993, 2000) investigated remodeling of right ventricular branches after hypertrophy in pigs.

Krombach et al. (1999) studied the effects of amlodipine in congestive heart failure in pigs at rest and after treadmill exercise.

Procedure

Left thoracotomy was performed in Yorkshire pigs under anesthesia. Catheters connected to a vascular access port were placed in the thoracic aorta, the pulmonary artery, and the left atrium. The access ports were then placed in a subcutaneous pocket. A 20-mm flow probe was placed around the pulmonary artery immediately distal to the pulmonary catheter and the electrical connection exteriorized through the thoracolumbar fascia. A shielded stimulating electrode was sutured onto the left atrium, connected to a programmable pacemaker, and buried in a subcutaneous pocket. The thoracotomy was closed in layers and the pleural space evacuated of air. After a 14–21-day recovery, measurements were performed under normal resting conditions and after exercise. The pacemakers were activated to 240 bpm for a period of 21 days. During the last 3 days, one group was treated with drug, the other served as control. At the day of the study, electrocardiograms were performed, and the pacemakers deactivated. After a 30-min stabilization period, 2-D and M-mode echocardiographic studies were used to image the left ventricle from the parasternal approach. Left ventricular fractional shortening was calculated as (end-diastolic dimension – end-systolic dimension)/diastolic dimension and was expressed as a percentage. The access ports were entered and pressures obtained using externally calibrated transducers. The flow probe was connected to a digital flowmeter. From the digitized flow signal, stroke volume was computed on a beat-to-beat basis and averaged for a minimum of 25 ejections. Pulmonary and systemic vascular resistances were computed as the mean pressure divided by cardiac output multiplied by the constant 80 to convert to resistance units of dyne $\cdot s \cdot \mathrm{cm}^{-5}$. Samples were drawn from the pulmonary artery and atrial catheters for measurement of oxygen saturation and hemoglobin content. The plasma samples were assayed for renin activity, endothelin concentration, and catecholamine levels.

Evaluation

Results were presented as mean $\pm$ SEM. Pairwise tests of individual group means were compared using Bonferroni probabilities.

Modifications of the Method

Zhang et al. (1996) studied functional and bioenergetic consequences of postinfarction left remodeling in a porcine model. Proximal left coronary artery occlusion was used to generate a myocardial infarction in young pigs. The animals were then followed over several months while remodeling of the left ventricle developed. Left ventricular wall thickness, ejection fraction, and wall stress were measured by MRI. Myocardial ATP, creatine phosphate, and inorganic phosphate levels were measured by spatially localized

^{31}P-NMR spectroscopy, and regional myocardial blood flow was measured with radioactive microspheres.

Procedure

MRI Protocols

All MRI studies were performed on the standard Siemens Medical System VISION operating at 1.5 T. The animals were anesthetized with sodium pentobarbital. A catheter was placed into the femoral artery and advanced into the LV chamber for LV pressure recording. Animals then were placed on their left side in a Helmholtz coil with a diameter of 18 cm, which was used to improve signal to noise. To compute LV wall stress, the image acquisition was triggered by the LV pressure through the fluid-filled LV catheter. All of the imaging sequences were synchronized to the LV pressure trace. The electronic LV pressure signal was recorded and fed to a comparator set to a threshold level of 10 % of the upslope of the LV pressure curve at the beginning of systole. The signal from the comparator was sent to a pulse former and then fed to the ECG port of the magnetic resonance system, where it was treated like the standard electrographic input to run the pulse sequences. Scout images were taken in the axial plane with a single-shot, ultrafast gradient echo sequence (McDonald et al. 1992; Wilke et al. 1993; Geiger et al. 1995). From the axial image, both horizontal and vertical long-axis images were obtained. By alternating back and forth several times, a true vertical long axis of the left ventricle was obtained. From the long-axis scout image, short-axis segmented cine turboflash slices were prescribed to cover the myocardium from apex to base. The double oblique, short-axis turboflash images cover the heart from apex to base with a slice thickness of 10 mm, with no interslice gap.

MRI Cine Technique

The parameters of the segmented cine sequence were TR/TE/flip angle = 33 ms/6.1 ms/25° with an FOV = 17.5 cm and a matrix of 87 × 128 (pixel size, 2 × 1.4 mm) and slice thickness of 7–10 mm (Atkinson and Edelman 1991). The sequence used segmented k-space acquisition such that three phase-encoded lines were gathered per cardiac phase per heartbeat. Total image acquisition required approximately 52 heartbeats for each slice location. The temporal image resolution (data acquisition window) of this sequence was 33 ms per cardiac image. Each myocardial level took <1.5 min to acquire, since two acquisitions were used and the average heart rate of the animals was 120 bpm. The average number of short-axis slices needed to image the entire myocardium from apex to base was 6–8. This 10-min protocol provided high signal-to-noise cine sequences covering the entire heart.

Spin-Echo Images

To obtain high-resolution anatomic heart images, multislice, single-phase spin-echo images trigged in the systolic phase were acquired to cover the entire heart. These images permitted the precise delineation of the extent of the scar region of the heart. Images were taken with a slice thickness of 5 mm and a FOV of 17.5 cm, resulting in a true spatial resolution of 2 × 1.4 mm pixel size. The TR for this sequence equals the RR interval (500 ms) and the echo time TE was set to 30 ms. Total measurement time for an average of 10–14 slices was 5 min.

Image Analysis of the MRI Cine Studies

The imaging data were archived to optical disk and copied to a SUN SPARC 10 workstation for evaluation with the use of an automatic segmentation program (ImageView, Siemens Cooperate Research). The program is based on robust deformable models of endocardial and epicardial border segmentation of ventricular boundaries in cardiac magnetic resonance images. This segmentation technique has been combined with a user interface that allows one to load, sort, visualize, and analyze a cardiac study in <20 min. The segmentation algorithm is based on the steepest descent as well as dynamic programming strategies integrated via multiscale analysis for minimizing the energy function of the resulting

contour. The ventricular boundaries are used to construct a three-dimensional model for visualization and to compute hemodynamic parameters. Automatic segmentation of endocardial and epicardial boundaries was performed for calculation of ventricular volumes, EF, LV diastolic and systolic volumes, and absolute myocardial mass from multislice, multiphase magnetic resonance cine images. Starting with a user-specified approximate boundary or an interior point of the ventricle for one starting image in one slice, the algorithm generated automatic contours corresponding to the epicardium and the endocardium and automatically propagated them to other slices in the cardiac phase (spatial propagation) and to other phases for a given slice location (temporal propagation) of the cardiac study. The observer then could make some manual corrections to the six or seven pairs of contours in the first column of the temporal-spatial matrix. Manual modifications generally were made on the apex and base levels.

Evaluation

Mean LV wall thickness for each short-axis ring was averaged from three measurements of the remote zone (anterior wall and septum wall). The thickness of the scar was averaged from three measurements of the scar area. LVSA measurement in each slice was computed by subtracting the total area enclosed by the endocardium from that enclosed by the subepicardium; the resultant area was multiplied by the slice thickness to obtain the volume of each slice; the total LV mass volume was calculated by adding up the volumes of all the short-axis slices. The total LVSA was obtained by dividing the total LV wall mass volume by the mean of LV wall thickness of each slice. Similarly, the LVSSA was obtained by dividing the total scar volume, which was the sum of the scar volume of each short axis, by the mean of the scar thickness of each short axis. LV mass was computed by the total LV wall mass volume multiplied by 1.05 (specific gravity of myocardium) to calculate the LV mass. The LV end-diastolic volume (V_d) and end-systolic volume (V_s) of each slice were represented by the area enclosed by the endocardium. The total LV volume was computed by adding the volumes of all slices. LVEF was calculated by $100 \times (V–V_s)/V_d\%$. Interobserver and intraobserver errors for the calculations of LV mass and LV volumes have been shown to be <3 mg and 3 ml, respectively (McDonald et al. 1994). Meridional wall stress was computed from the LV pressure and simultaneously obtained short-axis view of LV MRI (LV cavity diameter and average thickness the remote LV wall) as described by Grossman et al. (1975).

Spatially Localized ^{31}P-NMR Spectroscopic Technique

Measurements were performed in a 40-cm-bore, 4.7-T magnet interfaced with a SISCO (Spectroscopy Imaging Systems Corporation) console. The LV pressure signal was used to gate NMR data acquisition to the cardiac cycle, while respiratory gating was achieved by triggering the ventilator to the cardiac cycle between data acquisitions (Robitaille et al. 1990). ^{31}P and ^{1}H-NMR frequencies were 81 and 200.1 MHz, respectively. Spectra were recorded in late diastole with a pulse repetition time of 6–7 s. This repetition time allowed full relaxation for ATP and P_i resonances and ≈90 % relaxation for the CP resonance (Zhang and McDonald 1995). CP resonance intensities were corrected for this minor saturation; the correction factor was determined for each heart from two spectra recorded consecutively without transmural differentiation, one with 15-s repetition time to allow full relaxation and the other with the 6–7-s repetition time used in all the other measurements.

Radiofrequency transmission and signal detection were performed with a 25-mm-diameter surface coil. The coil was cemented to a sheet of silicone rubber 0.7 mm in thickness and ≈50 % larger in diameter than the coil itself. A capillary containing 15 μl of 3 M phosphonoacetic acid was placed at the coil center to serve as a reference. The proton signal from water detected with the

surface coil was used to homogenize the magnetic field and to adjust the position of the animal in the magnet so that the coil was at or near the magnet and gradient isocenters. This was accomplished with a spin-echo experiment and a readout gradient. The information gathered in this step also was used to determine the spatial coordinates for spectroscopic localization. Chemical shifts were measured relative to CP, which was assigned a chemical shift of −2.55 ppm relative to 85 % phosphoric acid at 0 ppm.

Spatial localization across the LV wall was performed with the RAPP-ISIS/FSW method (Hendrich et al. 1991). Signal origin was restricted with the use of B_0 gradients and adiabatic inversion pulses to a column coaxial with the surface coil perpendicular to the LV wall. The column dimensions were 17 × 17 mm. Within this column, the signal was further localized using the B_1 gradient to five voxels centered about 45°, 60°, 90°, 120°, and 135° spin rotation increments. FSW localization used a nine-term Fourier series expansion. The Fourier coefficients, the number of free induction decays acquired for each term in the Fourier expansion, and the multiplication factors used to construct the voxels have been reported previously. The position of the voxels relative to the coil was set using the B_1 magnitude at the coil center, which was experimentally determined in each case by measurement of the 90° pulse length for the phosphonoacetic acid reference located in the coil center. Each set of spatially localized transmural spectra was acquired in 10 min. A total of 96 scans was accumulated within each 10-min block.

Evaluation

Resonance intensities were quantified with the use of integration routines provided by the SISCO software. ATPγ resonance was used for ATP determination. Since data were acquired with the transmitter frequency being positioned between the ATPγ and CP resonance, the off-resonance effects on these peaks were negligible. The numeric values for CP and ATP in each voxel were expressed as ratios of CP/ATP. P_i levels were measured as changes from baseline values (ΔP_i) with the use of integrals obtained in the region covering the P_i resonance.

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Cardiac Failure in Sheep

Purpose and Rationale

Various methods have been used to induce cardiac failure in sheep: pressure overload after aortic banding (Aoyagi et al. 1993; Charles et al. 1996), volume loading after myocardial infarction (Charles et al. 2003), rapid ventricular pacing (Rademaker et al. 1997, 2002, 2005; Byrne et al. 2002; Moreno et al. 2005), coronary microembolization (Huang et al. 2004; Monreal et al. 2004), and thrombus-induced heart failure (Chandrakala et al. 2013).

Rademaker et al. (2002) studied combined angiotensin-converting enzyme inhibition and adrenomedullin in an ovine model of heart failure induced by rapid ventricular pacing.

Procedure

Surgical Preparation

Coopworth ewes (38 ± 47 kg) were instrumented via a left lateral thoracotomy. Under general anesthesia (induced by 17 mg/kg thiopentone; maintained with halothane/nitrous oxide), two polyvinyl chloride catheters were inserted in the left atrium for blood sampling and left atrial pressure (LAP) determination; a Konigsberg pressure-tip transducer was inserted in the aorta to record mean arterial pressure (MAP); an electromagnetic flow probe was placed around the ascending aorta to measure cardiac output (CO); a 7-French Swan-Ganz catheter was inserted in the pulmonary artery for infusions; and a 7-French His bundle electrode was stitched subepicardially to the wall of the left ventricle for left ventricular pacing. All leads were externalized through incisions in the back. A bladder catheter was inserted per urethra for urine collections.

The animals were allowed to recover for 14 days before commencing the study protocol. During the experiments, the animals were held in metabolic cages, had free access to water, and ate a diet of chaff and sheep pellets (containing 40 mmol/day sodium and 200 mmol/day potassium). A further 40 mmol of sodium was administered orally daily as NaCl tablets using an applicator.

Study Protocol

Heart failure was induced by 7 days of rapid left ventricular pacing (225 bpm) (Rademaker et al. 1997) and maintained by continuous pacing for the duration of the study. On four separate days with a rest day between each, the sheep received, in random order, a vehicle control (Haemaccel), human adrenomedullin alone (50 ng/min per kg infusion for 3 h), an ACE inhibitor alone (captopril: 25 mg bolus + 2 mg/h infusion for 3 h), and both agents combined. Infusions were administered in a total volume of 60 ml via the pulmonary artery catheter, commencing at 10:00 h.

Mean arterial pressure, left atrial pressure, cardiac output, and calculated total peripheral resistance (CTPR = mean arterial pressure/cardiac output) were recorded at 15-min intervals in the 1 h prior to infusion (baseline) and at 15, 30, 45, 60, 90, 120, and 180 min during both the 3-h infusion and post-infusion periods. Hemodynamic measurements were determined by online computer-assisted analysis.

Blood samples were drawn from the left atrium at 30 min and immediately pre-infusion (baseline) and at 30, 60, 120, and 180 min during the 3-h infusion and post-infusion periods. Samples were taken into tubes on ice, centrifuged at 3,939 g for 10 min at 4 °C, and stored at either −20 or −80 °C before assay for immunoreactive (ir-) adrenomedullin, cAMP, plasma renin activity, angiotensin II, aldosterone, atrial natriuretic peptide, brain natriuretic peptide, endothelin-1, catecholamines, and cortisol.

All samples from individual animals were measured in the same assay to avoid inter-assay variability. Plasma electrolytes and hematocrit were measured in every sample taken. Urine volume and samples for the measurement of urine cAMP, sodium, potassium, and creatinine excretion were collected every 1 h. Creatinine clearance was calculated as urine creatinine/plasma creatinine.

Evaluation

Results are expressed as mean ± SEM. Baseline hemodynamic and hormone values represent the means of the four and two measurements, respectively, made in the 1 h immediately pre-infusion. Statistical analysis was performed by repeated-measures ANOVA. Baseline data from all treatments were compared. Treatment- and time-related differences between all four study limbs were determined using a two-way ANOVA

(treatment–time interactions are quoted in the text). Statistical significance was assumed when $P < 0.05$.

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Cardiac Failure in Monkeys

Purpose and Rationale

Several authors used monkeys for studies of cardiac failure. Hollander et al. (1977) investigated the role of hypertension in ischemic heart disease in the **cynomolgus monkey** with coarctation of the aorta. Sieber et al. (1980) studied cardiotoxic effects of adriamycin in **macaques**.

Various studies were performed by the group of Hoit and Walsh in **baboons** (Hoit et al. 1995a, b, 1997a, b; Khoury et al. 1996). Hoit et al. (1997a) studied the effects of thyroid hormone on cardiac β-adrenergic responsiveness in conscious baboons.

Procedure

Animal Instrumentation

Adult male baboons (*Papio anubis*) weighing 21–30 kg were pre-instrumented for physiological monitoring in a lightly anesthetized, sedated state. Animals were pre-instrumented with a

Konigsburg micromanometer and a polyvinyl catheter in the LV apex, miniaturized sonomicrometer pairs (3 MHz, 6 mm) across the LV anteroposterior minor axis, a polyvinyl catheter in the right atrium for central venous access, and pacing wires on the right atrial appendage. Wires and tubes were tunneled subcutaneously into the interscapular area for later use. Postoperative pain was reduced by the use of Buprenex (0.01 mg/kg i.m., q 6 h), and postoperative antibiotic (Monocid 25 mg/kg) was administered for 5 days to reduce the risk of infection. Baseline hemodynamic studies were performed after a minimum of 1 week for postoperative recovery.

Hemodynamic Data Acquisition and Analysis The micromanometers and fluid-filled catheters were calibrated with a mercury manometer. Zero drift of the micromanometer was corrected by matching the LV end-diastolic pressure measured simultaneously through the LV catheter. The fluid-filled LV catheter was connected to a pre-calibrated Statham 23-dB transducer with zero pressure at the level of the mid-right atrium. The transit time of ultrasound between the ultrasonic dimension crystals was measured with a multichannel sonomicrometer (Triton Technology) and converted to distance assuming a constant velocity of sound in blood of 1.55 mm/ms.

The analog LV dP/dI signal was obtained online by electronic differentiation of the high-fidelity LV pressure signal. τ was derived from the high-fidelity LV pressure tracing by the method of Weiss et al. (1976), which assumes a monoexponential decay of LV pressure to a zero asymptote and has been shown to be directionally equivalent to other mathematical approaches for quantification of isovolumic pressure decay. τ is equal to the time in milliseconds for LV pressure to decay to 1/e; thus, decreases in τ reflect improved isovolumic ventricular relaxation.

Fractional shortening of the LV minor axis was calculated as (EDD–ESD)/EDD, where EDD is the LV end-diastolic dimension and ESD is the LV end-systolic dimension. LV end-diastole was defined as the time in which LV dP/d$t$max increased by $\geq$150 mmHg/s for 50 ms, and LV end-systole was defined as the time of the maximum ratio of LV pressure to LV minor-axis dimension. LV volumes were derived from minor-axis diameter (D) measurements:

$$\text{LV volume} = \frac{\pi}{6(D)^3}.$$

V_{cf} was calculated as LV fractional shortening divided by LV ejection time; LV ejection time was defined as the time from peak-positive to peak-negative dP/dt.

Analog signals for high-fidelity and fluid-filled LV pressures, LV short-axis dimension, LV dP/dt, and the ECG were recorded online on a Gould multichannel recorder at 25 and 100 mm/s paper speed and digitized through an analog-to-digital board (dual control systems) interfaced to an IBM AT computer at 500 Hz and stored on a floppy disk. Data were analyzed using an algorithm and software developed in our laboratory. Steady-state data were acquired over 5–10 s during spontaneous respiration and averaged.

Experimental Protocols

Hemodynamic studies were performed a minimum of 1 week after instrumentation and were repeated after 22–30 (26.8 $\pm$ 2.7) days of thyroid T_4 administration. Animals were tranquilized with Valium (1–5 mg) and ketamine (100 mg), and cholinergic blockade was achieved with atropine (0.4–0.8 mg i.v.); additional ketamine was administered as necessary, to a maximum cumulative dose of 40 mg/kg. Animals were atrially paced at a rate 40–50 % greater than the control heart rate in order to obtain data at matched heart rates after thyrotoxicosis was produced.

Dobutamine Group

After hemodynamic stability was ensured and baseline data were recorded, intravenous dobutamine was infused at 5-min intervals at upwardly titrated rates of 2.5, 5.0, 7.5, and 10.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ to examine the effects of β_1-adrenergic stimulation. The dose range of catecholamine for these studies was chosen to alter inotropic and lusitropic states without causing an untoward increase in heart rate. Steady-state hemodynamic measurements were made during minutes 4 and 5 of each

infusion period. At each level, the pacemaker was briefly turned off to determine the effect of dobutamine on the heart rate.

Four of the animals in this group were studied with incremental pacing both before and after β-adrenergic blockade with esmolol ($0.3\ mg \cdot kg^{-1} \cdot min^{-1}$ i.v.). The pacing protocol and the results from a larger group of animals studied before β-adrenergic blockade were detailed in a previous report. Briefly, atrial pacing was instituted at a rate above the intrinsic heart rate to avoid competing rhythms and was increased at 0.2-Hz increments until the critical heart rate was achieved. The critical heart rate was defined as the rate at which dP/dt_{max} and τ reached a maximum and minimum, respectively, during progressive increases in heart rate. We showed previously that hyperthyroidism significantly increases the critical heart rates for both dP/dt_{max} and τ.

The EC_{50} of dobutamine for LV dP/dt_{max} was determined by fitting log(dose)-transformed data to a sigmoidal relation with software from GraphPad.

Terbutaline Group

Additional animals were chronically instrumented so that we could examine the effects of β_2-adrenergic stimulation. One animal died suddenly after receiving thyroid hormone for 20 days. In the remaining three animals, the β_2-adrenergic agonist terbutaline was infused both before and after production of the hyperthyroid state. Incremental doses of terbutaline (15 min/dose) were infused over a dosing range of 25–300 $ng \cdot kg^{-1} \cdot min^{-1}$.

Thyroid Function Tests

Thyroid function tests were performed before the baseline experiment in the euthyroid state and before the terminal experiment (within 24 h of the last dose of T_4) in the hyperthyroid state. T_3 radioimmunoassay, T_4, and free T_4 levels were measured at each state.

Evaluation

Paired mean data were compared by Student's *t*-test. The effects of thyroid status, catecholamine dose, and β-blockade on hemodynamic and dimension variables were examined with repeated-measures ANOVA (SuperAnova, Abacus Concepts). When significant differences were found, group means were compared with contrasts. A value of $P < 0.05$ was considered significant. Unless specified, data are expressed as mean $\pm$ SD.

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Cardiac Failure in Other Species

Purpose and Rationale

Various species have been used to study experimental cardiac failure.

Breisch et al. (1984) studied the effects of pressure-overload hypertrophy in the left myocardium of young adult **cats**. Hypertrophy was induced by a 90 % constriction of the ascending aorta.

Genao et al. (1996) recommended dilated cardiomyopathy in **turkeys** as an animal model for the study of human heart failure.

Do et al. (1997) studied energy metabolism in normal and hypertrophied right ventricle of the **ferret** heart.

Wang et al. (1994) studied Ca^{2+} handling and myofibrillar Ca^{2+} sensitivity in **ferret** cardiac myocytes with pressure-overload hypertrophy.

Bovine hereditary cardiomyopathy was recommended as an animal model of human dilated cardiomyopathy by Eschenhagen et al. (1995).

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Hypertrophy of Cultured Cardiac Cells

Purpose and Rationale

Kojima et al. (1994), Komuro et al. (1990, 1991, 1993), and Yamazaki et al. (1993, 1994, 1996) described a method to induce hypertrophy of cardiomyocytes by mechanical stress in vitro.

Procedure

Primary cultures from cardiomyocytes are prepared from ventricles of 1-day-old neonatal Wistar Kyoto rats. According to the method of Simpson and Savion (1982), the cultures are treated for 3 days with 0.1 mM bromodeoxyuridine to suppress proliferation of non-myocardial cells. Elastic culture dishes ($2 \times 4 \times 1$ cm) are made by vulcanizing liquid silicone rubber consisting of methylvinyl polysiloxane and dimethyl hydrogen silicone resin using platinum as a catalyst. The bottom of the disk is 1 mm thick, and it is highly transparent because of no inorganic filler in either component. Cells are plated in a field density of 1×10^5 cells/cm^2 in culture medium consisting of Dulbecco's modified Eagle's medium with 10 % fetal bovine serum. Mechanical stress on cardiac cells is applied by gently pulling and hanging the dish on pegs. A 10 % change in length of the dish results in an almost identical change in

the length of the cell along a single axis (Komuro et al. 1990). Cardiocytes are stretched by 5 %, 10 %, or 20 %. Drugs, e.g., an angiotensin II receptor antagonist, are added 30 min before stretch.

For protein analysis, the silicone dishes are stretched for 24 h after 2 days of serum starvation and [^{3}H]phenylalanine (1 μCi/ml) is added for 60 min. At the end of each stress, the cells are rapidly rinsed four times with ice-cold phosphate-buffered saline and incubated for 20 min on ice with 1 ml of 5 %trichloroacetic acid. The total trichloroacetic acid-insoluble radioactivity in each dish is determined by liquid scintillation counting.

For the determination of mitogen-activated protein kinase, cardiomyocytes are lysed on ice and centrifuged. Aliquots of the supernatants of myocyte extracts are incubated in kinase buffer (25 mM/l Tris–HCl, pH 7.4, 10 mM/l $MgCl_2$, 1 mM/l dithiothreitol, 40 μM/l APT, 2 μCi [γ -^{32}P]ATP, 2 μM/l protein kinase inhibitor peptide, and 0.5 mM/l EGTA) and substrates (25-μg myelin basic protein). The reaction is stopped by adding stopping solution containing 0.6 %HCl, 1 mM/l ATP, and 1 % bovine serum albumin. Aliquots of the supernatant are spotted on P81 paper (Whatman), washed in 0.5 % phosphoric acid, dried, and counted.

For determination of*c-fos mRNA*, Northern blot analysis is performed.

Evaluation

Values are expressed as mean ± SEM. Comparisons between groups are made by one-way ANOVA followed by Dunnett's modified *t*-test.

Critical Assessment of the Method

The interesting approach to induce hypertrophy of cardiac cells in vitro has been used predominantly by one research group. Confirmation by other research groups including modifications of the mechanical procedures seems to be necessary.

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Positive Inotropic Activity (Cardiac Glycosides)

Michael Gralinski, Liomar A. A. Neves, and Olga Tiniakova

Contents

General Considerations

Biological standardization of cardiac glycosides was necessary as long as the drugs used in therapy were plant extracts or mixtures of various glycosides. They were standardized in units of an international standard. Some of the pharmacological methods used for these purposes and adopted by many pharmacopoeias have nowadays *historical interest* only. This holds true for the frog method and the pigeon method (Burn et al. 1950).

Particularly, the **frog method** was used for standardization. The method adopted by the US Pharmacopoeia X was the 1 h test. Healthy frogs (*Rana pipiens*) weighing 20–30 g were selected from the cold storage room. One hour before assay, their weight was recorded and they were placed in wire cages with a water depth of 1 cm. The doses of digitalis were calculated so that they approximated 0.015 ml/g body weight. Injections were made into the ventral lymph sac. One hour later, the animals were pithed and the heart removed and examined. Systolic arrest of the ventricle and widely dilated atrium indicated the typical result. Calculations of activity in terms of international units were made from the percentage of dead animals in the test group versus those in the group receiving the international standard.

The **pigeon method** introduced by Hanzlik (1929) and adopted by USP XVII depends on the observation that intravenously injected cardiac glycosides have an emetic action in pigeons. In the original test, adult pigeons weighing

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300–400 g are injected with a solution of the cardiac glycoside into a suitable wing vein in the axillary region. Vomiting occurring within 15 min is regarded as positive result. Two doses of test solution and standard are injected and percentage of vomiting pigeons registered. This four-point assay allows calculation of ED_{50} values and of the potency ratio compared with the standard.

Modifications of other methods, such as the **cat method** introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngaarden (1926), the **guinea pig method** described by Knaffl-Lenz (1926), and the **isolated cat papillary muscle** method introduced by Catell and Gold (1938) still being used for evaluation of synthetic cardiac glycosides and other positive inotropic compounds, are referenced in detail below.

Surveys on the evaluation of cardiac glycosides have been given by Bahrmann and Greef (1981), for the use of the isolated papillary muscle by Reiter (1981) and for other isolated heart preparations by Greef and Hafner (1981). Moreover, the influence on Na^+/K^+-ATPase, an in vitro model specific for cardiac glycosides (Gundert-Remy and Weber 1981), is described.

The mechanisms of action have been reviewed by Scholz (1984) and Grupp (1987).

Analogous to antiarrhythmic agents, Feldmann (1993) proposed a classification system that categorizes inotropic agents according to their supposed mode of action:

Class I: Inotropic agents that increase intracellular cyloAMP, including β-adrenergic agonists and phosphodiesterase inhibitors

Class II: Inotropic agents affecting sarcolemmal ion pumps and channels, in particular cardiac glycosides inhibiting Na^+/K^+-ATPase

Class III: Agents that modulate intracellular calcium mechanisms (no therapeutic inotropic agents in this kind yet available)

Class IV: Inotropic agents having multiple mechanisms of action

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In Vitro Tests

Ouabain Binding

Purpose and Rationale

Cardiac glycosides can be characterized by their binding kinetics (association process, equilibrium binding, and dissociation process) on the ouabain receptor.

Procedure

Heart sarcolemma preparations are obtained from rat or dog heart. From a canine heart or from rat hearts submitted to coronary perfusion, myocytes are isolated by collagenase digestion. The isolated membrane fractions consist mainly of myocyte sarcolemma. [^{3}H] ouabain with a specific radioactivity of about 20 Ci/mmol is incubated with ligands to be tested in 10 ml of binding medium consisting of 1 mM $MgCl_2$, 1 mM inorganic phosphate, and 50 mM Tris–HCl, pH 7.4 at 37 °C for 10 min.

Association process: After temperature equilibration in the presence of either 10 or 100 nM [^{3}H] ouabain, 200 μg of membrane preparation are added to initiate the reaction. At various times, 4.5 ml are removed and rapidly filtered.

Equilibrium binding: At the end of the temperature equilibration carried out in the presence of increasing concentrations of [^{3}H]ouabain ranging from 10 nM to 3 μ M, 40 μg of membranes is added. After 30 min, duplicate aliquots of 4.5 ml are removed and filtered.

Dissociation process: Once equilibrium has been achieved under the experimental conditions used to study association, 10 ml of prewarmed Mg^{2+} plus P_i Tris–HCl solution supplemented with 0.2 mM unlabeled ouabain is added to initiate dissociation of [^{3}H]ouabain. At various times, aliquots of 0.9 ml are removed and rapidly filtered.

All aliquots are filtered under vacuum on HAWP Millipore filters (0.45 μm) and rinsed three times with 4 ml of ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements are determined.

Evaluation

Kinetic parameters for the association and the dissociation process are calculated. The results of equilibrium binding are analyzed by Scatchard plots.

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Influence on Na^+/K^+-ATPase

Purpose and Rationale

The enzyme Na^+/K^+-ATPase is the transport system for Na^+ and K^+ in the cell membranes. The membrane-bound enzyme couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane through a series of conformational transitions between the E_1 and E_2 states of the enzyme. The enzyme is a heterodimer consisting of a catalytic subunit (110 kDa) associated with a glycosylated β subunit (55 kDa). Three alpha (α_1, α_2, α_3) subunits have been identified by cDNA cloning. In the heart, enzyme Na^+/K^+-ATPase is the target of the positive inotropic glycosides. Therefore, it is of interest for the characterization of positive inotropic compounds. The test is based on the determination of phosphate generated from ATP under special conditions. Inhibition of bovine cerebral Na^+/K^+-ATPase prepared according to Schoner et al. (1967) is measured after addition of various concentrations of the test compound compared with those of the standard (Erdmann et al. 1980).

Procedure

Solutions

1.00 ml 133 mM imidazole pH 7.3
0.04 ml 160 mM $MgCl_2$
0.02 ml DPNH (10 mg/ml)
0.04 ml 310 mM NH_4Cl
0.04 ml 100 mM ATP
0.02 ml 40 mM phosphoenol-pyruvate
0.05 ml pyruvate-kinase (1 mg/ml = 150 U/ml)
0.04 ml lactate-dehydrogenase
(0.5 mg/ml = 180 U/ml)
0.20 ml 1 M NaCl
0.01–0.02 ml bovine cerebral ATPase (depending on activity of the enzyme) up to 2.0 ml distilled water

Test

The enzyme activity is started by addition of the ATP solution at 37 °C. After 4 min the inhibitor (various concentrations of the cardiac glycoside) is added. Na^+/K^+-ATPase activity is measured by a coupled optical assay. The reaction is continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of Na^+/K^+-ATPase with 10^{-3} M ouabain.

Evaluation

Inhibition of ATPase is measured after addition of various concentrations of the test compound. Dose-response curves are established and compared with the standard (k-strophanthin). Potency ratios can be calculated.

Modifications of the Method

Brooker and Jelliffe (1972) and Marcus et al. (1975) described an in vitro assay based on displacement of radiolabeled ouabain bound to ATPase by various glycosides. Another method is based on the inhibition of rubidium uptake into erythrocytes (Lowenstein 1965; Belz 1981).

Erdmann et al. (1980) prepared Na^+/K^+-ATPase-containing cardiac cell membranes from rat hearts.

Maixent et al. (1987) described two Na^+/K^+-ATPase isoenzymes in canine cardiac myocytes as the molecular basis of inotropic and toxic effects of digitalis.

The effect of ouabain on Na^+/K^+-ATPase activity in cells of the human rhabdomyosarcoma cell line TE671 was studied by Miller et al. (1993) with a special equipment, the microphysiometer (McConnell et al. 1992).

Critical Assessment of the Method

The in vitro methods being used for determinations of plasma levels of glycosides (Maixent et al. 1995) have been largely substituted by radioimmunoassays specific for individual glycosides.

Nevertheless, the inhibition of Na^+/K^+-ATPase can be used as an indicator of activity of new semisynthetic cardiac glycosides.

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Tests in Isolated Tissues and Organs

Isolated Cat Papillary Muscle

Purpose and Rationale

Isolated cardiac tissue has been chosen to study the decrease of performance after prolonged electrical stimulation and during restoration of force under the influence of cardiac glycosides. Cattell and Gold (1938) described a method using cat papillary muscle.

Procedure

Cats of either sex weighing 2.5–3 kg are used. The animal is anesthetized with ether and the thorax is opened rapidly. The heart is removed, and a papillary muscle from the right ventricle is isolated and fixed in an organ bath containing oxygenated Ringer's solution at 36 °C. One end of the muscle is tied to a tissue holder and the other one to a strain gauge. The muscle is stimulated electrically with 4–6 V, 2 ms duration, and a rate of 30/min. The contractions are recorded on a polygraph. After 1 h, the muscle begins to fail and the force of contraction diminishes to a fraction of control. At this point, the cardiac glycoside is added to the bath, restoring the contractile force to levels approaching control. The standard dose is 300 ng/ml ouabain. The potency of natural and semisynthetic glycosides can be determined with this method. Catecholamines, like adrenaline (10 ng/ml) or isoprenaline (10 ng/ml), are active as well.

Evaluation

The increase of contractile force is calculated as percentage of the predose level. Dose-response curves can be established using various doses.

Critical Assessment of the Method

The use of isolated papillary muscle strips can be recommended for evaluation of inotropic compounds of various chemical classes.

Modifications of the Method

Instead of cat papillary muscle, the isolated left atrium of guinea pigs can be used (see chapter "▶ Studies in Isolated Organs", section "B1-Sympatholytic Activity in Isolated Guinea Pig"). For testing cardiac glycosides, the calcium content in the Ringer's solution is reduced to 50 %.

Andersom (1983) compared responses of guinea pig paced left atria to various positive inotropic agents at two different calcium concentrations (1.25 and 2.50 mM). Consistently good results were obtained at the lower calcium concentration with isoproterenol, ouabain, amrinone, and 3-isobutyl-1-methylxanthine.

Böhm et al. (1989) studied positive inotropic substances like isoprenaline and milrinone in isolated cardiac preparations from different sources. They used isolated papillary muscles from Wistar–Kyoto rats and from spontaneously hypertensive rats and also human papillary muscle strips from patients with moderate heart failure (NYHA II–III) and compared the effects with

papillary muscle strips from patients with severe heart failure (NYHA IV). They recommended that new positive inotropic agents should be screened in human myocardial tissue from patients with heart failure.

Labow et al. (1991) recommended a human atrial trabecular preparation for evaluation of inotropic substances.

Böhm et al. (1989) tested positive inotropic agents in isolated cardiac preparations from different sources, e.g., human papillary muscle strips from patients with severe heart failure (NYHA IV), human papillary muscle strips from patients with moderate heart failure (NYHA II–III), human atrial trabeculae, isolated papillary muscles from Wistar–Kyoto rats, and isolated papillary muscles from spontaneous hypertensive rats. They suggested that positive inotropic effects should be screened in isolated myocardium from patients with heart failure.

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Isolated Hamster Cardiomyopathic Heart

Purpose and Rationale

Special strains of Syrian hamsters develop cardiomyopathy. These animals can be used for evaluation of cardiotonic drugs

Procedure

Hamsters with cardiomyopathy (Bio 14/6) at the age of 50 weeks are used. Controls are normal Syrian hamsters (FIB hybrids) at the same age. The animals are pretreated with heparin (5 mg/kg i.p.), and 20 min later the heart is prepared according to the method of Langendorff and perfused with heart Ringer's solution under 75 mm H_2O hydrostatic pressure. The preparation is allowed to equilibrate in the isolated state for 60 min at 32 °C with a diastolic preload of 1.5 g. The force of contractions is recorded isometrically by a strain gauge

transducer on a polygraph, e.g., Heliscriptor He 19 recorder (Hellige GmbH, Freiburg, Germany). From these signals, the heart rate is measured by a chronometer. The coronary flow is measured by an electromagnetic flowmeter. Compounds are injected via the aortic cannula into the inflowing heart Ringer's solution.

Evaluation

The contractile force and the coronary flow in hearts from diseased and normal animals are registered before and after application of the test drugs. Mean values and standard deviation are calculated before and after drug application and statistically compared using Student's *t*-test.

Modification of the Method

Jasmin et al. (1979) showed after prolonged in vivo administration beneficial effects of a variety of cardiovascular drugs, including verapamil, prenylamine, dibenamine, and propranolol.

After chronic administration (4 or 12 weeks subcutaneously), Weishaar et al. (1987) found beneficial effects of the calcium channel blocker diltiazem but not by the administration of digitalis.

In contrast, in the experiments of Ottenweller et al. (1987), hamsters treated orally with digoxin survived and showed significant amelioration of the pathological syndrome of heart failure.

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Potassium Loss from the Isolated Guinea Pig Heart

Purpose and Rationale

Cardiac glycosides induce a net loss of potassium from cardiac tissue due to their inhibition of the Na^+/K^+-ATPase. Therefore, potassium is increased in the effluent of the isolated guinea pig heart. This phenomenon can be used as parameter for the activity of digitalis-like compounds (Lindner and Hajdu 1968).

Procedure

The isolated heart of guinea pigs according to Langendorff is prepared as described in chapter "▶ Coronary Drugs", section "Heart-Lung Preparation". The coronary outflow is measured by counting the drops of the effluent by a photocell. The effluent is collected in a funnel with a thin upward-shaped outlet allowing to withdraw small fluid samples for analysis by a flame photometer. A pump attached to a four-way valve changes the samples to the flame photometer every 15 s in the following sequence: effluent Tyrode solution from the heart, distilled water, Tyrode solution used for perfusion, and distilled water. The potassium content of affluent and effluent Tyrode solution is compared and registered on a Varian recorder. The difference is attributed to the potassium outflow from the heart. The dose-response curve is flat in the therapeutic range, much steeper in the toxic range.

Evaluation

The following parameters are recorded and calculated:

- Coronary flow [ml/min]
- Contractile force
- Potassium loss [mVal/min]

Critical Assessment of the Method

A good correlation was found between the measured potassium loss and the positive inotropic effect of cardiac glycosides. The method is suitable for the quick determination of efficacy of digitalis-like substances and facilitates the discrimination from other positive inotropic compounds like adrenaline.

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In Vivo Tests

Cardiac Toxicity in Cats (Hatcher's Method)

Purpose and Rationale

The purpose of the method, originally introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngaarden (1926), was to establish "cat units" for cardiac glycoside preparations. Hatcher and Brody defined "the cat unit as the amount of crystalline ouabain which is fatal within about 90 min to a kilogram of a cat when the drug in injected slowly and almost continuously into the femoral vein." Time to cardiac arrest after intravenous infusion of a solution with defined concentration of the standard was used as reference and the unknown solution of the test preparation compared with the standard. The method can be used for testing natural and semisynthetic glycosides.

Procedure

Cats of either sex weighing 2–3.5 kg are temporarily anesthetized with ether. Anesthesia is maintained with 70 mg/kg chloralose given intravenously. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula is inserted. ECG is recorded from lead II. Then, intravenous infusion of the test solution is started. The end point is cardiac arrest which should be reached within 30–60 min by proper adjustment of the concentration of the infused solution.

Modifications of the Method

Hatcher's original method has been modified by many authors. The method using **guinea pigs**, introduced by Knaffl-Lenz (1926), is in its essentials similar to the cat method.

Guinea pigs weighing 400–600 g are anesthetized with urethane (1.75 g/kg i.m.) The animal is secured on an operating table and the trachea is cannulated. The jugular vein is cannulated for infusion of the test preparation. Cardiac arrest is recorded from ECG lead II.

Dogs and guinea pigs were used by Dörner (1955).

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Decay Rate and Enteral Absorption Rate of Cardiac Glycosides

Purpose and Rationale

The basic principle of Hatcher's or Knaffl-Lenz's method is suitable to determine decay rates of cardiac glycosides. The decay of efficacy can be due to excretion or metabolic degradation of the glycoside.

Procedure

Beagle dogs of either sex weighing 820 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula inserted. The vena femoralis is cannulated for continuous infusion of a defined concentration (μg/kg/min) of the test compound. ECG is recorded from lead II. The signs of first toxic effects, e.g., extra systoles, AV-block, are recorded. At this time, the infusion is terminated and the total dose/kg of the applied glycoside registered. After 4, 8, 12, or 24 h the infusion procedure is repeated. Within this period of time the glycoside administered with the first dose is only partially metabolized or excreted. Therefore, the dose needed for observation of ECG changes during the second infusion will be lower than in the first experiment.

Evaluation

The dose required in the second experiment for induction of ECG changes is equal to the amount of metabolized or excreted glycoside. This value is expressed as percentage of the amount required in the first experiment and indicates the decay rate of the glycoside. Testing after various time intervals, the decay rate can be visualized graphically and half-life times be calculated.

Modifications of the Method

Rhesus monkeys have been used since their response to cardiac glycosides is more similar to that of man than that of dogs (Lindner et al. 1979).

The basic principle of Hatcher's or Knaffl-Lenz's method is also suitable to determine *enteral absorption of cardiac glycosides*. Again, for this purpose dogs are preferred instead of cats or guinea pigs. The dose to induce cardiac arrest is determined in 3–6 dogs. To other dogs, the same test compound is given intraduodenally at a dose below the intravenous lethal dose. Ninety or 180 min afterward, the intravenous infusion with the same infusion speed and the same concentration of the test compound as in the previous experiments is started and time until cardiac arrest determined. The higher the duodenal resorption of the compound, the lower the dose of the intravenous infusion will be. For evaluation, the intravenous dose needed in the second experimental series (with enteral predosing) is subtracted from the dose of the first series (without enteral predosing) and indicates the amount of absorbed compound. This value is expressed as percentage of the value of the first series and indicates the absorption rate.

The efficacy and safety of a novel Na^+/K^+-ATPase inhibitor has been tested in dogs with propranolol-induced heart failure by Maixent et al. (1992).

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Effects on Blood Supply and on Arterial and Venous Tonus

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Models for Stroke and Multi-Infarct Cerebral Dysfunction

Cerebral Ischemia by Carotid Artery Occlusion in Mongolian Gerbils

Purpose and Rationale

The Mongolian gerbil (*Meriones unguiculatus*) is extremely susceptible to carotid occlusion because of the peculiar anatomical occurrence of an incomplete circle of Willis without posterior communicating artery and a frequently rudimentary anterior communicating artery. Clamping of both carotid arteries induces a bilateral temporary brain ischemia (Levine and Sohn 1969; Bosma et al. 1981; Mršulja et al. 1983; Hossman et al. 1983; Chandler et al. 1985). This pathological animal model allows the simulation of circulatory disturbances in the human brain. The hippocampus is one of the most vulnerable regions of the brain to ischemia and anoxia. The gerbil is known to develop selective neuronal damage in the CA1 sector of the hippocampus following brief periods of forebrain ischemia. This damage differs from conventionally described ischemic neuronal injury because of its slow development (Ito et al. 1975; Kirono 1982; Hossman et al. 1983). The occlusion time can be varied allowing determination of various parameters, e.g., ischemia-induced amnesia.

Procedure

Male Mongolian gerbils (strain: Hoe GerK jirds) weighing 48–88 g are randomly divided into groups (10–15 animals for each test and control group). Prior to testing, the animals are housed in a climate-controlled environment (21 °C) with food and water available ad libitum. Fifteen minutes before surgery, the gerbils receive the test compound by oral or intraperitoneal administration. The control group is treated with vehicle alone.

The exposure of the common carotid arteries is performed under anesthesia with sodium pentobarbital (32 mg/kg i.p.), chloral hydrate (100 mg/kg i.p.), and atropine sulfate (0.8 mg/kg i.p.). The carotid arteries are isolated from the surrounding tissue and a loop of unwaxed dental floss is placed around each artery. A 2-cm length of double-lumen catheter is passed from the level of carotid artery through the muscle layers of the dorsal surface of the neck. Each end of the dental floss is threaded through a separate lumen, leaving a loose loop around the artery. Two days later, occlusion of each artery is produced by gently pulling the dental floss until the artery is completely occluded between the floss and the center wall of the catheter. Heifetz clips are placed on the floss against the exterior end of the tubing to maintain occlusion. After various intervals (5–30 min), the clips are removed and circulation is restored.

Complete bilateral occlusion of the arteries is confirmed by behavioral symptoms, i.e., depression of spontaneous motor activity, shallow and rapid respiration, and ptosis. Care is taken to avoid a drop of body temperature during any stage of the experiment. After experimental manipulations, animals are placed on a heating pad until complete recovery of motor activity.

Subjects are placed in individual observation glasses which are kept at a temperature of 29 °C. They are observed for neurological symptoms (such as circling behavior, jumping and rolling seizures, opisthotonus, tonic convulsions, etc.) for 90 min.

After various intervals, the gerbils are sacrificed and their brains are removed.

Evaluation

The following parameters are measured 2 h after occlusion:

Degree of brain edema: Water content (difference in weight of wet and dry brain)

Content of sodium and potassium. The hemispheres are separated and put on pre-weighed watch glasses to determine the wet weight. Then the hemispheres are dried in an open Petri dish for 2 days at a temperature of 95 °C. After cooling off, the dry weight is noted. Sodium and potassium concentrations in the dried brain hemispheres are determined by flame photometry. The Na^+/K^+ ratio is calculated.

For *histological examinations*, the animals are sacrificed at 2 or 4 days after ischemia under ether

anesthesia by decapitation. The brains are then removed and frozen in CO_2. Hippocampi are sectioned coronally with a cryostat at −14 °C. The section thickness for Nissl and glial fibrillary acid protein (GFAP) staining is 20 μm and for the histochemical localization of calcium 30 μm.

Nissl staining and its quantitative evaluation. The sections are mounted by thawing on glass slides, stained, and cover slipped with Permount. In order to standardize the histological procedure and to rule out the possibility that differences in the staining intensity were due to technical inconsistency, slides from control and experimental groups are processed together, stained for 5 min, and differentiated in a series of alcohols for 3 min each. The extent of hippocampal nerve cell damage (as reflected by cell loss and decreased stainability) is assessed by measuring the amount of Nissl-stained material in a predetermined representative region of the CA1 area with the aid of a guided densitometer (Leitz Texture Analysis System). The measuring field is 50 × 500 μm, fitting to the width of the CA1 soma layer (about 40 μm).

Calcium localization. A modification of technique described by Kashiwa and Atkinson (1963) is used for the cytochemical localization of ionic calcium. The principle of the technique is that calcium complexes with a chelating agent producing an insoluble chromophore.

Stock solutions: Two solutions were used: (a) Glyoxal-bis(2-hydroxyanil) (GBHA), 0.4 g/100 ml absolute ethanol (2 ml 0.4 % GBHA), and (b) NaOH, 5 g/100 ml distilled water (0.3 ml 5 % NaOH).

Staining procedure: First, cryostat sections are placed immediately in cold absolute acetone for rapid fixation for 5 min. Next, floating sections are transferred into 96 % alcohol for 5 min and then transferred to staining solution for 3–4 min. Sections are then placed in 96 % alcohol and mounted on glass slides. Because of quenching, it is necessary to view and photograph immediately.

GFAP (*glial fibrillary acid protein*) fluorescence microscopy. Following slide mounting, cryostat sections are fixed for 15 min in 3.5 % formaldehyde solution in 0.01-M phosphate-buffered saline (PBS). The sections are incubated with mouse primary antibody against GFAP (Boehringer, Mannheim, FRG) for 30 min diluted 1:50 PBS. This antibody shows cross-reactions also with GFAP from pigs and rats, indicating low species selectivity (Graeber and Kreutzberg 1986). The sections are rinsed in PBS and incubated to tetramethylrhodamine isothiocyanate (TRITC) specific for mouse immunoglobulin G (T-5393 from Sigma) diluted 1:50 in PBS. Control sections are incubated with PBS instead of primary antibody.

Measurements, expressed as extinction units per measuring field, are taken from three slides of each animal and averaged. Statistical analysis is done by Student's *t*-test.

Modifications of the Method

"Sensitive" gerbils can be selected according to the method of Delbarre et al. (1988). In this method, pupil dilatation is obtained with atropine sulfate (1 %) 20 min before anesthesia. The ocular fundus is examined with direct ophthalmoscope (Heine) before ligation and 5 min later. Only animals with an absence of retinal blood flow after ligation are considered as positive.

Using ^{31}P nuclear magnetic resonance spectroscopy, Sasaki et al. (1989) studied energy metabolism of the ischemic brain of gerbils in vivo.

An unanesthetized-gerbil model of cerebral ischemia was described by Chandler et al. (1985).

Using microdialysis, adenosine and its metabolites were measured directly in the brain of male gerbils by Dux et al. (1990). Two microdialysis probes (CMA/10, Carnegie Medicine, Sweden) were implanted stereotactically in the brain of the animals, one in the left dorsal hippocampus and one in the right striatum. The fibers were fixed to the cranium using dental wax. The dialysate was collected in 5-min intervals, and the concentrations of adenosine, hypoxanthine, and inosine were determined by HPLC (Zetterström et al. 1982; Fredholm et al. 1984).

Kindy et al. (1992) measured glial fibrillary acid protein and vimentin on mRNA level by Northern blot analysis and protein content by immunoblot analysis in the gerbil neocortex, striatum, and hippocampus after transient ischemia.

McRae et al. (1994) studied the effect of drug treatment on activated microglial antigens in

hippocampal sections after ischemia in gerbils with cerebral fluid from patients with Alzheimer's disease and the amyloid precursor protein.

Nurse and Corbett (1996) found neuroprotection in gerbils with global cerebral ischemia after several days of mild, drug-induced hypothermia. The protection by the AMPA-antagonist NBQX may be due to a decrease in body temperature. A protracted period of subnormal temperature during the postischemic period can obscure the interpretation of preclinical studies.

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Forebrain Ischemia in Rats

Purpose and Rationale

Smith et al. (1984a) described a model of forebrain ischemia in rats induced by transient occlusion of both carotid arteries and exsanguination to a blood pressure of 40 mmHg. This method has been used extensively (Nuglisch et al. 1990; Oberpichler et al. 1990; Rischke and Krieglstein 1990; Krieglstein and Peruche 1991; Nuglisch et al. 1991; Prehn et al. 1991; Rischke and Krieglstein 1991; Seif el Nasr et al. 1992; Peruche et al. 1995; Pradillo et al. 2009).

Procedure

Male Wistar rats weighing 250–300 g are anesthetized with 3.5 % halothane and then connected to a Starling-type respirator delivering 0.8 % halothane and 30 % O_2 in N_2O. The jugular vein and the tail artery are catheterized for withdrawal of blood and for monitoring blood pressure. Anticoagulation is achieved by intravenous heparin (200 IU/kg) application. Blood gases, blood pH, blood pressure, and blood glucose are measured 5 min prior to ischemia and 10 min after ischemia.

Halothane, but not N_2O, is discontinued and the rats are allowed to recover for 30 min. During this period, muscle paralysis is maintained with 5 mg/kg suxamethonium chloride, repeated every 15 min. After injection of trimethaphan camphor sulfonate (5 mg/kg), forebrain ischemia is induced by clamping of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. To prevent decay of intra-ischemic brain temperature, the environmental temperature is adjusted to 30 °C by means of an infrared heating lamp. After 10 min of ischemia, the carotid clamps are removed and blood pressure is restored by reinfusing the shed blood. To minimize systemic acidosis, the rats receive intravenously 50 mg/kg $NaHCO_3$. The animals are removed from the respirator when they regain spontaneous respiration.

Evaluation

Various parameters are used to evaluate the consequences of transient forebrain ischemia and the effectiveness of drug treatment:

- Local cerebral blood flow determination with the [^{14}C]-iodoantipyrine method (Sakurada et al. 1978)
- Histological assessment of ischemic cell damage in the hippocampus on day 7 after ischemia (Seif el Nasr et al. 1990; Nuglisch et al. 1990)
- Local cerebral glucose utilization using the [^{14}C]deoxyglucose method described by Sokoloff et al. (1977)
- Quantitative analysis of the electrocorticogram (Peruche et al. 1995)

Modifications of the Method

Kochhar et al. (1988) used two focal cerebral ischemia models in **rabbits**: a multiple cerebral embolic model by injection of microspheres into the internal carotid circulation and a spinal cord ischemia model by occluding the aorta for predetermined periods.

Gilboe et al. (1965) described the isolation and mechanical maintenance of the **dog** brain.

Andjus et al. (1967) and Krieglstein et al. (1972) described the preparation of the isolated perfused rat brain for studying effects on cerebral metabolism.

A cerebral ischemia model with conscious **mice** was described by Himori et al. (1990).

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Hypoxia Tolerance Test in Rats

Purpose and Rationale

The electrical activity of the brain is dependent on a continuous energy supply. Hypoxia is induced in test animals by inhalation of nitrogen. Marked hypoxia depresses cerebral metabolism resulting

in an electrical failure of the brain. The procedure is used to investigate the ability of test compounds to antagonize the hypoxia-induced electrical failure of the brain by measuring the hypoxia tolerance and the EEG recovery.

Procedure

Male Sprague–Dawley rats or stroke-prone rats weighing 250–300 g are used. They are anesthetized with hexobarbital sodium (100–120 mg/kg, i.p) and surgically implanted with 2 epidural EEG electrodes and a reference electrode to the parietal frontal cortex. After a minimum of a 1-week recovery period, testing can be started. The rats receive the test compound by intravenous or intraperitoneal administration. The control group is treated with vehicle alone. Thirty to sixty minutes after i.p. administration (immediately after i.v. administration), the animals are anesthetized by hexobarbital sodium at 100–120 mg/kg, i.p. When the stage of surgical anesthesia is reached, the animals are placed in a hypoxia chamber. EEG and ECG are recorded using a Hellige recorder.

Hypoxia is induced by inhalation of nitrogen (1,200 l/h). On reaching an isoelectric EEG, the nitrogen inhalation is terminated and the animals are allowed to breathe room air. The recording is continued until EEG potentials can again be registered.

Evaluation

The following parameters are measured in the test and control groups:

- Hypoxia tolerance (HT): The time from the start of nitrogen inhalation until the onset of isoelectric EEG.
- Latency of EEG recovery: The time from the end of nitrogen inhalation until the onset of EEG potentials.
- The values of test and control groups are compared.

The percent change is calculated.

Statistics: Student's *t*-test by unpaired comparison.

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Middle Cerebral Artery Occlusion in Rats

Purpose and Rationale

The permanent middle cerebral artery (MCA) occlusion technique in rats has been widely employed to evaluate various kinds of neuroprotective agents in cerebral ischemia (Ginsberg and Busto 1989). Focal cerebral infarction is achieved using a modification of the MCA occlusion model described by Tamura et al. (1981) and Park et al. (1992).

Procedure

Adult male Sprague–Dawley rats weighing 300–400 g are used in the experiments. The animals are anesthetized with a nitrous oxide–oxygen mixture (70:30 %) containing 1.0–1.2 % halothane. A mask is put on the nose and self-respiration is maintained during surgery, which takes around 20–30 min. The right femoral artery and vein are catheterized for monitoring blood pressure, blood sampling, and administration of the drug. Left MCA occlusion is performed via a subtemporal approach without removal of zygomatic arch or temporal muscle. Under high magnification of a surgical microscope, the left MCA is coagulated with a microbipolar coagulator from the olfactory tract to the most proximal portion of the MCA through a cranial window, about 3–4 mm in diameter, and cut afterward. Anesthesia is stopped just after MCA occlusion. The arterial catheter is removed soon after MCA occlusion, but the venous catheter is maintained for constant infusion of the drug by a swivel system (Harvard Apparatus, UK.). The animals are maintained normothermic (37 °C) by a homeothermic system (Homeothermic Blanket

System, Harvard Apparatus, UK). During the surgery, mean arterial blood pressure (MABP) is recorded continuously (Model 7E polygraph, Grass, USA). Arterial gases and pH (178 pH/blood gas analyzer, Corning, USA) and hematocrit and blood glucose are measured twice, once about 15 min before MCA occlusion and the other just following MCA occlusion. Bilateral temporal muscle temperature (Therm 2250–1, Ahlborn Mess- und Regelungstechnik, Germany) is monitored during the surgery and several minutes before sacrifice.

Two, six, 12, and 24 h after discontinuing anesthesia, each rat's level of consciousness and motor activity are evaluated using a grading scale of 0–3 (0: normal activity; 1: spontaneous activity +/−; 2: not arousable by tactile stimulation; 3: not arousable by painful stimulation, no spontaneous activity). Immediately before sacrifice, neurological status is examined using a grading scale of 0–3 (0: no observable deficit; 1: forelimb flexion; 2: decreased resistance to lateral push without circling; 3: the same status as grade 2, with circling). Four groups of rats are studied: vehicle-treated controls and drug-treated animals at three different doses.

All the rats are re-anesthetized 24 h after MCA occlusion and sacrificed by intravenous injection of KCl. Immediately after sacrifice the brain is removed and frozen at −10 °C for 10 min. The forebrain is cut into eight coronal slices by a rat brain slicer which are processed with the tetrazolium chloride (TTC) emulsion technique. Areas of the brain not stained by TTC are drawn on a diagram at 8 preselected coronal levels of forebrain without knowledge of the experimental treatment. The areas of ischemic damage in the cerebral hemisphere, cerebral cortex, and caudate nucleus, drawn in the diagram, are measured with a planimeter (KP-21, Koizumi, Japan) and integrated to determine the volumes.

Evaluation

Significance of the differences between the control and the treated groups is assessed by analysis of variance with subsequent intergroup comparison by Student's *t*-test with Bonferroni correction. $P < 0.05$ is required for significance.

Modifications of the Method

Hossmann (1982) reviewed the experimental models of cerebral ischemia.

Yamamoto et al. (1992) studied the inhibition of NO biosynthesis on the volume of focal ischemic infarction produced by occlusion of the middle cerebral artery in spontaneously hypertensive rats.

Shigeno et al. (1985) described a recirculation model following middle cerebral artery occlusion in rats. The trunk of the middle cerebral artery was isolated between the rhinocortical branch and the lenticulostriate artery and encircled with a loose-fitting suture of nylon thread. The thread was exteriorized through a small polyethylene catheter, which was previously introduced into the craniectomy site through a burr hole in the zygoma. The artery was occluded by retraction of the thread, which was then fixed with biological glue. Recirculation was achieved by cutting and removing the thread.

Bederson et al. (1986a) occluded the middle cerebral artery at different sites in six groups of normal rats and characterized the anatomical sites that produce uniform cerebral infarction. A neurological system was developed that can be used to evaluate the effects of cerebral ischemia.

Bederson et al. (1986b) evaluated the use of 2,3,5-triphenyltetrazolium chloride as a histopathological stain for identification and quantification of infarcted brain tissue after middle cerebral artery occlusion in rats.

Yang et al. (1992) found a reduction of Na, K–ATPase activity in the ischemic hemisphere shortly after middle cerebral artery occlusion in rats.

Du et al. (1996) induced transient focal cerebral ischemia in rats by a 90-min period of ligation of the right middle cerebral artery and both common carotid arteries.

Germano et al. (1987) found a decrease of stroke size and deficits in rats with middle cerebral artery occlusion after treatment with kynurenic acid, a broad spectrum antagonist of excitatory amino acid receptors.

Wu et al. (1999) reported that propentofylline attenuates microglial reaction in the rat spinal cord induced by middle cerebral artery occlusion.

Gotti et al. (1988) found a reduction of the volume of infarcted tissue due to occlusion of

the middle cerebral artery in **rats** and **cats** after administration of NMDA receptor antagonists.

Hossmann and Schuier (1980) studied experimental brain infarcts in **cats** after occlusion of the left middle cerebral artery.

Retro-orbital occlusion of the middle cerebral artery in cats was performed by Sundt and Waltz (1966) and by Gotti et al. (1988).

Welsh et al. (1987), Backhauß et al. (1992) described focal cerebral ischemia in **mice** after permanent occlusion of the middle cerebral artery.

Hara et al. (1997) found a reduction of ischemic and excitotoxic neuronal damage by inhibition of interleukin 1β-converting enzyme family proteases after occlusion of the middle cerebral artery in **mice** and **rats**. Nylon filaments were introduced from the carotid artery which were withdrawn after 2 h. One day later, the animals were tested for neurological deficits and the brains analyzed for infarct size and interleukin 1β levels.

Huang et al. (1994) produced **knockout mice** deficient in the neuronal isoform of NO synthase by targeted disruption of the neuronal nitric oxide synthase gene. In these mice, Hara et al. (1996) found reduced brain edema and infarction volume after transient middle coronary artery occlusion.

Nishimura et al. (1998) described an experimental model of thromboembolic stroke without craniotomy in **cynomolgus monkeys** by delivering an autologous blood clot to the middle cerebral artery. A chronic catheter was implanted in the left carotid artery in male cynomolgus monkeys. A 5-cm-long piece of an autologous blood clot was flushed into the internal carotid artery with physiological saline. A neurological score was assigned at 0.167, 0.5, 1, 2, 4, 6, and 24 h after embolization. In the acute phase after embolization, typical behavior consisted of circling gait and moderate deviation toward the side of embolization, long-lasting and strong extensor hypotonia of the contralateral lower and upper limbs, and mild to severe incoordination. Contralateral hemiplegia was observed over the following 24 h. At 24 h the animals were sacrificed immediately after the last neurological scoring, and the cerebral vasculature was inspected for the location of the clot. The brain was then cut into 2-mm-thick coronal sections. Cerebral infarction size and location were ascertained by the triphenyl-2H-tetrazolium chloride staining method. The lesions involved mostly the caudate nucleus, internal capsule, putamen, and thalamus.

Salom et al. (1999) subjected female **goats** to 20-min global cerebral ischemia under halothane/N_2O anesthesia. An episode of transient global ischemia was achieved by occlusion of the two external carotid arteries and simultaneous external compression of the jugular veins by a neck tourniquet. A reperfusion period started when the occlusions were released, and it was monitored for 2 h.

De Ley et al. (1988) studied experimental thromboembolic stroke induced by injection of a single autologous blood clot into the internal cerebral artery in dogs by **positron emission tomography**.

Shimamura et al. (2009) developed a new rat model of transient intraluminal suture middle cerebral artery occlusion without sacrificing the external carotid artery. This method uses a transfemoral approach to the internal carotid artery that is commonly used in neurointerventional examination and treatment.

Engel et al. (2011) described a modification of the middle cerebral artery occlusion model in mice. In this model a silicon-coated filament is introduced into the common carotid artery and advanced along the internal carotid artery into the circle of Willis, where it blocks the origin of the middle cerebral artery.

Christoforidis et al. 2011 refined the middle cerebral artery occlusion canine model to account for leptomeningeal collateral formation. This model used a retrievable coil allowing precise placement of the occlusion, reversal of the occlusion, and global assessment of pial collateral recruitment. Post-reperfusion infarct volume was assessed by MRI.

Zu et al. (2013) established an endovascular canine stroke model by occluding the middle cerebral artery with autologous clots followed by 2-h block of the ipsilateral internal carotid artery with a catheter. The model produced a large infarct volume with lower the risk of recanalization and survival for 7 days.

Van Winkle et al. (2013) developed a novel method for delivering substances directly into the ischemic brain during middle cerebral artery occlusion in the awake rat. This model allows delivering of drug intra-arterially to the target tissue minimizing loss in the liver.

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Photochemically Induced Focal Cerebral Ischemia in Rats

Purpose and Rationale

Focal cerebral ischemia in rats can be induced by irradiation with intensive green light via a fiber optic through the skull after injection of the dye rose bengal. The resulting cerebral infarct can be studied for various parameters, such as infarct volume, water content, local cerebral blood flow, and glucose utilization.

Procedure

Male Sprague–Dawley rats (300–350 g) are used. Anesthesia is induced with 3 % halothane in oxygen and is maintained with 1% halothane in oxygen applied via a face mask. A small incision is made in the skin over the right femoral vein and a thin catheter is inserted. One milliliter of the dye rose bengal (in a concentration of 5 mg/ml of saline) is injected. A midline head incision is made and the right side of the skull is exposed. An intense green light, produced by a xenon lamp (75 W, Zeiss, FRG) and then passed through a filter (wavelength 570 nm, Schott, Mainz, FRG) and a heat filter (Schott, Mainz, FRG), is directed on the skull at the level of the bregma for 15 min. The 3-mm diameter of the illuminated circle is determined by passing the light through a fiber optic (Schott, Mainz, FRG) while in close contact to the skull. The temperature of the skull underneath the fiber optic does not change during the time of illumination. At the end of the induction period, the temporary catheter is removed from the femoral vein which remains patent following the closure of the catheterization site with liquid

suture (Histoacryl, Braun, Melsungen, FRG), and the incisions in the leg and head are likewise sutured after liberal application of local anesthetic. The anesthetic gas mixture is discontinued, and the rats are allowed to recover consciousness in a warm environment until such times as the appropriate experiment is to be performed. Due to the noninvasive nature of this technique, it is not possible to measure blood pressure or blood gases during the ischemic period. However, rectal temperature and plasma glucose concentrations are controlled.

Measurement of Infarct Volume

Osmotically controlled minipumps (Model 2ML1 Alzet, USA) are placed into the peritoneum of two groups of six male Sprague–Dawley rats (body weight 300–350 g). The minipumps are fitted via thin polyethylene catheters to the femoral vein of the rats. Each pump contains either 2 ml of physiological saline or 2 ml of a solution of the drug to be studied. The animals are then given an ischemic insult as described in the previous section. Seventy-two hours after the induction of ischemia, the rats are sacrificed by decapitation and the brains removed and frozen at −50 °C. Coronal sections (20 μ) are cut in a cryotome at −20 °C, fixed in Heidenhain's Susa, and stained with cresyl violet. The ischemic area on 90 sections is measured and the volume of ischemic change is then calculated using a linear trapezoidal extrapolation of the areas measured.

Measurement of Brain Water Content

Three groups each of six rats are used. One group receives no ischemic lesion (the illumination with green light is omitted from the experimental protocol) and two groups are lesioned as described above. One lesioned group receives the test drug orally at 15 min, 30 min, and 1, 3, and 5 h after the induction of the ischemia or sham operation. The other group is treated with saline at the same time points. Twenty-four hours later the rats are sacrificed by decapitation. The brains are rapidly removed and placed on a cutting block with 1-mm gradations. Two cuts are made 1 mm or less anterior and posterior to the lesion. The thick section so produced is then divided into left (non-lesioned) and right (lesioned) halves and placed in pre-weighed vials and the wet weights of the tissue samples are carefully measured. The tissue is then frozen in liquid nitrogen and then left under vacuum (less than 0.1 Torr) for 24 h. On removal the vials are sealed to prevent rehydration and reweighed to obtain the dry weight of the tissue from which the water content (expressed as percentage of wet weight of tissue) is calculated.

Evaluation

All data are presented as mean ± SD of the mean. For left (contralateral to the lesion) against right (ipsilateral to the lesion) comparisons, a *t*-test with paired comparison was used ($P < 0.05$). Statistical differences between groups were calculated using the unpaired *t*-test.

Modifications of the Method

Boquillon et al. (1992) produced cerebral infarction in mice by intravenous injection of 10 mg/kg rose bengal and by focal illumination of the intact skull surface for 3 min with a laser source, operating at 570 nm with power levels of 2, 5, 10, and 20 mW.

Matsuno et al. (1993) used a similar model to induce cerebral ischemia in rats based on middle cerebral artery occlusion by the photochemical reaction of rose bengal after irradiation with high-intensity green light.

Stieg et al. (1999) studied neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. An excellent correlation between infarct size determined by magnetic resonance imaging (MRI) and histopathological analysis in the same animals was found.

Kim et al. (2014) described a new method for inducing a circumscribed subcortical capsular infarct by photothrombotic destruction of the internal capsule. In this study they used low-light energy to selectively disrupt circumscribed capsular fibers. They showed that the model is associated with a marked and persistent motor impairment and a decrease in metabolic activity, predominantly in the ipsilesional motor and sensory cortex.

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Microdialysis and Neuroprotection Experiments After Global Ischemia in Rats

Purpose and Rationale

Transient global ischemia can be induced in rats by electrocauterization of the vertebral arteries followed by clamping of the carotid arteries.

Procedure

Male Wistar rats weighing 280–340 g are used. The rats are anesthetized with methohexital sodium (60 mg/kg i.v.) and the vertebral arteries are electrocauterized (Pulsinelli and Brierley 1979). The rats are fasted overnight and re-anesthetized on the following day with halothane and intubated. The femoral artery and vein are cannulated to allow blood sampling, blood withdrawal, and recording of mean arterial pressure (MAP). Samples of blood are taken at regular time intervals and blood gas/acid base status is analyzed (Instrumentation Laboratory, 1306). Rectal temperature is measured with a thermistor and controlled at 37 °C by means of a heating lamp. Four-vessel occlusion ischemia is induced for 20 min by bilateral carotid clamping followed by a period of reflow.

Microdialysis Experiments

The head of the rat is fixed in a stereotactic frame. The skin is incised over the head and pulled apart and a 3 × 3-mm hole is drilled through the cranium. A microdialysis probe (Sandberg et al. 1986) is implanted into CA1 region of the hippocampus (2.2 mm lateral and 3.8 mm dorsal to bregma and the window of the dialysis membrane 1.4–2.9 mm below the cortical surface). The electroencephalogram (EEG) is measured continuously with a tungsten electrode attached to the dialysis probe. The probe is perfused at a rate of 2.5 μl/min with a modified Ringer's solution. Dialysates are sampled every 10 min and analyzed for purines (Hagberg et al. 1987) and amino acids (Lindroth et al. 1985). One group receives the test drug i.p. 15 min prior to ischemia, whereas the control group obtains saline. The animals are followed during 20 min of ischemia and 2 h of reflow.

HPLC analyses are carried out using a reverse-phase C_{18} column (Waters 10 10 μm mu;m μBondapak) with isocratic elution and a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine, and hypoxanthine, the mobile phase is 10-mM $NH_4H_2PO_4$, pH6.0, 13 % methanol.

Neuroprotection Experiments

During ischemic insult and for 20 min of reflow, the temperature of the temporalis muscle is controlled at 37 °C. Immediately following ischemia the rats are divided into two groups. One group is treated with the test drug and the other with saline. A bolus injection is administered i.p. 15 min after ischemia and a mini-osmotic pump is implanted into the abdominal cavity which delivers the test

drug for 7 days. Control animals receive a bolus of saline and mini-osmotic pumps filled with saline. Seven days later the rats are anesthetized with pentobarbital and perfusion-fixed with formol saline. The histological evaluation is done "blind." The hippocampal damage is semiquantified according to the following scoring system:

0 = no damage
1 = scanty damage
2 = moderate damage
3 = severe damage
4 = complete loss of pyramidal cells in the hippocampus

Evaluation

The purine and amino acid data are expressed as mean ± SEM and differences are evaluated with the nonparametric Mann-Whitney U-Test. The neuroprotective efficacy of the test drug is evaluated with two-tailed Student's *t*-test.

Modifications of the Test

In addition to the assessment of neuronal damage, Block et al. (1996) tested spatial learning of treated rats 1 week after 4-vessel occlusion in a Morris water maze.

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Hypoxia/Hypoglycemia in Hippocampal Slices

Purpose and Rationale

The in vitro release of adenosine, inosine, and hypoxanthine from rat hippocampal slices can be determined with and without drug.

Procedure

Male Sprague–Dawley rats weighing 150–275 g are decapitated, the hippocampi isolated and cut into 400 μm thick slices which are placed into KRB (4 °C) containing (mM) NaCl (118), KCl (4.85), $MgSO_4$ (1.15), KH_2PO_4 (1.15), $NaHCO_3$ (25), glucose (5.5), and $CaCl_2$ (1.3) equilibrated with 95 % O_2/5 % CO_2. The incubation medium is brought up to room temperature over a period of 30 min. The KRB is then replaced with fresh medium and the slices are incubated for a further 30 min at room temperature, followed by 30 min at 37 °C in fresh KRB. Following the initial

incubations, slices are labeled for 45 min with ^{3}H-adenine (5 μCi/ml) at 37 °C. Two labeled slices are transferred into plastic cylinders which have nylon net bases, and these, together with the slices, are placed into glass superfusion chambers. Slices are superfused at a flow rate of 0.5 ml/min with KRB at 37 °C. After a 60 min wash, collection of 5 min fractions begins, which continues throughout the remainder of the experiment. A 1.25 ml aliquot of the fractions is taken for determination of radioactivity using scintillation spectrometry (scintillation fluid: Picofluor 15). The remaining 1.25 ml is taken for HPLC analysis of purines and amino acids.

Hypoxia/hypoglycemia is induced by superfusion with KRB containing no glucose and 95%N_2 and 5 % CO_2 for 35 min followed by recovery. All other procedures are as described above. The test drug is added to the perfusion fluid at an appropriate concentration. The 1.25-ml aliquots taken for HPLC analysis are pooled with two other aliquots (total 3.75 ml), lyophilized and concentrated tenfold before analysis. Samples are analyzed for adenosine, inosine, and hypoxanthine with HPLC. The radioactivity associated with each of these fractions is also determined by collecting the eluent from the column at the appropriate times. HPLC analyses are carried out using a reverse-phase C_{18} column (Waters 10 10 μm mu;m μBondapak) with isocratic elution and a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine, and hypoxanthine, the mobile phase is 10-mM $NH_4H_2PO_4$, pH6.0, 13 % methanol.

Evaluation

Values for adenosine, inosine, and hypoxanthine can be expressed in two ways: (1) release rate per slice (pmol/min × slice) and (2) percentage of the total amount of released radioactivity (% total $_3$H-label released). The purine data are expressed as mean ± SEM and differences are evaluated with the nonparametric Mann-Whitney U-Test.

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Measurement of Local Cerebral Blood Flow and Glucose Utilization in Rats

Purpose and Rationale

Cerebral glucose utilization can be determined using [^{14}C]2-deoxyglucose according to Sokoloff et al. (1977). Local cerebral blood flow using [^{14}C]-iodoantipyrine is measured as described by Sakurada et al. (1978).

Procedure

Animal Preparation

The experiments are performed on male Sprague–Dawley rats (260–300 g). Catheters are placed, under light halothane (1 %) anesthesia, into the femoral vein and artery of each hind limb (for the measurement of mean arterial pressure, the sampling of arterial blood and the intravenous administration of drugs and radioisotopic tracer). The wound sites are then infused with local anesthetic, sutured, and protected by pads. The lower abdomen is covered by an elastic stocking, followed by a loose-fitting plaster coat. Anesthesia is discontinued and the rats are allowed at least 2 h to recover before any further manipulations are performed.

Experimental Protocol

The rats are given an intravenous infusion (50 μl/min) of either saline or the test drug dissolved in saline. This infusion is maintained throughout the measurement of local cerebral blood flow or local cerebral glucose utilization.

Local Cerebral Glucose Utilization

A full description of the method for measuring local cerebral glucose utilization using

[^{14}C]2-deoxyglucose has been published (Sokoloff et al. 1977). Five minutes after the administration of the test drug, the experiment is started with the intravenous administration of [^{14}C]2-deoxyglucose (125 μCi/kg). Fourteen timed arterial blood samples are taken during the following 45 min. These samples are centrifuged and the plasma is measured for glucose concentration (using an automated glucose analyzer) and [^{14}C] levels (by liquid scintillation counting). At the end of this period, the rats are sacrificed by decapitation, the brain rapidly removed and frozen at −45 °C. Twenty-micron-thick coronal sections are cut in a cryostat (−22 °C), and autoradiograms are prepared by placing these sections in an array against Kodak SB-5 X-ray film along with precalibrated plastic standards range (55–85 1 nCi/g) for 7 days in light-tight cassettes.

Local Cerebral Blood Flow

The autoradiographic measurement of local cerebral blood flow using [^{14}C]-iodoantipyrine is carried out as described by Sakurada et al. (1978). [^{14}C]-iodoantipyrine (125 μCi/kg) is administered 15 min after the infusion of the test drug has commenced. In a period of 60 s, 18 timed arterial samples are collected in pre-weighed filter paper disks from a free-flowing arterial catheter. The disks are reweighed and the [^{14}C] concentration of each is measured by liquid scintillation counting. At the end of 1 min, the rat is decapitated and autoradiograms are prepared in the same manner as for the measurement of local cerebral glucose utilization.

Densitometric Analysis of Autoradiograms

Tissue [^{14}C] concentrations were determined using a densitometer system (Zeiss, FRG) by reference to the images of the precalibrated standards. For each structure of interest, bilateral determination of optical densities are made on six different autoradiographic images in which the structure is best defined.

The mean optical density is used to calculate [^{14}C] concentrations. From this value, and the history of [^{14}C] in the blood, values of local cerebral blood flow and glucose utilization are obtained using the respective operational equations of these methods (Sakurada et al. 1978; Sokoloff et al. 1977).

Evaluation

Groups of data are statistically compared by *t*-test with unpaired comparison using the Bonferroni correction factor for multiple group analyses. Linear regression data for comparing cerebral blood flow (CBF) and glucose utilization (GU) undergo log transformation as recommended by McCulloch et al. (1982).

Modifications of the Method

Ito et al. (1990) measured glucose utilization in the mouse brain by the simultaneous use of [^{14}C] 2-deoxyglucose and [^{3}H]3-*O*-methylglucose.

High-resolution animal positron emission tomography was recommended by Magata et al. (1995) for noninvasive measurement of cerebral blood flow with ^{15}O-water and glucose metabolic rate with ^{18}F-2-fluoro-2-deoxyglucose.

The effect of ginseng pretreatment on cerebral glucose metabolism in ischemic rats using animal positron emission tomography (PET) with 2-[^{18}F] fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) was described by Choi et al. (1997).

Hawkins et al. (1993) developed a method for evaluating tumor glycolytic rates in vivo with nude mice injected with 2-[^{18}F]fluoro-2-deoxy-D-glucose and a dedicated animal positron emission tomography scanner.

Positron emission tomography has been used with specific ligands for CNS imaging (De la Sayette et al. 1991; Jones et al. 1991; Kung 1993).

Rogers et al. (1994) synthesized ^{18}F-labeled vesamicol derivates to be evaluated in small-animal positron emission tomography.

Hume et al. (1997) measured in vivo saturation kinetics of two dopamine transporter probes using a small-animal positron emission tomography scanner.

Ku and Choi (2012) demonstrated the use of optical imaging using the near-infrared fluorescence dye, indocyanine green, combined with a time-series analysis of the molecular dynamics for non-invasive analysis of cerebral blood flow in mice.

Bouwens et al. (2014) demonstrate high-resolution quantitative imaging of the blood flow

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Cerebrovascular Resistance in Anesthetized Baboons

Purpose and Rationale

Cerebral blood flow and cerebrovascular resistance can be measured by injection of inert radioactive gas (^{133}xenon) and evaluation of the ^{133}Xe clearance curve in anesthetized baboons.

Procedure

Animal Preparation

The experiments are performed on baboons (*Papio cynocephalus*) weighing 9–13 kg. Initial sedation with phencyclidine (10 mg i.m.) is followed by an intravenous injection of sodium thiopental (75 mg/kg). The animals are intubated to a positive pressure ventilator delivering 70:30 % nitrous oxide and oxygen in open circuit. A continuous intravenous infusion of phencyclidine (0.01 mg/kg/min) is given throughout the course of the experiment. Suxamethonium (50 mg i.m.) is administered every 30 min in order to assist control of ventilation with the respiratory pump.

During the experiments, the end-tidal concentration of CO_2 is continuously measured and the ventilating pump adjusted to maintain an arterial CO_2 tension ($PaCO_2$) of between 37 and 40 mmHg for the control measurements. Arterial blood samples are taken during every measurement of cerebral blood flow (CBF) to measure $PaCO_2$, PaO_2, and pH by direct reading electrodes (Corning). Body temperature is maintained around 37 °C by means of an electrically heated blanket and infrared heating lamps.

Catheters are inserted into the aorta via femoral arteries for the continuous measurement of arterial blood pressure and for the withdrawal of arterial blood samples. Both femoral veins are cannulated, one for the continuous infusion of phencyclidine and the other for the infusion of the test drug. A catheter is inserted into the right lingual artery so that its tip lays just distal to the carotid bifurcation. This catheter is flushed at regular intervals with heparinized saline to prevent platelet aggregation at the tip. In the studies with required intravenous administration of test drug, the other branches of the external carotid artery are ligated. Where the requirement is for intracarotid administration of test drug, another catheter is retrogradely advanced into the external carotid artery so that its tip lays next to the tip of the lingual catheter. This catheter is then attached to a constant-rate infusion pump (Sage Instruments). Heparinized saline is infused at a rate of 0.2 ml/min to act as a control for drug infusion.

A burr hole is made over the midline fissure and a catheter inserted into the sagittal sinus for the withdrawal of cerebral venous blood samples. The hole is sealed with plaster of Paris. The scalp and temporalis muscle are removed with diathermy down to the level of the zygomatic arch.

Measurement of Cerebral Blood Flow, Cerebral Oxygen Utilization, and Cerebrovascular Resistance

A collimated scintillation crystal is placed over the temporal region of the exposed skull on the right side and angled in such a way that viewed only are the brain and overlying skull.

Approximately 260 μCi of ^{133}xenon dissolved in 0.5-ml sterile heparinized saline (500 IU) is injected over 1 s into the catheter in the lingual branch of the carotid artery. The gamma-ray emission of the ^{133}Xe are detected by the scintillation counter attached to a photomultiplier. The pulses are amplified and subjected to pulse height analysis (peak 81 $\pm$ 8 KeV) to reduce Compton scatter before fed into a rate meter and scaler. The output from the rate meter is displayed on a chart recorder. Cerebral blood flow is calculated from the height/area equation (Høedt-Rasmussen et al. 1966). The formula used is

$$F = (H_2 - H_{10}) \times 100/\lambda A_{10},$$

where F is CBF in ml blood per 100 g brain tissue per min; λ = brain tissue/blood partition coefficient for ^{133}Xe (the figure of 1.1 is used [Veall and Mallett 1966]); H_i = maximum initial height of the ^{133}Xe clearance curve in counts per min as taken from the chart recorder; H_{10} = height of the clearance curve 10 min after the peak height in counts per s; and A_{10} = total integrated counts over the 10 min of clearance as taken from the scaler and corrected for background activity over that period.

Cerebral oxygen consumption is measured from the product of the DBF and the difference in oxygen content between the arterial blood and cerebral venous blood sampled from the sagittal sinus. Blood oxygen is measured by a charcoal-fuel cell system (Lex-O_2-Con).

An estimate of cerebrovascular resistance is obtained by subtracting the mean sagittal sinus pressure from the mean arterial pressure and dividing this pressure difference by the CBF.

EEG Recording

Electroencephalographic readings are recorded bilaterally throughout the experiment. A series of holes are drilled in the calvarium 10 mm apart in two rows. Each row is 14 mm lateral to the sagittal suture. The holes are threaded to receive nylon screws in which silver–silver chloride ball electrodes are fixed loosely. The electrodes are positioned epidurally and the free ends are soldered to a multichannel socket which is mounted on the calvarium with plaster of Paris.

Experimental Procedure

Following completion of the surgery, the animals are left undisturbed for 1 h. At least three control estimations of CBF and other parameters are made until steady conditions of flow, arterial blood pressure, and blood gas tensions are obtained.

Protocols

Intravenous administration. After stable control values have been established, the infusion is started. The CBF is measured at 5 min after the start and again at 25 min. The infusion is stopped 10 min after this flow period, giving a total infusion time of 35 min. Further flow measurements are made at 10, 30, and 50 min after stopping the infusion.

Intracarotid administration. After establishing control values, the intracarotid infusion of the test drug is begun. The CBF is measured at 10, 30, 50, and 70 min. The infusion is stopped 80 min after commencing, and post-infusion measurements are made after 20, 40, and 60 min.

Evaluation

Data are presented as mean values ± SEM. Evaluation of statistical significance is performed by means of Student's *t*-test with Bonferroni correction.

Modifications of the Method

Kozniewska et al. (1992), Wang et al. (1992) measured cerebral blood flow in **rats** by intracarotid injection of ^{133}Xe.

Solomon et al. (1985) and Clozel and Watanabe (1993) induced cerebral vasoconstriction by injection of autologous blood in the cisterna magna of rats. Cerebral blood flow was measured with the radioactive microsphere technique.

Lin et al. (2003) described a model of subarachnoid hemorrhage-induced cerebral vasospasm in **mice**. Adult mice received injections of autologous blood into the cisterna magna. The diameters of large intracranial vessels were measured 1 h to 7 days after the subarachnoid hemorrhage. A diffuse blood clot was evident in both the anterior and posterior circulations. Vascular wall thickening, lumenal narrowing, and corrugation of the internal elastic lamina were observed. Both acute (6–12 h) and delayed (1–3 days) phases of vasoconstriction occurred after subarachnoid hemorrhage. Overall mortality was only 3 %. The model is recommended for screening of therapeutic candidates.

Delayed cerebral vasospasm was induced in anesthetized **dogs** by removal of 4-ml cerebrospinal fluid and injection of the same volume of fresh autologous arterial nonheparinized blood into the cisterna magna by Varsos et al. (1983) as a model of subarachnoid hemorrhage. The procedure was repeated on the 3rd day and angiograms were taken of the vertebral–basilar vessels. The reduction of diameter of the basilar artery was taken as endpoint.

Imaizumi et al. (1996) produced experimental subarachnoid hemorrhage by intracisternal injection of arterial blood in **rabbits**. The degree of vasospasm and the effect of calcitonin gene-related peptide were evaluated angiographically by measuring the basilar artery diameter.

Inoue et al. (1996) produced experimental subarachnoid hemorrhage in **cynomolgus monkeys** by placing a clot around the internal carotid artery. A series of angiographic analyses were performed, before subarachnoid hemorrhage and on days 7 and 14 after treatment with calcitonin gene-related peptide to examine changes in the diameter of the ipsilateral internal carotid artery, middle cerebral artery, and anterior cerebral artery.

Hughes et al. (1994) adapted the ^{133}xenon clearance technique for simultaneous measurement of cutaneous blood flow in **rabbits** at a large number of skin sites within the same animal.

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Effect on Cerebral Blood Flow in Cats (Fluvography)

Purpose and Rationale

Cerebral blood flow in anesthetized cats can be measured by fluvography. The heat transfer

coefficient of the brain can be measured with a probe representing an indirect blood flow value.

In this method, the measuring device consists of a thermo-probe, which is attached to the tissue in order to record continually the heat transport (Betz 1965). The device depends on having an electrically heated part and an unheated reference point. The difference in temperature between the heated part of the device and the unheated reference point is a function of local blood flow. An increase in flow tends to lower the local temperature by carrying away the heat gain and vice versa.

Procedure

Cats of either sex weighing 2.5–4.0 kg are anesthetized by intraperitoneal administration of pentobarbital sodium (35 mg/kg) and intubated with a tracheal tube. The left femoral vein and the right femoral artery are cannulated for i.v. drug administration and determination of arterial blood pressure, respectively. The arterial cannula is connected to a Statham transducer P 23Db.

For intraduodenal drug administration the duodenum is cannulated following laparotomy.

Before actually starting the experiment, the arterial blood gas concentrations are determined.

Animals are only used for further testing if they show normal blood gas concentrations. During the course of the experiment, blood flow, blood pressure, and blood gas concentrations are regularly monitored.

The head of the animal is fixed in a stereotactic device. The skull cap and the dura are opened, the probes are placed on the surface of the cortex in the region of the marginal frontal gyrus, and the exposed brain is covered with moist swabs. The fluvograph (Hartmann + Braun, Frankfurt) is used with the appropriate thermo-probes.

To test the correct position of the thermocouple and the response of the animal, inhalations of carbon dioxide/air or injections of epinephrine (adrenaline) are used, leading to a distinctive increase in cerebral blood flow. Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is administered.

Standard compound:

- Pentoxifylline
- 1 and 3 mg/kg (i.v. administration)
- 10 and 30 mg/kg (i.d. administration)

Evaluation

The percent change in the heat transfer coefficient is used as an indirect measure for the change of cerebral blood flow. Statistics: Student's *t*-test is performed by unpaired comparison.

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Effect on Cerebral Blood Flow and in Ischemic Skeletal Muscle in Rats (Laser-Doppler Effect)

Purpose and Rationale

The principle of the laser-Doppler effect is based on the fact that a laser light beam directed on a tissue is scattered in static structures as well as in moving cells. Light beams scattered in moving red cells undergo a frequency shift according to the Doppler effect, while beams scattered in static tissues alone remain unshifted in frequency. The number of Doppler shifts per time is recorded as a measure for erythrocyte flow in a given volume. This means that the direction of flow cannot be determined, but relative changes in microcirculatory blood flow can be recorded. The procedure can be used to detect test compounds that improve the blood supply of the brain or the flow of red blood cells in the ischemic skeletal muscle.

Procedure

Male Sprague–Dawley rats weighing 300–500 g are anesthetized with pentobarbital sodium (60 mg/kg i.p.). The trachea is exposed and intubated with a short tracheal tube to allow ventilation. The following vessels have to be cannulated: The femoral vein is cannulated for test drug administration. The femoral artery is cannulated for blood pressure recording and blood gas analysis.

Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further testing if they show normal blood gas concentrations (pa CO_2: 32–42 mmHg; pa O_2: 70–110 mmHg). The mean arterial blood pressure should not drop below 100 mmHg. During the course of the experiment, blood flow, blood pressure, and blood gas concentrations are regularly monitored.

For Cerebral Blood Flow

The head of the animal is fixed in a stereotactic device. After trepanation of the skull (opening 3 mm in diameter), the laser-Doppler probe is placed 1 mm above the surface of the brain. Values are measured with the laser-Doppler apparatus (Periflux F2, Perimed KB, Stockholm).

Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is infused. Following two administrations of the test compound, the standard compound is administered again. Duration of the effect is measured as half-life in seconds.

For Peripheral Blood Flow

A small area of the femoral artery of the right hind limb is exposed and the laser-Doppler probe is placed 1 mm above the muscle surface. Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further proceeding if they show normal blood gas concentrations. During the course of the experiment, the blood pressure is recorded. The RBC flux is recorded continuously and after stabilization of the output signal, the femoral artery is occluded leading to underperfusion of the muscles of the right pelvic limb. At this stage, the test compound is administered by intravenous infusion for 10 min (0.05 ml/min).

The standard compound for cerebral blood flow is propentofylline (1 mg/kg, i.v.). The percent increase in blood flow produced by propentofylline ranges between 40 % and 60 %.

Evaluation

The percent increase in blood flow after test drug administration is determined (compared to the value before drug administration).

Statistics: Student's *t*-test by unpaired comparison, test substance versus standard

Modifications of the Method

Iadecola (1992), Prado et al. (1992), and Raszkiewicz et al. (1992) measured the influence of nitric oxide on cortical cerebral blood flow in anesthetized rats by laser-Doppler flowmetry.

Benessiano et al. (1985) measured aortic blood flow with range-gated Doppler flowmeter in anesthetized rats.

Partridge (1991) measured nerve blood flow in the sciatic nerve of anesthetized rats with a laser-Doppler flowmeter after application of local anesthetics and of epinephrine.

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Traumatic Brain Injury

Purpose and Rationale

A major goal in research into mechanisms of brain damage and dysfunction in patients with severe head injury and in discovery of potential therapies is the development of a suitable animal model. While a variety of experimental techniques have been developed (Denny-Brown and Russell 1941; Gurdjian et al. 1954; Ommaya and Gennarelli 1974; Sullivan et al. 1976; Nilsson et al. 1977; Hayes et al. 1984; Marmarou et al. 1994; Mésenge et al. 1996), the most widely employed technique is fluid percussion, which produces brain injury by rapid injection of fluid into the closed cranial cavity (Sullivan et al. 1976; McIntosh et al. 1989, 1990; Faden et al. 1989; Dixon et al. 1991; Sun and Faden 1995; Petty et al. 1996; Laurer et al. 2000; Maegele et al. 2005). Most authors used rats; however, other species, such as cats (Sullivan et al. 1976; Hayes et al. 1983, 1984), rabbits (Lindgren and Rinder 1969), and mice (Hall et al. 1988; Mésenge et al. 1996), were employed.

Procedure

Surgical Preparation

Male Sprague–Dawley rats weighing from 400 to 500 g are anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). During surgical preparation and throughout the experiment, all wounds are infused with a topical anesthetic (lidocaine hydrochloride 2.0 %). Catheters are inserted into the femoral vein for drug administration and into the femoral artery for blood pressure/blood gas monitoring. A 2.0-mm hollow female Luer-Lock fitting (to induce trauma) is rigidly fixed with dental cement to the animal's skull in a craniectomy centered over the left parietal cortex 5 mm from lambda, 5 mm from bregma, and 4 mm from sagittal suture. The dura is left intact at this opening. Immediately following surgical preparation, a constant i.v. infusion of sodium barbital (15 mg/kg/h) is begun and maintained for the duration of the studies.

Drug Administration

Drugs or equal volumes of saline are administered through the femoral vein over 10 min by constant infusion beginning 15 min before trauma.

Fluid-Percussion Injury

The fluid-percussion device consists of a Plexiglas cylindrical reservoir, 60-cm-long and 4.5 cm in diameter, bounded at one end by a Plexiglas, cork-covered piston mounted on O-rings. The opposite of the reservoir is fitted with a 2-cm-long metal housing on which a transducer is mounted and connected to a 5-mm tube (2-mm inner diameter) that terminates with a male Luer-Lock fitting. At the time of injury, the tube is connected with the female Luer-Lock fitting that has been chronically implanted over the exposed dura of the rat. After the entire system is filled with 37 °C isotonic saline, injury is induced by a metal pendulum which strikes the piston of the device from a predetermined height. The device produces a pulse of increased intracranial pressure of fairly constant duration (21–23 ms) by injecting various volumes of saline into the closed cranial

cavity. Brief displacement and deformation of neural tissue results from the rapid epidural injection of saline. The magnitude of injury is regulated by varying the height of the pendulum, which results in corresponding variations of the intracranial pressure expressed in atmospheres. The pressure pulses are measured extracranially by a transducer and recorded on a storage oscilloscope.

Evaluation

Behavioral Outcome

Posttraumatic deficits are evaluated at 24 h, 1 week, and 2 weeks following trauma. Outcomes include forelimb flexion (right and left), lateral pulsion (right and left), and angle score (left, right, and vertical position). Scores range from 0 (maximal deficits) to 5 (normal) for each task; by combining scores of all tests, a composite neuroscore is determined, ranging from 0 to 35 (Faden 1993).

Histopathology

At 2 weeks, following final neurological scoring, the rats are sacrificed by decapitation. The brain is removed, quickly frozen in isopentane and stored in a −80 °C freezer until sectioning. Coronal brain sections are selected to span the longitudinal axis of the dorsal hippocampus between −3.2 and −3.8 bregma. Sections (16-mm thick) are cut at −18 °C in a microtome-cryostat and thaw-mounted onto chrome-gelatin rubbed microscope glass slides and kept at −80 °C for histological study.

Sections are stained with crystal violet. CA1 and CA3 pyramidal cells with a distinct nucleus and nucleolus are counted as viable neurons, in one reticle within CA3 and in three reticles (R1, R2, and R3) within the subfield of the hippocampus, in both the right and left hemispheres. The number of viable neurons is counted twice at 400× microscope field.

An immunochemical method is used to detect glial fibrillary acidic protein (GFAP)-positive astrocytes in the hippocampus (Faddis and Vijayan 1988). Counting of the number of cells is done under 400× light microscopy in the dorsal CA1 subfield between medial and lateral regions.

Statistical Analysis

Neuroscores from forelimb flexion tests, lateral pulsion tests, and angle board tests are statistically analyzed by nonparametric Mann-Whitney U-Test. Histological data are analyzed by one-way ANOVA test, followed by Scheffe's test.

Modifications of the Method

Shohami et al. (1995) tested the effect of a non-psychotropic cannabinoid which acts as a noncompetitive NMDA antagonist on motor and memory functions after closed-head injury in the **rat**.

Fox et al. (1998) developed a **mouse** model of traumatic brain injury using a device that produces controlled cortical impact, permitting independent manipulation of tissue deformation and impact velocity and resulting in sustained sensory/motor and cognitive defects.

Tang et al. (1997) reported impairment in learning and memory in an experimental model of concussive brain injury in **mice**.

Bemana and Nago (1998) induced acute intercranial hypertension in **cats** by continuous inflation of an extradural balloon with physiological saline at a constant rate of 0.5 ml/h for 3 h. At this point, inflation was discontinued and the balloon remained expanded for an additional hour after which it was deflated.

A model of **traumatic injury to the spinal cord** was used by Springer et al. (1997). Female Long–Evans rats weighing 200–250 g were anesthetized with pentobarbital, and a dorsal laminectomy was performed to expose the spinal cord at thoracic level T 10. The vertebral column was stabilized by clamping the column at vertebra 8 and 11. The New York University (NYU) impactor device was used which produces accurate and reproducible damage to the rat spinal cord (Gruner 1992; Basso et al. 1996). This device is a weight-drop apparatus that uses optical potentiometers to record the movement of a 10-g impact rod and the vertebral column following impact

and is connected to a PC that monitors rod and vertebral movements during impact.

Morganti-Kossmann et al. (2010) reviewed traumatic brain injury models available in small and large animals. Kooijman et al. (2014) review the animal models of subarachnoid hemorrhage and focus on the diagnostic and mechanistic similarities and distinctions between the endovascular puncture model in animals and the human pathology.

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Cerebral Blood Flow Measured by MRI

Purpose and Rationale

Over the past several years, magnetic resonance imaging (MRI) has become an established tool in the drug discovery and development process. The main advantages of MRI are its high resolution, noninvasiveness, and versatility, which allow comprehensive characterization of a disease state and the effects of drug intervention. Recent advances now allow the application of this technique to the characterization of models of lung inflammation in rats and to the profiling of anti-inflammatory drugs. Repeated measurements can be carried out on the same animal, and time courses of events can be easily assessed. Furthermore, the prospect of using MRI to detect noninvasively a sustained mucus hypersecretory phenotype induced by endotoxin brings an important new perspective to models of chronic obstructive pulmonary disease in animals. Importantly, it might be possible to extend the use of this technique to the clinical study of inflammation in the lung and the consequences of drug treatment (Beckmann et al. 2003, 2004).

Functional magnetic resonance imaging has been developed as pharmacological magnetic resonance imaging for pharmacodynamic assays and to establish brain-penetrating parameters (Leslie and James 2000). Although the regional cerebral metabolic rate of glucose is strongly increased during cerebral activity, the cerebral metabolic rate of O_2 is not increased in direct proportion. The result is that the relative uptake of O_2 from blood actually decreases. The veins and capillaries draining from the activated region are “arterialized” and their deoxyhemoglobin concentration is reduced. Deoxygenated and oxygenated hemoglobin have different magnetization properties; thus, the changes in the ratio of these two entities can be detected by

blood-oxygen-level-dependent (BOLD) magnetic resonance imaging. At least for studies in animals, these methods may be preferred to positron emission tomography (Cherry and Phelps 1996).

Petty et al. (2003) studied the in vivo neuroprotective effects of a systemically active antagonist of the NMDA receptor glycine site by magnetic resonance imaging in ischemic stroke in rats.

Procedure

Transient Model of Focal Ischemia in Rats

In the right common carotid artery was ligated. The transient model of middle cerebral artery occlusion was based on the techniques described by Zea Longa et al. (1989) and Belayev et al. (1996). By means of an operating microscope, the right common carotid artery was exposed through a midline neck incision. The right superior thyroid, occipital, and pterygopalatine arteries were ligated and cut. A poly-L-lysine-coated 3–0 nylon monofilament with a heat-blunted tip was inserted through the proximal external carotid artery into the internal carotid artery and pushed forward a distance of 19 or 20 mm from the carotid bifurcation, depending on the weight of the rat, so as to occlude the origin of the middle cerebral artery. After suture placement, the neck incision was closed and the animal was allowed to regain consciousness. Two hours following occlusion of the artery, the rats were tested on a standard neurological battery to confirm the presence of a neurological deficit. Animals that did not exhibit a forelimb flexion were excluded from further study. At this time, the rats were re-anesthetized and the intraluminal suture was completely withdrawn to restore the blood supply.

Magnetic Resonance Imaging (MRI) Measurements

Measurements were performed 30 min, 3 h, and 24 h after the onset of ischemia on a 7-T Bruker BIOSPEC experimental scanner (DBX; Bruker Medical, Ettlingen, Germany) with a 30-cm bore magnet and actively shielded gradient coils (200 mT/m; rise time < 80 µs).

A 72-mm resonator was used for *rf* transmission; signals were detected with a 35-mm inductively coupled surface coil placed over the skull of the animal. The *rf* coils were decoupled from each other – the transmitter coil actively and the receiver coil passively.

Using gradient-echo imaging, sagittal pilot scans were performed to ensure accurate positioning of the animal in the magnet. For this purpose, the coronal plane 5.9 mm posterior to the rhinal fissure was placed in the isocenter of the magnet, thus focusing on the center of the ischemic territory resulting from the middle cerebral artery occlusion.

For the determination of the temporal evolution of the ischemic lesion, two NMR imaging modalities were used. A field of view of 3.2 cm, a slice thickness of 1.5 mm, and an interslice distance of 2 mm were used for both sequences. Multislice packages were recorded by placing the center of the multislice imaging packet 5.9 mm posterior to the rhinal fissure.

Diffusion-weighted imaging was performed using a Stejskal–Tanner spin-echo sequence [echo time (TE) = 37.2 ms, repetition time (TR) = 2,325 ms, eight slices] in six rats per group. To enable quantification of the apparent diffusion coefficient (ADC) of brain water, three b values were used (b = 50, 825, and 1,600 s/mm^2). ADC maps were calculated pixelwise using the monoexponential model (Le Bihan et al. 1986).

Perfusion-weighted imaging was performed with an ultrafast version of the arterial spin tagging technique (Kerskens et al. 1996; Franke et al. 2000) in four rats per group. In independent experiments, three coronal slices were recorded, thus covering the central part of the ischemic lesion. Measurement parameters were TE = 3.5 ms, TR = 7.4 ms, MATRIX = 128 × 64, AVERAGE = 8. Each experiment consisted of two image acquisitions separated by a recovery period. During the first acquisition, blood flowing through the neck was adiabatically inverted (preparation TIME = 3 s; z-gradient = 5 mT/m; B1 FIELD = 150 mG; off-resonance FREQUENCY = 6,000 Hz; mean preparation DISTANCE = 2.8 cm upstream from the imaging plane); in the

second acquisition, inflowing spins were left undisturbed. Both phases were separated by a recovery period of 10 s. In each perfusion experiment, the two images suffered the same signal loss due to magnetization transfer effects but differed in the magnetization of the inflowing blood. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion. In the second acquisition, inflowing spins were left undisturbed. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion.

Evaluation

Data were transferred to a PC and image analysis was carried out using the image processing software Scion Image for Windows (Scion Corporation, Frederick, Md., USA). Lesion volumes were calculated using ADC maps, as the ischemic lesion area was estimated by summing up all pixels with a relative ADC < 80 % compared to the healthy, contralateral hemisphere (Hoehn-Berlage et al. 1995). Perfusion signal intensities are referred to the homologous contralateral regions and given as ratios of ipsilateral to contralateral values.

Modifications of the Method

Edema following middle cerebral artery occlusion in spontaneously hypertensive rats was measured by magnetic resonance imaging (Seega and Elger 1993; Elger et al. 1994a). Magnetic resonance imaging was also used to determine the size of intracerebral hemorrhage in rats induced by stereotactic microinfusion of collagenase into the caudate putamen (Elger et al. 1994b).

Reese et al. (2000) visualized regional brain activation by bicuculline by functional magnetic resonance imaging. Time-resolved assessment of bicuculline-induced changes in local cerebral blood volume was achieved using magnetite nanoparticles as an intravascular contrast agent.

Pevsner et al. (2001) described a photothrombotic method of acute small stroke induction in rats with histopathologic and in vivo magnetic resonance imaging (MRI) observations from 3 to 6 h after irradiation, which is homologous to a human autopsy specimen. Utilizing 30 min of irradiation with minimal beam intensity (0.1 W/cm^2) cold white light in conjunction with 20 mg of intravenous (i.v.) rose bengal as a rapid infusion, small infarcts were induced photochemically in the frontal lobes of rats.

Using in vivo and ex vivo magnetic resonance imaging, Ohlstein et al. (2000) evaluated the effects of tranilast, an antiallergic drug, on neointima formation following balloon angioplasty of the rat coronary artery.

Swain et al. (2003) employed T-two-star (T_2*)-weighted and flow-alternating inversion recovery (FAIR) functional magnetic resonance imaging to assess chronic changes in blood volume and flow as a result of exercise in rats. Prolonged exercise induced angiogenesis and increased cerebral blood flow in primary motor cortex.

Using MRI evaluation of brain damage, Banfi et al. (2004) demonstrated that pentoxifylline prevents spontaneous brain ischemia in stroke-prone rats.

Henderson et al. (2004) studied functional magnetic resonance imaging during hypotension in young and adult cats.

In a magnetic resonance imaging study, Shirhan et al. (2004) found that spermine reduces infarction and neurological deficit in a rat model of middle cerebral artery occlusion.

Paczynski et al. (2000) studied the effects of fluid management on edema volume and midline shift in a rat model of ischemic stroke. MRIs were obtained 24 h after the onset of ischemia so that the ratio of hemispheric volumes ipsilateral and contralateral to the infarct and the extent of midline shift could be obtained.

Cash et al. (2001) evaluated the effectiveness of aminoguanidine as a neuroprotective agent in a rat model of transient middle cerebral artery occlusion using serial magnetic resonance imaging.

The MRI protocol consisted of three interleaved imaging regimens: proton density-weighted imaging (PDWI), T_2-weighted imaging (T_2WI), and diffusion-weighted imaging (DWI). Repetition time (TR) was 3 s for each of the three regimens and echo times (TE) were 70 ms for the T_2WI and DWI and 28 ms for the PDWI. For DWI, diffusion-sensitizing gradients

(b-value 590 $\times$ 10^{-3} s/mm^2) were applied along the inferior to superior axis of the brain. Two averages were acquired per phase encode step. The images were collected at an in-plane resolution of 0.31 $\times$ 0.31 mm from 18 contiguous, 1-mm-thick slices, with a total acquisition time of 43 min.

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Peripheral Blood Supply

Perfused Hindquarter Preparation with Sympathetic Nerve Stimulation in Rats

Purpose and Rationale

Perfusion of the hindquarter in rats with a constant flow rate allows the evaluation of the effect of drugs on the peripheral vascular bed. Since constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

Procedure

Male Wistar rats weighing between 250 and 300 g are pretreated with heparin (1,000 units/kg i.v.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.) The animals are intubated with a tracheal tube and positive pressure ventilation is maintained with a Harvard rodent respirator at 4–6 ml/stroke and 50 strokes/min. The right jugular vein is cannulated with polyethylene tubing for administration of drugs.

The lumbar sympathetic chain is isolated dorsal to the inferior mesenteric branches via an abdominal midline incision. The aorta is freed from the vena cava and two silk ligatures are placed around the aorta. The aorta is ligated and cannulated proximal as well as distally with polyethylene tubings. A short piece of rubber tubing is inserted at the distal end to allow intra-arterial injections of drugs. Two "T" junctions allow the measurement of arterial pressure and perfusion pressure by Statham P 23Db pressure transducers being recorded through a Hellige physiological recorder. From the proximal part of the aorta, blood is forced to its distal part by a peristaltic pump (Desaga) through a glass coil kept at 40 °C. Flow rate is adjusted to produce a stable perfusion pressure as close to the systemic pressure as possible. After initial adjustment, flow rate is not altered for the remainder of the experiment.

Following perfusion pressure stabilization, the sympathetic chain is isolated and a small (1-mm-wide, 2-mm-long) curved bipolar electrode is placed around the nerve for electrical stimulation. Square-wave pulses from a Grass stimulator are used to activate the nerve with a constant current of 2.5 milliamps and supramaximal voltage and varying frequencies of 5 ms duration.

A dose–response curve is established for norepinephrine by giving doses of 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 μg intra-arterially and measuring perfusion pressure changes. Similarly, a frequency–response curve to nerve stimulation is established by stimulation at 3, 6, and 10 Hz for

30 s. Two predrug readings are taken to insure consistent responses.

A minimum of four animals is used for each test compound.

Evaluation

The first predrug dose–response curves are compared with the second predrug, 5-min and 60-min postdrug dose–response curves. From regression equations for norepinephrine and nerve stimulation, mean responses and potency values with 95 % confidence limits are calculated.

Modifications of the Method

Folkow et al. (1970) perfused the hindquarters of spontaneously hypertensive rats and control rats at a constant rate of flow with an oxygenated plasma substitute in order to study the increased flow resistance and vascular reactivity. The hindquarters were isolated from the upper part of the body by standardized mass ligatures at identical levels until the aorta, and the inferior caval vein provided the only intact circulatory connections between the two parts of each animal.

Thimm et al. (1984) described reflex increases in heart rate induced by perfusing the hind leg of the rat with solutions containing lactic acid.

Thimm and Baum (1987) obtained spike recordings from chemosensitive nerve fibers of groups III and IV of the rat nervus peroneus. Applications were performed either by perfusion of the circulatory isolated hind leg or by superfusion of the isolated musculus extensor digitorum longus.

Kitzen et al. (1978) used the perfused hind limb of the **dog** with sympathetic nerve stimulation for cardiovascular analysis.

Reitan et al. (1991) developed a near anesthetic-free isolated hind-limb model in the **dog** and studied the effects of halothane and atropine sulfate on vascular resistance.

Wiegershausen and Deptalla (1969) used the isolated perfused hind limb of the **cat** to study the influence of local anesthetics on the vasoconstrictor actions induced by bradykinin, epinephrine, and histamine.

Santiago et al. (1994) analyzed the responses to bradykinin in the hindquarter vascular bed of the cat.

Champion et al. (1996, 1997) analyzed the responses of human synthetic adrenomedullin, an analog of adrenomedullin and calcitonin gene-related peptides in the hind-limb vascular bed of the cat.

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Effect on Peripheral Blood Flow in Rats

Purpose and Rationale

Various methods exist to measure peripheral blood flow in rats, such as the microsphere technique, electromagnetic flowmetry, and Doppler ultrasonic flowmetry.

Radioactive microspheres are used to calculate the distribution of blood from the heart to various organs and tissues before and after the administration of test compounds. In this method, microspheres are injected into the left cardiac ventricle. It is postulated that the first contraction of the cardiac muscle will expel these spheres into the circulation. Consequently the microspheres can be trapped in different organs according to the organ's perfusion rate. It is not a primary screening method, but it is a useful test for distinguishing compounds with blood flow altering activities.

The microspheres used are 14 μ in diameter. They are marked with isotopes. In this test, four different radioactive elements are used (Cr^{51}, Sr^{85}, Sc^{46}, Ce^{141}), allowing the determination of blood flow before dosing and after the administration of 3 different compounds or 3 different doses (of the same compound).

Procedure

Male Wistar rats weighing 500–550 g are anesthetized with pentobarbital. The trachea is exposed and intubated with a short endotracheal tube to allow ventilation. Prior to testing, the jugular vein is cannulated for administration of test drugs. The carotid artery is cannulated and later on the catheter is passed retrograde into the ventricle. Ventricular pressure is recorded to assure the correct emplacement of the catheter tip in the ventricle. This catheter is connected to another catheter allowing the injection of microspheres into the left ventricle later on. The right arteria brachialis is cannulated and connected to a Hellige blood pressure recorder. During the course of the test, blood pressure will be measured continuously. The left femoral artery is cannulated and connected to an infusion pump. During the experiment blood will be withdrawn from this artery.

After these preparations the rat is allowed 15–30 min to recover. Before actually starting the experiment, the arterial blood gas concentration of each animal is measured. Animals are only used for the experiment if they show normal blood gas concentrations. During the following procedure, the blood pressure, the ventricular pressure, and the heart rate are continuously recorded. To determine baseline blood flow, animals receive 0.2 ml vehicle/min over a 3-min period. In the

fourth min rats receive the first injection of microspheres (Cr^{51}). Simultaneously 0.5 ml/min blood is withdrawn from the femoral artery catheter for 1 min, the pump thus being used as a reference organ. The animals are allowed 20 min to recover before the administration of drugs. The test compound is infused into the jugular vein at a rate of 0.2 ml/min for 3 min followed by injection of the second microsphere (Sr^{85}). The same procedure is repeated using the other two microspheres (Sc^{46} and Ce^{141}) following administration of the second and third test compound.

At the end of the experiment, blood gas concentrations are measured. The animals are killed and their organs are removed. Usually blood flow is determined in the following organs:

- Brain (right and left hemisphere; right hemisphere showing slight ischemia due to cannulation of the carotis)
- Cerebellum
- Lungs
- Heart
- Kidney (right and left)
- Skeletal muscle (right hind extremity)
- Duodenum
- Stomach
- Spleen
- Diaphragm
- Adrenal gland (right and left)

To determine effects of test compounds on the blood flow in the underperfused skeletal muscle, the same experiment can be performed with the right femoral artery being clamped. In this way effects of the test drug on the ischemic and normal skeletal muscle (left thoracic limb) can be compared in the same animal.

Evaluation

The rate of blood flow/tissue at a certain time is determined by measuring radioactivity in the different tissues and comparing the results to that of the blood sample.

Modifications of the Method

Blood flow in various peripheral organs, e.g., renal blood, can be measured with electromagnetic flowmeters (e.g., Transflow601, Skalar Medical, Holland) or with Doppler ultrasonic flowmetry (Shaffer and Medvedev 1991).

Lappe et al. (1986) studied regional vascular resistance in conscious spontaneously hypertensive rats which were chronically instrumented with pulse Doppler flow probes to allow measurement of renal, mesenteric, and hind quarter blood flow.

Hartman et al. (1994) validated a transit-time ultrasonic volume flowmeter by simultaneous measurements with an electromagnetic flowmetering method.

Lepore et al. (1999) used electron paramagnetic resonance to investigate the time course of nitric oxide generation and its susceptibility of nitric oxide synthase in ischemia-reperfusion injury to rat skeletal muscle in vivo. Total hindlimb ischemia was applied for 2 h using a rubber band tourniquet method. At the end of ischemia, the tourniquet was removed and the limb allowed to reperfuse for various time intervals.

Beattie et al. (1995) measured carotid arterial vascular resistance in anesthetized **rabbits**. Carotid blood flow was measured by a Doppler flow probe placed around the right common carotid artery. Dose–response curves of reduction of carotid arterial vascular resistance were constructed after injection of various doses of substance P-methyl ester via the right lingual artery. Intravenous injection of various doses of a selective tachykinin NK_1 receptor antagonist inhibited this effect dose dependently.

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Effect on Peripheral Blood Flow in Anesthetized Dogs

Purpose and Rationale

Effects on blood pressure have to be analyzed whether they are mediated by central, cardial, or peripheral action. By injecting small doses of the test compound directly into a vascular bed, thus avoiding changes of central hemodynamics, peripheral vasodilating activity of a compound can be tested. This test is used to evaluate direct vasodilating or constricting activities of drugs in in vivo measurements of blood flow.

Procedure

Male or female Beagle or Labrador–Harrier dogs weighing 15–25 kg are used. The dogs are premedicated intravenously with heparin (bolus of 500 IU/kg and successive injections of 50 IU/kg every 30 min) and anesthetized by intravenous injection of thiobarbital sodium (0.5 mg/kg i.v.), chloralose (20 mg/kg i.v.), and urethane (250 mg/kg i.v.). Respiration is maintained with room air through a tracheal tube using a positive pressure respirator. Blood gas analysis is performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

To measure peripheral blood flow and to administer the test substance, a bypass is inserted into a femoral artery incorporating an electromagnetic flow probe and a port for injections. The other femoral artery is also equipped with a bypass used for the administration of a reference compound or a second test drug.

For recording of peripheral blood pressure and heart rate, one of the bypasses is connected to a pressure transducer (Statham P 23BD).

All parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 20 min, the vehicle is administered (control) and 10 min later the test compound. Immediately after each administration, the port is flushed with physiological saline. Successive doses are administered after recovery to baseline values.

Readings are taken at times 0, 0.5, 1, 2, 5, and 10 min and, if necessary, at additional 10-min intervals following drug administration.

Standard compound:

- Carbocromene 1 mg/kg characteristics:
- Blood pressure
  - Systolic, BPs
  - Diastolic, BPd
- Heart rate, HR
- Peripheral blood flow, PF

Evaluation

Changes in blood pressure, heart rate, and peripheral blood flow at different times after drug

administration are compared to vehicle control values obtained in the 10-min predrug period.

With $n > 2$, results are presented as mean ± SEM. Statistical significance is assessed by means of the paired *t*-test. Scores are compared to the efficacy of standard compounds for intensity and for duration of the effect.

Modifications of the Method

Regional blood flow can be determined by the use of microspheres (Rudolph and Heyman 1967). The method is based on the principle that biologically inert microspheres will be trapped due to their diameter in the microvasculature (Hales and Cliff 1977). The use of radioactive microspheres has some disadvantages (Buckberg et al. 1971). The use of fluorescent-labeled microspheres for measurement of regional organ perfusion has been recommended (Glenny et al. 1993; Prinzen and Glenny 1994; van Oosterhout et al. 1995). Raab et al. (1999) and Thein et al. (2000) described the automation of the use of fluorescent microspheres using a special sample processing unit. A Zymate robotic system (Zymark, Idstein, Germany) was modified to handle a special filtration device.

Ebara et al. (1994) measured renal blood flow in dogs after intrarenal arterial infusion of adrenomedullin.

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Effect on Peripheral Blood Supply Measured by Local Oxygen Pressure

Purpose and Rationale

Local oxygen pressure is directly related to oxygen supply to peripheral organs, e.g., the muscle (Luebbers et al. 1969; Kessler and Grunewald 1969). The local oxygen pressure (PO_2) is recorded directly on the muscle surface. In the following procedure the effect of test compounds on the local oxygen pressure (PO_2) of the normal and the ischemic skeletal muscle is determined.

Procedure

Male Beagle dogs weighing 15–20 kg are used. The dog is anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal). Prior to testing, the following vessels have to be cannulated: The V. femoralis of the left pelvic limb is cannulated for administration of test compounds. The A. femoralis of the left pelvic limb is cannulated for blood pressure recording. The V. femoralis of the right pelvic limb is cannulated. During the

course of this test, blood will be withdrawn from this vein to monitor lactate concentrations.

Small areas of muscles of the right pelvic limb and the right thoracic limb are exposed. Muscle relaxation is induced by intravenous injection of 0.1 mg/kg alcuronium chloride (Alloferin) and maintained by i.p. administration of 0.05 mg/kg Alloferin at 30 min intervals. The trachea is exposed and intubated to assist the dog's respiration.

A PO_2 electrode is placed on the exposed muscle area of the right hind limb. After stabilization of PO_2 curves, the femoral artery of the right hind limb is occluded by putting a clip around the vessel. Muscle PO_2 drops rapidly. Following stabilization, test compounds are given by intravenous infusion for 10 min or by intraduodenal administration at this stage. The PO_2 of the nonischemic muscle is recorded simultaneously via a second electrode on the right thoracic extremity. The clip is removed after maximally 1 h. This procedure can be repeated up to four times in one animal. Blood gas concentrations and pH are determined at the beginning and end of each experiment.

Standard compound:

- Pentoxifylline

Evaluation

The following parameters are determined:

- Maximal increase in PO2 (mm Hg) after administration of test drug
- Duration of effect by determining the half-life

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Effect on Mesenteric Blood Flow in Rats

Purpose and Rationale

Blood flow in the mesenteric vascular bed in situ (Bhattacharya et al. 1977; Eikenburg 1984; Randall et al. 1989; Jackson and Inagami 1990) can be studied in rats in a way similar to that of the perfused hindquarter.

Procedure

Wistar rats of either sex weighing 250–300 g are anesthetized with a combination of urethane (500 mg/kg i.v.) and sodium pentobarbitone (30 mg/kg). The abdomen is opened by a midline incision and a segment of the superior mesenteric artery is exposed by careful dissection of the surrounding tissue. Care is taken to avoid damage to the accompanying nerve terminals. One cannula is inserted into the carotid artery and the other into the superior mesenteric artery. Blood from the carotid artery is forced by a peristaltic pump (Desaga) into the superior mesenteric artery, using a glass coil kept at 40 °C. Blood required to fill the tubing initially is obtained from donor rats. Heparin is administered intravenously to the animals prior to cannulation of the mesenteric artery. The blood pressure and the perfusion pressure are measured by Statham P 23Db pressure transducers and recorded through a 2-channel Hellige recorder. The pump speed is initially adjusted so that the perfusion pressure equals the

systemic blood pressure. Intra-arterial injections into the mesenteric vascular bed are made by puncturing the tubing going toward the periphery. Intravenous injections are made through a cannula inserted into the external jugular vein.

Evaluation

Changes in the vascular resistance are measured by comparing perfusion pressure before and after drug administration. If constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

Modifications of the Method

Reactivity in the mesenteric vascular bed can be tested in an isolated preparation (McGregor 1965; Kawasaki and Takasaki 1984; Laher and Triggle 1984; McAdams 1984; Foy and Nuhu 1985; Longhurst and Head 1985; Soma et al. 1985; Hsueh et al. 1986; Longhurst et al. 1986; Manzini and Perretti 1988; Nassar et al. 1988; Randall and Hiley 1988). The abdomen of the anesthetized rats is opened and the superior mesenteric artery is separated from the surrounding tissue in the region of the aorta. A cannula is inserted into the superior mesenteric artery at its origin from the abdominal aorta. The cannula is filled with heparinized Krebs solution. The ileocolic branch of the artery is tied off and the intestine separated from the mesentery by cutting close to the intestinal border of the mesentery. The cannulated artery and its vascular bed are dissected out and mounted in an organ bath. The preparation is perfused with oxygenated Krebs–bicarbonate buffer (pH 7.4) at 37 °C. Perfusion pressure is recorded via a side arm of the arterial cannula using a Statham pressure transducer. The flow rate is adjusted to give a baseline perfusion pressure of 20–30 mm Hg. The test substances are infused into another side arm of the arterial cannula for 15 s using an infusion pump. After three stimuli with norepinephrine (1 μg) or potassium chloride (1 mg), the test drugs are infused followed by further stimulation. The inhibition of increase of perfusion pressure after test drugs is expressed as percentage of control.

Nuki et al. (1994) compared the vasodilating activity of chicken calcitonin gene-related peptide with human α-CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats.

The **rabbit** isolated arterially perfused intestinal segment preparation was used by Brown et al. (1983) as a model for vascular dopamine receptors.

Komidori et al. (1992) recommended the isolated rat mesenteric vascular–intestinal loop preparation as an excellent model for demonstrating resistance changes in isolated vascular beds while simultaneously measuring endogenous catecholamine overflow.

Pelissier et al. (1992) showed that perfusion with hypotonic solutions removed the endothelial layer in the isolated perfused mesenteric vascular bed of the rat, allowing the study of endothelial-dependent vascular responses.

Santiago et al. (1993) used the mesenteric vascular bed of the **cat** to study the inhibitory effects of the bradykinin receptor antagonist Hoe 140 on vascular responses to bradykinin.

The responses of adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat were compared by Santiago et al. (1995).

Chu and Beilin (1994) studied the mesenteric vascular reactivity which is reduced in pregnant rats after application of bradykinin and the bradykinin receptor antagonist Hoe 140.

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Effect on Pulmonary Blood Flow

Purpose and Rationale

During controlled pulmonary blood flow, lobar arterial pressure can be measured in anesthetized cats (Lippton et al. 1984; Hyman et al. 1989; McMahon and Kadowitz 1993; DeWitt et al. 1994a).

Procedure

Adult cats of either sex weighing 2.5–4.5 kg are sedated with ketamine hydrochloride (10–15 mg/kg i.m.) and anesthetized with pentobarbital sodium (30 mg/kg i.v.). The animals are fixed in supine position and supplemental doses of anesthetic are administered to maintain a uniform level of anesthesia. The trachea is intubated and the animals breathe room air enriched with 95 % O_2/ 5 % CO_2. Systemic arterial pressure is measured from a catheter inserted into the aorta from a femoral artery, and intravenous injections are made from a catheter positioned in the inferior vena cava from a femoral vein.

For perfusion of the left lower lung lobe, a special designed 28-cm 6-F triple-lumen balloon perfusion catheter (Arrow International, Reading, PA) is passed under fluoroscopic guidance from the left external jugular vein into the artery to the left lower lobe. The animal is heparinized by 1,000 IU/kg i.v., and the lobar artery is isolated by distension of the balloon cuff on the perfusion catheter. The lobe is then perfused by way of the catheter lumen beyond the balloon cuff, with blood withdrawn from a femoral artery with a perfusion pump. Lobar arterial pressure is measured from a second port 5 mm from the cuff on the perfusion catheter. The perfusion rate is adjusted so that lobar arterial perfusion pressure approximates mean pressure in the main pulmonary artery. Left atrial pressure is measured with a 6-F double-lumen catheter passed transseptally into the vein draining the left lower lobe. The catheter tip is positioned so that the left atrial pressure port on the distal lumen is 1–2 cm into the lobar vein and the second catheter port is near the venoatrial junction.

Lobar arterial pressure can be elevated to a high steady-state level by the administration of Nω-Nitro-L-arginine, followed by an intralobar infusion of the stable prostaglandin/endoperoxide analog U-46619.

Evaluation

Dose–response curves after administration of graded doses of drugs, e.g., decrease of lobar arterial pressure after various doses of bradykinin, are established. The effects of antagonists, e.g., HOE 140, can be studied.

Modifications of the Method

Liu et al. (1992) used a blood-perfused rat lung preparation to study pulmonary vasoconstriction or endothelium-dependent relaxation.

Byron et al. (1986) studied the deposition and airway-to-perfusate transfer of disodium fluorescein from 3- to 4-μm solid aerosols in an isolated perfused lung preparation of rats.

Mor et al. (1990) determined angiotensin-converting enzyme activity in the isolated perfused **guinea pig** lung.

Franks et al. (1990) used in Beagle **dogs** a single-breath technique employing freon-22 as the soluble marker gas simultaneously with measurement of aortic blood flow by an electromagnetic flowmeter.

Tanaka et al. (1992) measured lung water content in dogs with acute pulmonary hypertension induced by injection of glass beads.

Drake et al. (1978) studied filtration characteristics of the exchange vessels in isolated dog lung by calculating the volume conductance with the use of different components of the weight-gain curve following changes in capillary pressure.

Heaton et al. (1995) studied the effects of human adrenomedullin on the pulmonary vascular bed of isolated, blood-perfused rat lung.

DeWitt et al. (1994b) and Lippton et al. (1994) investigated the effects of adrenomedullin in the pulmonary and systemic vascular bed of the **cat**.

Nossaman et al. (1995) compared the effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat.

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Effect on Contractile Force of Ischemic Muscle

Purpose and Rationale

The skeletal muscle is stimulated until it reaches its maximal force of contraction. This means that the muscle is forced to function at a level near exhaustion and has to use maximally the substrate supplied by the circulation. Then, the femoral artery is occluded, leading to underperfusion and a subsequent lack of substrate. As a consequence, the muscle's force of contraction decreases rapidly. Measuring the change in contractile force caused by drug administration reveals a drug's ability to restore ischemic muscle functions. In the following

procedure, the drugs are tested for their effect on the force of contraction of the ischemic skeletal muscle.

Procedure

Male Wistar rats weighing 400–450 g are anesthetized by intraperitoneal administration of pentobarbital (Nembutal) (35 mg/kg). A tracheal tube is placed to assist the rat's ventilation. The left carotid artery is cannulated for blood pressure recording and the left jugular vein is cannulated for the i.v. administration of test drugs. An incision is made to the skin of the right pelvic limb distal to the groin and the skeletal muscle is exposed down to the ankle. The skin is carefully trimmed away from the muscle to assure that contractions cannot be impaired by retraction of the skin. The major nerve supply is severed and a small length of the descending branch of the femoral artery is prepared free. The freely hanging muscle is attached to the force transducer (range 0–500 g, Z 6, Rhema, Germany) and a resting tension of 50 g is placed on the muscle. To prevent dehydration, the skin is left attached to the muscle and the muscle is kept moist by the continuous drip of a 0.9 % NaCl solution.

After these preparations, the rat is allowed to recover at least 30 min. Two needle electrodes are inserted into the muscle. Square impulses of 40 ms are generated with Stimulator 1 (Hugo Sachs Elektronik, Freiburg, Germany). The muscle is stimulated with a frequency of about 80 contractions per minute. The amplitude is increased gradually up to the muscle's maximal contractile force (usually between 2.0 and 3 mA). Following stabilization, the femoral artery is occluded with a clip for 5 min and subsequently reopened. After at least 15 min, test drugs are administered by intravenous infusion (0.075 ml/min) for 10 min. Five minutes after starting drug infusion, the artery is clamped again (for 5 min), while drug infusion is still going on. The force of contraction is continuously recorded. After declamping the artery, the rat is allowed to recover for at least 30 min before the whole procedure is repeated with another test drug. In this way, 3 different compounds can be tested in the same animal.

Standard compound:

- Pentoxifylline

Evaluation

The following parameters are measured:

- The percent inhibition of contractile force before drug administration (artery being clamped)
- The percent inhibition of contractile force after drug administration (artery being clamped)

The percent increase in contractile force after drug administration is calculated.

Critical Assessment of the Method

An attempt is made to measure not only the effects of the drug on vasculature tonus but also on muscle metabolism.

Modifications of the Method

Weselcouch and Demusz (1990) studied drug effects in the ischemic hind limbs of ferrets. The hind limb was stimulated to contract isometrically via supramaximal electrical stimulation of the sciatic nerve. Ischemia was induced by partial occlusion of the abdominal aorta. Pentoxifylline attenuated the loss of function in a dose-related manner.

Okyayuz-Baklouti et al. (1992) studied the functional, histomorphological, and biochemical changes in atrophying skeletal muscle using a novel immobilization model in the rat.

Le Tallec et al. (1996) reported the effects of dimethylformamide on in vivo fatigue and metabolism in rat skeletal muscle measured by 31P nuclear magnetic resonance spectroscopy.

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Effect on Perfusion of Rabbit Ear (Pissemski Method)

Purpose and Rationale

The procedure was described as early as 1914 by Pissemski, based on experiments of Krawkow (1913) in fish gills. It can be used to elucidate vasoactive properties (both vasoconstrictive and vasodilating) of compounds. The isolated rabbit's ear is used to determine the effect of test compounds on its perfusion rate. Administration of norfenephrine induces vasoconstriction leading to a decrease in perfusion rate. A compound with vasodilatory properties will inhibit the norfenephrine-induced fall in perfusion rate, whereas a vasoconstrictor will potentiate this effect.

Procedure

A rabbit of either sex weighing 1.5–3 kg is sacrificed by CO_2 narcosis and its ears are severed immediately. The ear is placed on a glass disk and the posterior auricular artery is exposed and cannulated. The cannula is connected to a tubing with a T-branch allowing the infusion of different solutions. Ringer's solution, kept at room temperature, is infused under 40-cm water column pressure via the cannula. The perfusion flow volume is recorded using a time-ordinate recorder and a CONDON tipper or a photoelectric drop counter.

Prior to drug administration, the pH of the Ringer's solution (containing test compound) must be determined. If the pH is greater than 8.5 or smaller than 6.5, it should be adjusted by adding a diluted NaOH or HCl solution.

Testing for Vasodilatory (Norfenephrine Antagonistic) Activity

Norfenephrine is infused at a concentration of 0.5 μg/ml until the maximal contraction is reached. The test compound is prepared in Ringer's solution at a concentration of 100 μg/ml. A volume of 30 ml is infused via the cannula over a 15-min period under constant pressure. The change of perfusion rate is determined. If there is a positive response (increase in perfusion rate), the test may be repeated using lower concentrations. If there is a negative response (further decrease in perfusion rate), the compound can be tested for vasoconstrictive activity.

Testing for Vasoconstrictive Activity

This test is repeated as described above without administration of norfenephrine.

Standard compounds:

- As vasodilator
  - Dihydralazine
  - Theophylline
  - Pentoxifylline 100 μg/ml
- As vasoconstrictor
  - Norfenephrine (Novadral)

Evaluation

Testing for Vasodilatory Activity

The perfusion rate of the ear vessel is determined during the course of the test:

R = perfusion rate of vehicle perfused vessel

RN = perfusion rate of norfenephrine constricted vessel

RNP = perfusion rate of norfenephrine constricted vessel following compound administration

The percent inhibition of norfenephrine-induced decrease in perfusion pressure is calculated using the following formula:

$$\%\,\text{inhibition} = \frac{(RN - RNP)}{R - RN} \times 100$$

Testing for Vasoconstrictive Activity

The normal perfusion rate (ear vessel perfused with Ringer's solution) is taken as 100 %. The percent inhibition of perfusion rate following compound administration is determined.

Modifications of the Method

Schlossmann (1927) used the isolated rabbit ear preparation according to Pissemski for determination of the adrenaline content in blood.

De la Lande and Rand (1965) and de la Lande et al. (1967) described a method of perfusing the isolated central artery of the rabbit ear. Small segments of the artery, taken from the base of the ear, were perfused at a constant rate with Krebs solution at 37 °C. To enable drugs to be applied either to the intima or the adventitia, the artery was double cannulated so that the intraluminal and extraluminal perfusion media did not mix. Constrictor responses were measured by the maximum rise in perfusion pressure.

Steinsland et al. (1973) studied the inhibition of adrenergic transmission by parasympathetico-mimetics in the isolated central ear artery of the rabbit. Perfusion was performed at a constant flow rate with Krebs solution and perfusion pressure was recorded with a transducer.

Allen et al. (1973) incubated isolated segments of rabbit ear artery with (^{3}H)-(−)-noradrenaline and measured the amount of tritium released into the luminal perfusate and into the extraluminal superfusate.

Budai et al. (1990) used isolated proximal 3- to 4-cm segments of the rabbit ear artery or rat tail artery in a low-volume perfusion–superfusion system for measurement of transmitter release from blood vessels in vitro.

Miyahara et al. (1993) used arterial ring rabbit ear arteries in vitro which were contracted by perivascular nerve stimulation or 5×10^{-7} noradrenaline or high-potassium (29.6-mM) solution. High doses of dexamethasone or clobetasol-17-propionate decreased the amplitude of contractions. Furthermore, the authors performed in vivo experiments in albino rabbits, whereby the fur was removed from the distal parts of the ear by applying a depilatory cream at least 24 h before the experiments. The apical regions of the ear were then stripped with adhesive tape seven times to remove the keratinous epidermal layer. The rabbit was anesthetized and the experimental parts of the ear were placed under a high-resolution magnifying camera and immobilized using bilayer adhesive tapes. The vascular reactions induced by topical application of corticosteroids were recorded chronically using videotapes.

Aoki and Chiba (1993) described a method for separate intraluminal and extraluminal perfusion of the **basilar artery** in dogs. A polyethylene roof was designed to cover the canine basilar artery so that an extraluminal superfusion stream could pass over the artery that simultaneously received an intraluminal perfusion.

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Effect on Venous Tonus In Situ in Dogs

Purpose and Rationale

Veins can be classified into two groups: those that respond and those that do not respond to epinephrine, acetylcholine, and sympathetic nerve stimulation. As studies in dogs have shown (Rice et al. 1966), the reactive veins have a considerable amount of smooth muscle, whereas the nonreactive ones lack any appreciable amount of smooth muscle fibers in the tunica media. A special preparation allows the registration of localized venous vasoconstriction.

Procedure

Dogs weighing 20–30 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The trachea is cannulated and the dog ventilated with a respiration pump. The femoral vein is cannulated for systemic injections. After administration of 5 mg/kg heparin sodium i.v., the saphenous vein and the femoral artery are cannulated. The venous cannula is placed approximately 1 cm distal to its junction with another vein. After the non-perfused branch of the junction is ligated, constant blood flow is maintained from the femoral artery by using a Sigmamotor pump. The flow is adjusted so that a normal physiologic pressure in the vein is maintained. Perfusion pressure is measured between the pump and the vein so that any changes in pressure reflect changes in venous resistance. The peak changes in perfusion pressure are used to measure pressure changes from recorded data. The blood flow is maintained on a constant level. Therefore, changes in pressure must reflect changes in resistance. Pressure is recorded with a polygraph using a Statham pressure transducer (P23AA). In addition to recording perfusion pressure, venous pressure is measured

at two additional points along the vein. In order to record venous pressure centrally from the site of perfusion, the shaft of a 27-gauge needle is placed into the end of a 10-cm piece of a thin Silastic tubing. At the other end, a 27-gauge needle is inserted and attached to a Statham pressure transducer (P23B). Pressure is recorded on a polygraph. One needle is inserted into the vein just proximal of the junction of the two veins. The second needle is placed into the vein so that the distance between the tip of the perfusion cannula and the first needle is the same as the distance between the two needles. In this way pressure decreases across the junction and an adjacent segment can be measured simultaneously. Injections of test compounds are made into the cannula between the pump and the saphenous vein. Changes in pressure measured by the three transducers are recorded. As standard, doses of 0.1–1.0 μg norepinephrine are injected.

Evaluation

Responses to test drugs are measured in mm Hg and calculated as percentage of response to norepinephrine.

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Arterial Aneurysms

General Considerations

Abdominal aortic aneurysms represent a life-threatening condition characterized by chronic inflammation, destructive remodeling of the extracellular matrix, and segmental dilatation of the aortic wall (Dobrin 1989; Ernst 1993). Several authors described animal models of aneurysms (Carrell et al. 1999; Dobrin 1999; Daugherty and Cassis 2004). Most studies were performed in mice. Abdominal aortic aneurysms are evoked by genetically defined defects in extracellular matrix maturation, increased degradation of elastin and collagen, aberrant cholesterol homeostasis, or enhanced production of angiotensin peptides.

The **blotchy mouse** has an X-linked trait that leads to aortic aneurysms and subsequent fatal rupture in nearly all affected male mice (Brophy et al. 1988; Reilly et al. 1990).

Maki et al. (2002) showed that inactivation of the **lysyl oxidase gene Lox** leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice.

Aneurysm development has been noted in a number of mice with deficiencies in the components of the matrix metalloproteinase system (Silence et al. 2001, 2002).

The use of mice in atherosclerosis research was escalated by the development of mice that are deficient in either apoE or LDL receptors susceptible to aneurysm development (Ishibashi et al. 1994; Prescott et al. 1999).

Accelerated atherosclerosis, aortic aneurysms formation, and ischemic heart disease were found in apolipoprotein E and endothelial nitric oxide synthase double-knockout mice (Kuhlencordt et al. 2001). ACE inhibition reduces some symptoms of vascular pathology in apoE and eNOS compound-deficient mice (Knowles et al. 2000). Mice with deficiency of the LDL receptor-related protein (LRP) showed pathological changes in the aortic arch and abdominal aorta with substantial lengthening, dilatation, thickening, and large aneurysms (Herz and Strickland 2001; Boucher et al. 2003).

Salt-sensitive aortic aneurysms and rupture in hypertensive transgenic mice that overproduce angiotensin II were described by Nishijo et al. (1998).

The infusion of elastase into the infrarenal segment has been used as model for abdominal aorta aneurysms in rats (Anidjar et al. 1990) and mice (Pyo et al. 2000).

Lee et al. (2001) described abdominal aortic aneurysms in mice lacking expression of inducible nitric oxide synthase.

Periarterial application of calcium chloride was used to induce aneurysms of the **rabbit** common carotid artery (Gertz et al. 1988) and in the rabbit aorta (Freestone et al. 1997).

This technique has been used in **mice** by Chiou et al. (2001) and Longo et al. (2002).

Modifications of the Method

Nomoto et al. (2003) described the effects of two inhibitors of renin–angiotensin system on attenuation of postoperative remodeling after **left ventricular aneurysm** repair in rats.

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Angiotensin II-Induced Aortic Aneurysm in Mice

Purpose and Rationale

Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice (Daugherty et al. 2000, 2001;Knowles et al. 2000; Wang et al. 2001; Manning et al. 2002, 2003; Deng et al. 2003; Martin-McNulty et al. 2003; Saraff et al. 2003).

Wang et al. (2005) reported that a Rho-kinase inhibitor attenuated angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis.

Procedure

Animal Preparation

Osmotic minipumps (model 2004, Alzet) containing either PBS or Ang II (1.44 mg/kg per day) in PBS were implanted subcutaneously in 6-month-old apoE-KO male mice (Jackson Laboratories). Two days before saline and Ang II infusion, mice were provided with water (Ang II group) or water containing test compound at a concentration of 0.5 mg/ml (low-dose group) or 1.0 mg/ml (high-dose group). Age-matched apoE-KO mice without any treatment were used as naïve controls. At the end of the 30-day treatment period, mice were euthanized, and the hearts were perfused with DEPC in saline. The arterial tree was rapidly dissected from fat and connective tissue and snap frozen in liquid nitrogen.

Morphological Examination

Quantification of Aneurysm Formation

After the aorta was dissected free from the surrounding connective tissue, images were recorded with a digital camera and later used to measure the outer diameter of the suprarenal aorta at the midpoint between the diaphragm and right renal artery. A commonly used clinic standard to diagnose abdominal aortic aneurysm is an increase in aortic diameter of ≈ 50 % (Johnston et al. 1991). The average diameter of the normal suprarenal aorta in naïve control mice is 0.8 mm. A threshold of 1.22 mm was set as evidence of an incidence of aneurysm formation. Aneurysm severity was assessed with a scoring system described by Daugherty et al. (2001): type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from naïve apoE-KO mice without Ang II treatment); type I, a dilated lumen with no thrombus; type II, remodeled tissue often containing thrombus; type III, a pronounced

bulbous form of type II containing thrombus; and type IV, multiple aneurysms containing thrombus. To analyze this measurement semiquantitatively, the numerical score assigned to the type of aneurysm for each animal in a group was averaged to generate a pathology score for statistical comparison.

Quantification of Atherosclerotic Lesion Area

The left and right carotid arteries and the aortic arch were dissected, excised, opened longitudinally, and pinned down on wax-coated Petri dishes. Atherosclerotic lesions were visible without staining. Images of the open luminal surface of the vessels were captured with a digital camera (Sony) mounted on a dissecting microscope. The atherosclerotic lesion area was quantified by use of the C-Simple system (Compix) and expressed as a percentage of the total luminal surface area (Wang et al. 2000, 2001; Martin-McNulty et al. 2003).

Histology and Immunohistochemical Staining

Two representative suprarenal aortas from each group were fixed in formalin, embedded in paraffin, and cut into 5-µm-thick sections. The adjacent sections were stained with hematoxylin and eosin or by the immunohistochemical method. To identify macrophages in the aortic wall, a purified rat anti-mouse Mac-3 monoclonal antibody (BD Pharmingen) was used for immunochemistry staining (PhenoPath Laboratories). To localize cells undergoing nuclear DNA fragmentation, in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using an in situ apoptosis detection kit (Roche Biochemicals) (Song et al. 2000; Feng et al. 2002). Paraffin sections were deparaffinized and rehydrated. Sections were then washed with PBS and incubated with proteinase K (20 µg/ml) for 20 min. Endogenous peroxidase was inactivated with 3 % hydrogen peroxide in methanol at room temperature. TdT, which catalyzes a template-independent addition of deoxyribonucleotide to 3-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragment in situ. The TUNEL signal was then detected with an anti-digoxigenin antibody conjugated with peroxidase and developed with diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and cleared before coverslips were placed. Both positive and negative control slides were processed at the same time in each experiment. The presence of apoptotic cells was scored as nuclear staining, with a distinctive morphological appearance associated with cell shrinkage and chromatin condensation.

Evaluation

Results are presented as mean ± SE for the number of animals used. Statistical comparison for the incidence of abdominal aortic aneurysms was performed by χ^2 analysis. Multiple comparison of mean values was performed by ANOVA, followed by a subsequent Student–Newman–Keuls test for repeated measures. Differences were considered statistically significant at a value of $P < 0.05$.

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Angiogenesis and Antiangiogenesis

General Considerations

Regulation of new blood vessel formation, angiogenesis, is precisely programmed throughout the lifetime of vertebrates. Besides the role of angiogenesis in normal function, it is an essential component of disease processes, including tumor growth, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Folkman and Shing 1992). Multiple factors that stimulate angiogenesis either directly or indirectly have been described, including the fibroblast growth factor family (Esch et al. 1985), vascular endothelial growth factor (Leung et al. 1990; Thomas 1996; Ferrara and Davis-Smyth 1997), epidermal growth factor (Gospodarowicz et al. 1979), transforming growth factor-α and transforming growth factor-β (Schreiber et al. 1986; Yang and Moses 1990), tumor necrosis factor-α (Leibovich et al. 1987), angiogenin (Fett et al. 1985), CYR61, a product of a growth factor-inducible immediate early gene (Babic et al. 1998), etc.

The pharmacological inhibition of angiogenesis is of considerable interest in the development of new therapeutic modalities for the treatment of diseases such as diabetic retinopathy, atherosclerosis, hemangiomas, rheumatoid arthritis, and

cancer, in which pathological angiogenesis occurs (Ezekowitz et al. 1992; Folkman and Shing 1992; Fan and Brem 1992; O'Brien et al. 1994). Several natural inhibitors of angiogenesis were described, such as thrombospondin (Good et al. 1990); somatostatin (Barrie et al. (1993); angiostatin, isolated from a subclone of Lewis lung carcinoma (O'Reilly et al. 1994, 1996); endostatin, a 20 kDa angiogenesis inhibitor from a murine hemangioendothelioma which is a C-terminal fragment of collagen XVIII (O'Reilly et al. 1997; Dhanabal et al. 1999); and vasostatin (Pike et al. 1998).

Angiogenesis was studied ex vivo by culturing rat or mouse aortic rings in collagen gel (Zhu et al. 2003). Unlike rat aorta explants, unstimulated mouse aortic rings were unable to spontaneously produce an angiogenic response under serum-free conditions. They, however, responded to basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), generating networks of branching neovessels.

Couffinhal et al. (1998) published a mouse model of angiogenesis. The femoral artery of one hind limb was ligated and excised. Laser-Doppler perfusion imaging was employed to document the consequent reduction in hind-limb blood flow, which typically persisted for up to 7 days. Neovascularization was shown to develop in association with augmented expression of VEGF mRNA and protein from skeletal myocytes as well as endothelial cells from the ischemic hind limb.

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Endothelial Cell Proliferation

Purpose and Rationale

Human umbilical vein endothelial cells (HUVEC) were used by various authors to study endothelial cell proliferation (Bussolino et al. 1992; Benelli et al. 1995; Danesi et al. 1997; Hu 1998; Iurlaro et al. 1998; Vacca et al. 1999; Xin et al. 1999).

Procedure

The HUV-EC-C human endothelial cells (American Type Culture Collection, Rockville, MD) are cultured at 37 °C and 5%CO_2 in 90 % Ham's F12K, 10%fetal bovine serum, 30 μg/ml endothelial cell growth factor, 100 μg/ml heparin, and 4-mM L-glutamine. The effect of test compound on HUV-EC-C cell proliferation is evaluated on 3×10^3 cells/well in 24-well plates. After 24 h, the test compound in various concentrations of the vehicle are added, and plates are incubated for 72 h. Cells are then harvested with trypsin/EDTA and counted by a hemocytometer.

Evaluation

Results are expressed as number of cells in vehicle- and compound-treated cultures and are the mean of three separate experiments ± SE.

Modifications of the Method

In addition to human umbilical veil endothelial cells, Pike et al. (1998) used fetal bovine heart endothelial cells and measured DNA synthesis by [^{3}H]thymidine deoxyribose uptake.

Oikawa et al. (1991) used vascular cells from bovine carotid arteries and tested cell proliferation in a collagen gel and cell migration in a Boyden chamber.

Bovine capillary endothelial cells were used by Folkman et al. (1979), Clapp et al. (1993), O'Reilly et al. (1997), and Cao et al. (1999).

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Chorioallantoic Membrane Assay

Purpose and Rationale

The chick chorioallantoic membrane assay, originally described by Auerbach et al. (1974), has been used with some modifications by several authors to test angiogenesis and inhibition of angiogenesis, e.g., by Taylor and Folkman (1982), Crum et al. (1985), Vu et al. (1985), McNatt et al. (1992, 1999), Barrie et al. (1993), Clapp et al. (1993), Gagliardi and Collins (1993), Benelli et al. (1995), Ribatti et al. (1995), Klauber et al. (1996), Oikawa and Shimamura (1996), Danesi et al. (1997), O'Reilly et al. (1997), Iurlaro et al. (1998), Cao et al. (1999), and Vacca et al. (1999).

Procedure

Fertilized White Leghorn chicken eggs are incubated at 37 °C at constant humidity. On incubation day 3, a square window is opened in the shell and 2–3 ml of albumen is removed to allow detachment of the developing chorioallantoic

membrane (CAM). The window is sealed with a glass and the eggs are returned to the incubator. On day 8, 1-mm^3 gelatin sponge loaded with 3-µl phosphate-buffered saline alone as the negative control or containing 3 µg (1 mg/ml) of the angiogenic recombinant basic fibroblast growth factor alone as positive control, or together with various doses of test compound, is implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAMs are examined daily until day 12, when the angiogenic response peaks. On day 12, blood vessels entering the sponge within the focal plane of the CAM are recognized microscopically, counted by two observers in a double-blind fashion under a Zeiss SR stereomicroscope and photographed in ovo with the MC63 camera system (Zeiss, Oberkochen, Germany). To better highlight vessels, the CAM are injected into a large allantoic vein with India ink solution, fixed in Serra's fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate. On day 12, after microscopic counting, the embryos and their membranes are fixed in ovo in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions are removed, embedded in paraffin, sectioned at 8 pm along a plane parallel to the CAM surface, and stained with a 0.5 % aqueous solution of toluidine blue.

Evaluation

Angiogenesis is measured by a planimetric point count method (Ribatti et al. 1999): four to six 250× magnification fields covering almost the whole of every third section within 30 serial slides of each sponge per sample are analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm^2. Only transversely sectioned microvessels, i.e., capillaries and venules with or without a 3- to 10-µm lumen occupying the intersection points, are counted and calculated as the mean ±1 SD per section, per CAM, and groups of CAM. Statistical significance of differences is calculated by comparing the data from each experiment to their controls using Student's *t*-test.

Modifications of the Method

Oh et al. (1997) studied the lymphatics of differentiated avian chorioallantoic membrane using microinjection of Mercox resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α-smooth muscle actin, and in situ hybridization with vascular endothelial growth factor VEGFR-2 and VEGFR-3 probes.

Using the chick chorioallantoic membrane assay, Giannopoulou et al. (2003) showed that amifostine, an inorganic thiophosphate-cytoprotective agent, inhibits angiogenesis in vivo.

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Cornea Neovascularization

Purpose and Rationale

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (Gimbrone et al. 1974; Crum et al. 1985; BenEzra et al. 1987, 1997; Klauber et al. 1996; McNatt et al. 1999; Joussen et al. 1999).

Procedure

New Zealand white rabbits are anesthetized for surgery and quantification of newly developed blood vessels with 5 mg/kg xylazine hydrochloride and 35 mg/kg ketamine hydrochloride i.m. Corneal blood vessels are induced by basic fibroblast growth factor which is applied in carrier pellets. These pellets are produced by dispersing 50 µl of 2 % methylcellulose containing 500-ng human recombinant basic fibroblast growth factor diluted in 10-µl phosphate-buffered saline in plastic rods with a diameter of 4 mm. Dried pellets are folded twice and implanted intrastromally in the 12 o'clock position into a corneal tunnel. This tunnel is created by a central cut of approximately 50 % depth and extended into the peripheral cornea to a point 2.0 mm away from the limbus. Following implantation, the central entrance of the tunnel is closed with a single 10–0 nylon suture in order to ensure that the tear film does not dissolve the

pellet and uncontrolled liberation of the growth factor is prevented.

The test substance is dissolved in a viscous gel containing 0.002 % polyacrylic acid, 0.04 % sorbitol, and 0.001 % cetrimide in a watery base. The eyes are treated once daily with 0.1 ml of this gel which is applied in the lower conjunctiva sac. The eyes are closed for several seconds to avoid loss of the substance. Each animal's contralateral eye receives gel without test substance and serves as control. Control animals receive the viscous gel without test substance.

Animals are observed daily under an operating microscope, and vascular growth is documented on days 6, 9, 12, and 16 after surgery. The number of blood vessels, their length, and the dimension of the vascularized area are quantified with a caliper under the operating microscope as well as on standardized photographs. On every observation day the corneas are stained with fluorescein in order to show epithelial irregularities due to the topical treatment.

Evaluation

Differences between treated eyes and controls are tested for significance using unpaired Student's *t*-test.

Modifications of the Method

Damms et al. (1997) characterized the neovascularization that follows the intracorneal injection of bovine albumin in rabbits as a model of angiogenesis. New Zealand white rabbits received intracorneal injections of phosphate-buffered saline with and without various amounts of bovine albumin. The rabbits were co-sensitized or pre-sensitized by intramuscular bovine albumin. The corneal response was quantified by ranking photographs taken periodically after the injection.

Babic et al. (1998) tested an angiogenesis promoter in the corneal pocket angiogenesis assay in **rats**.

Xin et al. (1999) studied inhibitors of angiogenesis in the corneal angiogenesis assay in rats. A 1.5-mm incision was made approximately 1 mm from the center of the cornea in anesthetized Sprague–Dawley rats. Using a curved spatula, the incision was bluntly dissected through the stroma toward the outer canthus of the eye. A hydron pellet (2 × 20 mm) containing 200-ng vascular endothelial growth factor and 100-ng sucralfate was inserted into the base of the pocket.

Foschi et al. (1994), Benelli et al. (1995), and Danesi et al. (1997) studied neovascularization of rat cornea induced by **chemical injury**. Both eyes of ether-anesthetized rats were cauterized by applying a $AgNO_3/KNO_3$ (1:1, w/w) applicator to the surface of the cornea eccentrically at a point approximately 2 mm from the corneoscleral limbus. Rats were treated four times daily for 6 days with eyedrops. The eyes were examined by slit-lamp microscopy daily for 6 days to evaluate the growth of the vessels. On the 6th day after cauterization, the rats were anesthetized and the upper body perfused through a cannula inserted in the ascending aorta with Ringer's solution until the normal pink color of the fundi disappeared and then with a mixture of 10 % India ink/6%gelatin in Ringer's solution. The eyes were enucleated and placed in 4 % formaldehyde. The cornea and a 1-mm rim of adjacent scleral tissue were dissected from the rest of the globe and three full-thickness peripheral radial cuts were made to allow flattening of the cornea. The corneas were then placed on a glass slide in mounting media, magnified, and photographed. The area occupied by blood vessels was calculated and the area vascularization of drug-treated animals was compared to that of control rats.

Kenyon et al. (1996) and Cao et al. (1999) performed the corneal micropocket assay in **mice**.

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Rat Subcutaneous Air Sac Model

Purpose and Rationale

Lichtenberg et al. (1997, 1999) recommended the subcutaneous air sac model in rats as a simple method for in vivo screening of antiangiogenesis. Subcutaneous injection of air in the rat results in the formation of an air pouch. If air pouches are kept inflated by repeated injections of air, they develop into structures with the features of synovial lining (Edwards et al. 1981). The subcutaneous air pouch appears more like a bursal cavity than a synovial joint (Kowanko et al. 1986), and after 8–10 days the cells of the air sac appear as a transparent membrane on which the formation of new vessels can be studied.

Procedure

Under anesthesia 10–15 ml of air is introduced dorsally to female Sprague–Dawley rats weighing 150–180 g by subcutaneous injections using a 25-gauge needle to produce an air sac located approximately 4–5 cm behind the head of the animal. The air sacs are reinflated every 4th day. The wall of the air sac becomes progressively thicker with time and after approximately 10 days a sufficient lining of cells has been established. For sponge implantation, the animals are anesthetized again. A 1.5-cm incision is made through the clipped skin covering the air sac, and blunt dissection is used to open a 2-cm deep cavity toward the cranial base of the air sac by careful separation of the skin from the membrane. A cellulose implant (Spontex sponge) with a diameter of 8 mm is carefully pressed into the cavity of the membrane away from the incision site and the incision closed by sutures. The animals are treated for 10 days with various doses of

test compound in a volume of 10 ml/kg or vehicle. The subcutaneous injection is made under light CO_2/O_2 anesthesia into the hind leg 5–7 cm away from the air sac. This injection site is chosen to eliminate any risk of inducing irritative side effects on the membrane. After 10 days of treatment, the animals are sacrificed after having received 20 min before an injection of 1 μCi of ^{125}I-labeled immunoglobulin via the tail vein. The overlying skin of the air sac is removed to expose the transparent membrane. The extent of vascular proliferation is scored in situ:

1+: Slight background vascularization.
2+: Few new vessels reach the sponge.
3+: Many new vessels reach and penetrate the implant.
4+: Very intense formation of new vessels which reach and penetrate the implant.

Following in situ scoring, the implant and the membrane from each animal are placed in the same plastic vial containing 10 % formalin and the radioactivity is measured in a γ-counter. The implants are examined microscopically after staining with hematoxylin and eosin.

Evaluation

The extent of vascular proliferation scored in situ is compared between vehicle and treated animals by the Wilcoxon test. The angiogenic response measured by ^{125}I activity in cpm is subjected to analysis of variance followed by Dunnett's *t*-test to compare each dose with the vehicle. The cpm's are log transformed to obtain variance homogeneity. The correlation between in situ scores and cpm is estimated by Spearman's rank correlation coefficient after ranking cpm values.

Modifications of the Method

In a further study, Lichtenberg et al. (1999) inoculated vascular endothelial growth factor producing tumor cells subcutaneously directly on the membrane, and the formation of vessels was measured 8 days later. Furthermore, slow-release pellets containing angiogenic factors, basic fibroblast growth factor, or vascular endothelial growth factor were implanted on the subcutaneous membrane.

Nakamura et al. (1999) studied suppression of angiogenesis induced by S-180 mouse tumor cells in the dorsal air sac assay in **mice**.

Funahashi et al. (1999) developed a mouse dorsal air sac model for quantifying in vivo tumor-induced angiogenesis which is determined by measuring the blood volume in an area of the skin held in contact with a tumor cell-containing chamber, using ^{51}Cr-labeled red blood cells.

Schreiber et al. (1986) described the **hamster cheek pouch assay** for testing angiogenic/antiangiogenic activity.

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Mesenteric Window Angiogenesis Model

Purpose and Rationale

Norrby et al. (1986, 1990, 1995) described the mesenteric window assay in rats for quantitative measurement of induction and inhibition of angiogenesis. The tissue being used is the membranous, "window"-like parts of the mesentery which is normally vascularized and appears to lack significant physiologic angiogenesis. Since the mesenteric window natively measures only 5–10 μm in thickness, the vasculature is virtually two dimensional. Due to the structural and metabolic simplicity of the test tissue, the mesenteric window microvasculature is regarded as an ideal test system for establishing the functional influences of defined factors (Zweifach 1973).

Procedure

Angiogenesis is induced by i.p. injection of the mast cell secretagogue compound 48/80 twice daily for 4.5 days to male Sprague–Dawley rats weighing about 225 g. Test compounds or saline are injected s.c. 1 h before each injection of compound 48/80.

Angiogenesis is quantified by microscopically counting the number of vessel profiles per unit length of the mesenteric window in 4 microtome sections per specimen, cut perpendicularly to the surface, from the central part of the window. This reflects the degree of branching, the degree of tortuosity, and the degree of spatial expansion of the vasculature. Four specimens per animal are analyzed.

Four mesenteric window specimens are spread, fixed on objective slides, and stained with toluidine blue to measure the relative vascularized area. Three randomly selected vascular view fields per mesenteric window spread are analyzed for microvascular length per unit area of vascularized tissue. The total microvascular length is computed from the vascularized area of each animal multiplied by the mean microvascular length for the corresponding treatment group.

Evaluation

The nonparametric two-tailed Mann–Whitney U rank sum test for unpaired observations is used for statistical analysis.

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Quantification of Vascular Endothelial Growth Factor-C

Purpose and Rationale

Vascular endothelial growth factor (VEGF) has achieved considerable therapeutic interest (Clauss 1998; Enholm et al. 1998; Ferrara et al. 2004).

Weich et al. (2004) described an ELISA for quantification of vascular endothelial growth factor-C.

Procedure

Materials and Methods

The fully processed rat protein dNdC-VEGF-C and dNdC-VEGF-D were produced in insect cells and purified from supernatants (Kirkin et al. 2001). Human dNdC-VEGF-C and soluble VEGFR-3 production was achieved according to (Joukov et al. 1997; Hornig et al. 1999). A polyclonal antibody against rat VEGF-C was developed in rabbits (antibody 4080;

BioGenes Berlin). A total amount of 1.2-mg rat VEGF-C (containing a C-terminal 6His-tag) was used for immunization of two New Zealand white rabbits. After immunization with 0.1-mg protein, each rabbit was boosted on days 7, 14, 28, 56, and 84 with the same amount. The dilution of the serum for half-maximal titer was 1:10,000. The total IgG from rabbit serum was isolated using HiTrap Protein-A Sepharose columns (Amersham Bioscience, Freiburg).

Generations of Cell Lines, Serum-Free Cell Culture Supernatants, Lysates, and Tissue Sample Preparation

Experiments were conducted using three human (PC-3 cells, 293 cells, COLO 800 cells) and four rat (10AS, ARIP, BRL3A, MT-450) tumor cell lines and 1640 medium (Gibco-BRL, Bethesda, Md., USA). Stable transfected clones were selected and tested for VEGF-C expression and secretion using a polyclonal anti-VEGF-C antiserum.

The cells were grown to 80 % confluence in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Conditioned media was collected under low-serum (2 %) growth conditions. Tumor tissues were snap frozen in liquid nitrogen and homogenized in RIPA buffer (0.1 % SDS, 1 % IGEPAL CA-630, 0.5 % Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline).

VEGF-C Sandwich ELISA

The VEGF-C antibody 4080 was isolated from serum using HiTrap Protein-A Sepharose columns. Then, depletion of the anti-his antibody fraction was done by antigen-affinity purification using 10 mg of immobilized 6H-tagged TxnTb protein [Tryparedoxin (Txn) from *Trypanosoma brucei* (Tb)]. Antigen-affinity purification for the antibody 4080 was performed by immobilizing 1-mg rat VEGF-C on an NHS-activated HiTrap column (Amersham Bioscience, Freiburg). The development of a highly sensitive and specific sandwich ELISA for VEGF-C was done using standard methods. Rabbit IgG 4080 (10 μg/ml) was used for coating and the antigen-affinity purified and biotinylated antibody 4080 at 1 μg/ml was used as a detector antibody. Biotinylation of antibody 4080 was done with 6-mg IgG in 100-mM carbonate buffer, pH 8.5 at 3 mg/ml with using biotin-amidohexanoic acid NHS (Sigma, St. Louis, Mo., USA). The molecular ratio biotin/protein was 30:1. As a standard, human and rat dNdC-VEGF-C was used over a concentration range between 0.1 and 6.25 ng/ml. For visualization of the detector, streptavidin-enzyme conjugate was used (Endogen, Woburn, Mass., USA) followed by the addition of TMB (tetra-methyl-benzidine; Roche Mannheim, Germany).

Evaluation

After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 450 and 620 nm with an ELISA plate reader (Labsystems, Finland). Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicate. Samples were diluted at least 1:2 with sample diluent (BenderMedSystems, Vienna).

Modifications of the Method

Rissanen et al. (2003) found that VEGF-D is the strongest angiogenesis and lymphangiogenetic factor among VEGFs delivered into the skeletal muscle via adenoviruses.

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Inhibitors of Vascular Endothelial Growth Factor

Purpose and Rationale

Inhibitors of vascular endothelial growth factor have gained therapeutic interest (Whittles et al. 2002; Gingrich et al. 2003; Hamma-Kourbali et al. 2003; Verheul and Pinedo 2003; Fernandez et al. 2004; Roberts et al. 2004; Baka et al. 2006). In particular, VEGF-Trap, a soluble decoy receptor comprising portions of VEGF receptors 1 and 2, has been studied (Wulff et al. 2001; Holash et al. 2002; Kim et al. 2002; Hood and Cheresh 2003; Saishin et al. 2003; Fukusawa and Korc 2004; Fraser et al. 2005; Hu et al. 2005; Lau et al. 2005).

Byrne et al. (2003) found that vascular endothelial growth factor-Trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model.

Procedure

Cell Lines

Ascites fluid from athymic mice previously inoculated with OVCAR-3 cells was used. Cells from the SKOV-3 human cystadenocarcinoma cell line were grown in McCoy's 5a medium with 1.5-mM L-glutamine, penicillin, and streptomycin, supplemented with 10 % FCS. Cells were grown to confluence and harvested by trypsinization with 0.25 mg/ml trypsin/EDTA and suspended in PBS before inoculation into mice.

Animals

Athymic Balb c *nu/nu* mice were housed under pathogen-free conditions and fed autoclaved pellets and water.

Retroviral Constructs

The pLZR Phoenix vector was modified by the addition of an MCS followed by an internal ribosome entry sequence-GFP cassette (Rommel et al. 1999). Thus, genes subcloned into the MCS produce bicistronic constructs under the control of the viral 5′ long terminal repeat. The entire coding sequence of $mVEGF_{164}$ was inserted into the MCS for the construct used to transduce cells with VEGF, whereas the MCS was left empty for the GFP-only vector. Constructs were transfected into amphotropic packaging lines to produce infective virus using standard techniques (Grignani et al. 1998).

SKOV-3 Model

SKOV-3 cell lines were infected with amphotropic viruses encoding either $mVEGF_{164}$ and GFP or GFP only. Cells that were successfully transduced with the

retroviruses were collected by FACS using a Cytomation MoFlo (Fort Collins, Colo., USA) with fluorescence emission from GFP measured with a 530/540-nm band-pass filter. More than 50 % of the cells were GFP positive after infection, allowing $> 4.0 \times 10^5$ cells to be collected and used to establish cell lines. To verify viral transduction, cells were resorted several days later and found to be > 80 % positive for GFP expression. Cells were then expanded, aliquoted, and frozen. All experiments were performed with an aliquot expanded by four to five passages and tested for viability before injection.

In Vivo Adenoviral and SKOV-3 Studies

Adenoviral constructs were achieved according to Thurston et al. (2000). Adenoviral plaque-forming units (5.0×10^8) or 1.0×10^7 SKOV-3 cells were suspended in a volume of 300–400 µl of PBS or serum-free cell culture medium and injected i.p. into female nude mice. VEGF-Trap or control buffer was delivered twice weekly at 25 mg/kg via s.c. injection in a volume of 50–200 µl. Mice were assessed daily for general health and development of ascites and weighed at least twice weekly. Animals were sacrificed if they had lost > 10 % of body weight or had persistent ascites on three consecutive assessments. After sacrifice, ascites was removed with a sterile thin caliber plastic transfer pipette and quantified, and hematocrit was measured.

OVCAR-3 Model

OVCAR-3 cells obtained from ascites fluid were prepared (Hu et al. 2002). Briefly, 2×10^6 cells in 500 µl of RPMI 1640 were injected i.p. into athymic Balb/C nude (*nu/nu*) mice. Fourteen days after inoculation, blinded administration of VEGF-Trap or human Fc as control was initiated at a dose of 25 mg/kg. Injections were given s.c. in the nape of the neck using a 28.5-gauge needle and a 0.5-ml insulin syringe. Injections (0.05 ml) were administered twice weekly throughout the experimental period. Body weight and abdominal circumference were quantified twice weekly. In addition, animals were monitored daily for evidence of advanced disease (listlessness, extensive swelling of the abdominal cavity). At the end of the experiment, all remaining mice underwent euthanasia with CO_2 followed by cervical dislocation. The volume of ascites was measured, and tumors were excised and weighed. Immediately before sacrifice, mice received i.v. injection with FITC lycopersicon lectin (see below).

Tumor Vasculature

According to Holash et al. (2002) s.c. tumors were established. After small s.c. tumors became palpable (1 week after implantation), treatment with the VEGF-Trap was initiated. VEGF-Trap or an equivalent volume of vehicle was delivered twice weekly s.c. at the nape of the neck. Tumor vasculature was visualized by using antibodies to platelet–endothelial cell adhesion molecule for immunohistochemistry.

VEGF-Trap-treated OVCAR-3 tumor-bearing mice and control, untreated tumor-bearing mice were anesthetized by i.m. injection with ketamine (87 mg/kg) and xylazine (13 mg/kg), followed by i.v. injection with 100 µl of FITC lycopersicon lectin or 100 µg of Cy3 albumin (Jackson Immunology Research, West Grove, Pa., USA). Then 10 min later, mice were perfused through the ascending aorta with 4 % paraformaldehyde in PBS for 2 min. Tumors and control organs were extracted and placed in fixative for 1–2 h followed by immersion in 30 % sucrose/PBS overnight, embedded in OCT, cryostat sectioned, and viewed by fluorescence microscopy.

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General Considerations

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α- and β-Adrenoreceptor Binding

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α1-Adrenoceptor Binding

Purpose and Rationale

α1-Adrenoceptors are widely distributed and are activated either by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla.

Receptor activation mediates a variety of functions, including contraction of the smooth muscle, cardiac stimulation, cellular proliferation, and activation of hepatic gluconeogenesis and glycolysis. In the CNS, the activation of α1-adrenoceptors results in depolarization and increased neuronal firing rate. α1-Adrenergic receptors are members of the G protein-coupled receptor superfamily with three α1-adrenoceptor subtypes – α1A, α1B, and α1D.

The α-adrenoceptor population of plasma membranes from rat heart ventricles consists only of the α1-adrenoceptor subtype. A constant concentration of the radioligand ^{3}H-prazosin (0.2–0.3 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM to 1 mM) in the presence of plasma membranes from rat heart ventricles. If the test drug exhibits any affinity to α-adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug. The assay is used to evaluate the concentration binding characteristics of drugs at the α1-adrenoceptor (Morrow and Creese 1986; Minneman and Esbenshade 1994).

Procedure

Solutions

Preparation buffer A	
Tris–HCl	5 mM
$MgCl_2 \times 6H_2O$	1 mM
D(+)-sucrose	250 mM
pH 7.4	

(*continued*)

Preparation buffer B (= rinse buffer)	
Tris–HCl	50 mM
$MgCl_2 \times 6H_2O$	10 mM
pH 7.4	
Incubation buffer	
Tris–HCl	50 mM
$MgCl_2 \times 6H_2O$	10 mM
Ascorbic	Acid 1.6 mM
Catechol	0.3 mM
pH 7.4	
Radioligand:	
^{3}H-prazosin × HCl	
Specific activity	
0.37–1.11 TBq/mmol	
(10–30 Ci/mmol) (NEN)	

Tissue Preparation

Male Sprague–Dawley rats (200–300 g) are sacrificed by decapitation, and the dissected hearts are placed in ice-cold preparation buffer A. After removal of the atria, the ventricles (approx. 30 g from 40 rats) are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/20 ml preparation buffer A); the homogenate is filtered through gauze and centrifuged at 2,000 *g* (4 °C) for 10 min. The pellets are discarded; the supernatant is collected and centrifuged again at 40,000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml preparation buffer B, homogenized by Ultra-Turrax, and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in preparation buffer B, corresponding to 1 g ventricle wet weight/4 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at −77 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 *g* (4 °C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane

suspension with a protein content of 1.0–1.5 mg/ml. After homogenization by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

For each concentration, samples are prepared in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

Total binding:

- 50 µl ^{3}H-prazosin (12 concentrations, 5×10^{-11}–5×10^{-9} M)
- 50 µl incubation buffer

Nonspecific binding:

- 50 µl ^{3}H-prazosin (4 concentrations, 5×10^{-11}– 5×10^{-9} M)
- 50 µl phentolamine (10^{-5} M)

Competition Experiments

- 50 µl ^{3}H-prazosin (1 constant concentration, 2–3 $\times 10^{-10}$ M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1.0–1.5 mg protein/ml). The samples are incubated for 30 min in a shaking bath at 25 °C. The reaction is stopped by withdrawing the total incubation volume by rapid vacuum filtration over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding
- Nonspecific binding
- Specific binding = total binding − nonspecific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-prazosin versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D\,{}^{3}\mathrm{H} \times \mathrm{IC}^{50}}{K_D\,{}^{3}\mathrm{H} \times [{}^{3}\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which competes 50 % of specifically bound ^{3}H-prazosin in the competition experiment.

[^{3}H] = concentration of ^{3}H-prazosin in the competition experiment.

$K_D\,{}^{3}\mathrm{H}$ = dissociation constant of ^{3}H-prazosin, determined from the saturation experiment.

The K_i value of the test drug is the concentration, at which 50 % of the receptors are occupied by the test drug. The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Modification of the Method

Binding of ^{3}H-WB 4101 to α1-adrenergic receptors in brain is used to test hypotensive activity as a possible side effect of neuroleptic drugs. The test E.5.1.6 is described in the chapter on "► Neuroleptic Activity."

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α1-adrenoceptor similar to that found in the vas deferens. To overcome some limitations of conventional binding methods such as a low yield of receptors and changes in receptor environment, the tissue segment binding method has been developed (Muramatsu et al. 2005; Tanaka et al. 2004). Both groups discuss extensively advantages and limitations of the method.

Subtypes of the α1-Adrenoceptor

The first attempt to distinguish α1-adrenoceptors using radioligand binding method revealed existence of α1A and α1B subtypes. The decision to discriminate was based on the affinities of different agonists and antagonists, especially WB4101 and prazosin, and also on the ability of chlorethylclonidine to inactivate the α1B but not α1A subtype (Ahlquist 1948; Morrow and Creese 1986; Han et al. 1987, 1990; Johnson and Minneman 1987; Minneman et al. 1988; Bylund et al. 1994; Sayet et al. 1993; Endoh et al. 1992; García-Sáinz et al. 1992; García-Sáinz 1993; Ohmura et al. 1992; Regan and Cotecchia 1992; Satoh et al. 1992; Schwinn and Lomasney 1992; Veenstra et al. 1992; Aboud et al. 1993; Oshita et al. 1993; Vargas et al. 1993; Ruffolo et al. 1994; Minneman and Esbenshade 1994; Alexander et al. 2001).

Later, with the advance of molecular biology and cloning techniques, the three subtypes of α1-adrenoceptors have been cloned – α1B (cloned α1b), α1A (cloned α1c and previously designated by some authors α1a/c), and novel subtype with unique pharmacological properties α1D adrenoceptor (cloned α1d and previously designated by some authors α1a/d) (Cotecchia et al. 1988; Esbenshade et al. 1995; Hieble and Ruffolo 1997; Schwinn et al. 1990, 1995; Perez et al. 1991; Hirasawa et al. 1993; Forray et al. 1994).

Initial confusion in nomenclature was resolved with acceptance of three subtypes of α1-adrenoceptors – α1A, α1B, and α1D (Ford et al. 1994; Calzada and de Artiñano 2001; Muramatsu et al. 2008; Zhong and Minneman 1999; Bylund et al. 1994; 1998; Hieble and Ruffolo 1997; Alexander et al. 2001).

Binding of the radioligand [3H]-prazosin to the α1A-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat submaxillary glands (Michel et al. 1989).

Binding of the radioligand [3H]-prazosin to the α1B-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat livers (Adolfo et al. 1989).

According to Eltze and Boer (1992), the adrenoceptor agonist SDZ NVI 085 discriminates between α1A and α1B adrenoceptor subtypes in the vas deferens, kidney, and aorta of the rat and may therefore be used as a tool either to detect (rat vas deferens or kidney) or exclude (rat aorta) the functional involvement of "α1A-adrenoceptors in smooth muscle contraction."

Stam et al. (1998) found that (+)-cyclazocine, which behaves as a selective, high-affinity α1B-adrenoceptor ligand in binding experiments, did not show the profile of a α1B-adrenoceptor antagonist in functional tissues.

Decreased blood pressure response in mice deficient of the α1b-adrenergic receptor was found by Cavalli et al. (1997).

Kenny et al. (1995) used the contractile response of rat aorta to adrenaline after the application of various α1-adrenoceptor antagonists for characterization of a α1D-adrenoceptor.

Understanding the role of each α1-adrenoceptor subtypes and linking it to particular physiological function is clearly important. The main obstacles on this path are the partially overlapped tissue distribution of the subtypes α1-adrenoceptors and the lack of sufficiently subtype-selective agonists and antagonists (Docherty 2010; Perez 2007; Chen and Minneman 2005).

The animal models with genetically manipulated α1-adrenoceptors have been successfully used to identify receptor subtypes, their tissue distribution, subcellular localization, and involvement in a specific physiological function or drug effect (Philipp and Hein 2004; Koshimizu et al. 2002; Link et al. 1995; Rokosh and Simpson 2002; Tanoue et al. 2002a, b; O'Connell et al. 2003; Koch et al. 2000; Cavalli et al. 1997; Muramatsu et al. 2008; Hague et al. 2003).

Multiple studies demonstrate that the levels of α1-adrenoceptors in the heart determined by ligand binding are relatively constant across the most species, including mice, guinea pig, rabbit, pig, and cow (Steinfath et al. 1992a; Yang et al. 1998; Lin et al. 2001; O'Connell et al. 2003; Rokosh and Simpson 2002). The exception is rat's heart tissue, in which the α1-adrenoceptors are approximately tenfold higher (Steinfath et al. 1992a; Michel et al. 1994; Noguchi et al. 1995; Stewart et al. 1994).

α1-Adrenoceptor expression levels in the human heart (by ligand binding methods) are similar to mouse and other species but rat (Bristow et al. 1988; Steinfath et al. 1992b; Hwang et al. 1996; Jensen et al. 2009), suggesting that careful interpretation of the results from studies in rats is required and also that the mouse could be more appropriate model to investigate cardiac α1-adrenoceptor function than the rat (O'Connell et al. 2014).

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*α*2-Adrenoceptor Binding

Purpose and Rationale

*α*2-Adrenoceptors are widely distributed and are activated by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla or from some neurons in the CNS. The most extensively characterized action is the prejunctionally mediated inhibition of the release of neurotransmitters from many peripheral and central neurons. *α*2-Adrenoceptors are also present at postjunctional sites, where they mediate actions such as the smooth muscle contraction, platelet aggregation, and inhibition of insulin secretion. Activation of postsynaptic *α*2-adrenoceptors in the brain stem results in an inhibition of sympathetic outflow in the periphery.

Clonidine is a nonselective α2-adrenoceptor agonist and centrally acting antihypertensive agent, which lowers blood pressure mostly through reducing sympathetic tone by acting at the nucleus tractus solitarius in the brain stem (Kobinger and Walland 1967). Clonidine can also weakly activate α1-adrenoceptors; therefore, the evaluation of the effects of this compound in complex systems may be difficult (Minneman 1988; Bylund 1992).

The purpose of this assay is to assess the interaction of hypotensive agents with central

$\alpha 2$-receptors and determine possible clonidine-like mechanisms of action. Clonidine binding may also be relevant to the activity of other classes of drugs such as antidepressants that interact with $\alpha 2$-receptors.

Procedure

Reagents

1. Tris buffer pH 7.7
   (a) 57.2 g Tris–HCl q.s. to 1 liter (0.5 M Tris buffer, pH 7.7) 16.2 g Tris base.
   (b) Make a 1:10 dilution in distilled H_2O (0.05 M Tris buffer, pH 7.7).
2. Tris buffer containing physiological ions
   (a) Stock buffer.

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g

q.s to 100 ml in 0.5 M Tris buffer

   (b) Dilute 1:10 in distilled H_2O.

This yields 0.05 M Tris–HCl, pH 7.7, containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (1 mM).

3. [4-^{3}H]-Clonidine hydrochloride (20–30 Ci/mmol) is obtained from New England Nuclear.

   For IC_{50} determinations: ^{3}H-Clonidine is made up to a concentration of 120 nM and 50 µl is added to each tube (yielding a final concentration of 3 nM in the 2 ml volume assay).
4. Clonidine–HCl is obtained from Boehringer Ingelheim.

   A stock solution of 0.1 mM clonidine is made up to determine nonspecific binding. This yields a final concentration of 1 µM in the assay (20 µl to 2 ml).
5. Test compounds:

   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay, and higher or lower concentrations can be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation, and the cortical tissue is rapidly dissected. The tissue is homogenized in 50 volumes of 0.05 M Tris buffer pH 7.7 (buffer 1b) with the Brinkmann Polytron and centrifuged at 40,000 *g* for 15 min. The supernatant is discarded and final pellet rehomogenized in 50 volumes of buffer 2b. This tissue suspension is then stored on ice. The final tissue concentration is 10 mg/ml. Specific binding is 1 % of the total added ligand and 80 % of total bound ligand.

Assay

100 µl	0.5 M Tris – physiological salts pH 7.7 (buffer 2a)
830 µl	H_2O
20 µl	Vehicle (for total binding) or 0.1 mM clonidine (for nonspecific binding) or appropriate drug concentration.
50 µl	^{3}H-clonidine stock solution
1,000 µl	Tissue suspension

Tissue homogenates are incubated for 20 min at 25 °C with 3 nM ^{3}H-clonidine and varying drug concentrations and immediately filtered under reduced pressure on Whatman GF-B filters. The filters are washed with three five ml volumes of 0.05 M Tris buffer pH 7.7 and transferred to scintillation vials. Specific clonidine binding is defined as the difference between total bound radioactivity and that bound in the presence of 1 µM clonidine.

Evaluation

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

Modifications of the Method

Motulsky et al. (1980), along with Perry and U'Prichard (1981), described [^{3}H]yohimbine and its isomer [^{3}H]rauwolscine as a specific radioligand for brain α2-adrenergic receptors, with [^{3}H]yohimbine showing slightly greater α2A-receptor selectivity (Bylund 1988; Boyajian et al. 1987).

Cheung et al. (1982) and Broadhurst et al. (1988) used [^{3}H]rauwolscine and [^{3}H] yohimbine radioligand binding to rats cerebral and human platelet membranes to propose heterogeneity of α2-adrenoceptors.

Goldberg and Robertson (1983) reviewed yohimbine as a pharmacological probe for the study of the α2-adrenoceptor.

Bylund et al. (1988) used [^{3}H]-yohimbine and [^{3}H]-rauwolscine in five different tissues and cell lines containing only one subtype of α2-receptors to demonstrate distinct pharmacological profile for α-2A and α-2B adrenoceptors.

Blaxall et al. (1991) and Murphy and Bylund (1988) confirmed that OK (opossum kidney) cells and membranes from opossum kidney express a novel subtype of α2-adrenergic receptors, which they termed α2C subtype.

The compound UK 14,304 appears to activate all known α2-adrenoceptors and has little action on α1- and β-adrenoceptors, thus could be considered a selective full α2-adrenoceptor agonist (Beckeringh et al. 1984).

Marjamäki et al. (1993) recommended the use of recombinant human α2-adrenoceptors to characterize subtype selectivity of antagonist binding.

Uhlén et al. (1994) found that the α2-adrenergic radioligand [3H]-MK912 is α2C selective among human α2A-, α2B-, and α2C-adrenoceptors.

Uhlén et al. (1998) tested the binding of the radioligand [3H]RS79948–197 to human, guinea pig, and pig α2A-, α2B-, and α2C-adrenoceptors and compared the values with MK912, RX821002, rauwolscine, and yohimbine. [3H] RS79948–197 was nonselective for the α2-adrenoceptor subtypes, showing high affinity for all three.

Bücheler et al. (2002) used atipamezole on transgenic mouse lines lacking different α2-receptors subtypes to determine the localization and density of brain α2-receptors (Bücheler et al. 2002).

The advantages and obstacles in using different α2-adrenoceptor agonists/antagonists for radioligand binding studies have been discussed in the following reviews: Piascik et al. (1996), Starke (2001), and Calzada and de Artiñano (2001).

The binding of the radioligand [^{3}H]-rauwolscine to the α2A-adrenoceptor subtype can be measured in membranes prepared from rabbit spleens (Michel et al. 1989).

Binding of the radioligand [^{3}H]-yohimbine to the α2B-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat kidney cortices (Connaughton and Docherty 1989).

Subtypes of the α2-Adrenoceptor

α2-Adrenoceptors are one of the three types of adrenoceptors that belong to the same superfamily of G protein-coupled receptors. Three distinct α2-adrenoceptor subtypes – α2A, αB, and α2C – have been characterized and cloned (Cheung et al. 1982; Feller and Bylund 1984; Neylon and Summers 1985; Bylund 1992; Bylund et al. 1994; Kobilka et al. 1987; Regan et al. 1988; Lomasney et al. 1990; Murphy and Bylund 1988; Blaxall et al. 1991; Ruffolo et al. 1993; Lorenz et al. 1990; Ruffolo 1990; Uhlén and Wikberg 1990; Satoh and Takayanagi 1992; Takano et al. 1992).

For the extensive reviews on the subtypes of α2-adrenoceptors, see the following publications: Docherty (1998), Starke (2001), Fairbanks et al. (2009), Philipp and Hein (2004), Gilsbach and Hein (2012), and Knaus et al. (2007).

All three subtypes differ in tissue distribution and pharmacological properties and show different radioligand biding characteristics. However, without specific subtype-selective agonists/antagonists, it has been difficult to assign physiological responses to individual α2-adrenergic receptor subtypes.

As an alternative to using pharmacological ligands, genetically modified mice with inactivated or modified genes of α2-adrenoceptor subtypes have been used (Philipp et al. 2002; Bücheler et al. 2002; Starke 2001; Kable et al. 2000; MacMillan et al. 1996; Link et al. 1995, 1996; Altman et al. 1999; Hein et al. 1999; MacDonald and Scheinin 1995; MacDonald et al. 1997; Hein 2001; Trendelenburg et al. 2001, 2003; Knaus et al. 2007).

α2A-adrenoceptor is the main presynaptic inhibitory autoreceptor regulating norepinephrine release from central and peripheral sympathetic nerves. Mice with mutated or deleted α2A-receptor subtype do not exhibit hypotensive, sedative, antinociceptive, or hypothermic effects in response to α2-adrenergic agonists and have higher resting systemic blood pressure and heart rate (Altman et al. 1999; Guyenet 1997; Lakhlani et al. 1997; Flordellis et al. 2004; Bücheler et al. 2002; Stone et al. 1997). α2A-Adrenoceptor is alternatively called α2A-/D-adrenoceptor because the rodent version of this receptor (α2D) differs pharmacologically from the human version (α2A).

α2B Subtype plays a major role in peripheral vasoconstriction in response to α2-adrenergic agonist (Altman et al. 1999; Link et al. 1996; MacDonald et al. 1997) development of salt-induced hypertension (Makaritsis et al. 1999) and the development of placental vascular system (Philipp et al. 2002).

The blood pressure response to intravenous injection of α2 agonist consists of two phases – short hypertensive phase, which is a result of activation peripheral of α2B-receptors, and long-lasting hypotensive phase, mediated by α2A-receptors (Altman et al. 1999; Hein et al. 1999; Philipp et al. 2002).

Adrenoceptors of α2C subtype also participate to presynaptic inhibition of norepinephrine release; however, they are particularly efficient at low stimulation frequencies, comparing with high stimulation frequency of α2A-adrenoceptors. (Hein et al. 1999; Scheibner et al. 2001; Trendenelburg et al. 2001; Bucheler et al. 2002; Blaxall et al. 1991).

Sallinen et al. (1999) demonstrated that α2C-adrenoceptors affect a number of behavioral functions (for the reviews, see Philipp et al. 2002; Scheinin et al. 2001).

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Electrically Stimulated Release of [^{3}H] Norepinephrine from Brain Slices

Purpose and Rationale

The existence of presynaptic receptors which regulate the evoked release of neurotransmitters has been functionally demonstrated in both peripheral and central nervous system (Langer 1981; Starke 1981, 2001; Raiteri et al. 1984; Miller 1998; Philipp et al. 2002; Docherty 1998). Presynaptic adrenergic α2-receptors regulate the evoked release of norepinephrine, comprising a short negative feedback loop. Alpha-2 agonists, such as clonidine and guanabenz, inhibit evoked release, and alpha-2 antagonists, such as yohimbine and idazoxan, enhance evoked release.

The assay is used as a biochemical screen for agents which enhance or inhibit release of [^{3}H] norepinephrine (^{3}H-NE) and is particularly useful for testing receptor function of α2-adrenergic agonists and antagonists. The method was described in details in the following publications: Reynolds et al. (2005), Dooley et al. (2002), Vizi et al. (2004), Trendelenburg et al. (2003), Scheibner et al. (2001), Trendelenburg et al. (2001), and Bücheler et al. (2002).

The procedures used emphasize delicate care of slices. By treating slices with great care, one is able to incubate at low tracer concentrations of ^{3}H-NE (25 nM), thus minimizing nonspecific labeling of releasable pools other than those in noradrenergic nerve terminals. It also permits the use of low (and more physiological) stimulation parameters, which allow the neurons to recover easily between stimulations and do not flood the synaptic cleft with released NE, which would compete with any applied drug thus decreasing sensitivity.

Procedure

This assay is based on the method described by Zahniser et al. (1986).

Reagents

1. Krebs–Henseleit bicarbonate buffer, pH 7.4 (KHBB):

NaCl	118.4 mM
KCl	4.7 mM
$MgSO_4 \times 7\ H_2O$	1.2 mM
KH_2PO_4	2.2 mM
$NaHCO_3$	24.9 mM
$CaCl_2$	1.3 mM
Dextrose (added prior to use)	11.1 mM

   The buffer is aerated for 60 min with 95 % O_2 and 5 % CO_2 on ice and pH is checked.
2. Levo-[ring-2,5,6-^{3}H]-norepinephrine (specific activity 40–50 Ci/mmol) is obtained from New England Nuclear.

   The final desired concentration of ^{3}H-NE is 25 nM. 0.125 nmol is added to 5 ml KHBB.
3. Test compounds

   For most assays, a 1 mM stock solution of the test compound is made up in a suitable solvent and diluted such that the final concentration in the assay is 1 μM. Higher or lower concentrations may be used depending on the potency of the drug.

Instrumentation

Neurotransmitter release apparatus consisting of:

- Oscilloscope B8K, Precision Model 1420, dual-trace microscope (DynaScan Corp.)
- Constant current unit, Grass model CCU1 (Grass Instr. Co.)
- Stimulator, model S44, solid state square wave stimulator (Grass Instr. Co.)
- Pump, Watson–Marlow, model 502 SHR, standard drive module; model 501 M multichannel pumphead (Bacon Technical Instr.)
- Circulator, Haake D8 immersion circulator (Haake Buchler Instr. Inc.)
- Fraction collector, Isco Retriever IV fraction collector (Isco Inc.)

Tissue Preparation

Male Wistar rats (100–150 g) are decapitated and cortical tissue removed on ice, and 0.4 mm slices are prepared with a McIlwain tissue chopper.

The slices are made individually and removed from the razor blade by twirling an artist's paint brush underneath the slice. Care should be taken not to compress the slice or impale it on the bristles. The slices are placed in cold, oxygenated buffer (10–20 ml) and incubated at 35 °C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 5 ml of cold oxygenated buffer is added and enough [^{3}H]NE to bring the final concentration to 25 nM. This is then incubated and shaken for 30 min at 35 °C under oxygen. After this step, the buffer is decanted and the "loaded" slices are rapidly placed on the nylon mesh in the stimulation chambers using a cut-off Pipetman tip.

Assay

To establish a stable baseline, control buffer is pumped through the chamber for 1 h at a flow rate of 0.7 ml/min before the first stimulation. One hour is allowed to pass before the second stimulation. When drugs are used, each concentration is prepared in a separate flask in control buffer and allowed to equilibrate with the tissue slice 20 min before the second stimulation. The experiment is stopped 40 min after the second stimulation.

Stimulation parameters are set at 5 Hz (2 ms duration) for 60 s, with 1 ms delay and voltage setting of 440 SIU (250 Ω).

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, then 200 ml of 20 % methanol in distilled water, and then distilled water again for at least 20 min.

Evaluation

After conversion of dpm, percent fractional release is calculated for each fraction, using the spreadsheet program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue at that moment in time. "Spontaneous release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from each animal.

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Imidazoline Receptor Binding

Purpose and Rationale

Imidazoline receptors constitute a family of nonadrenergic high-affinity binding sites for clonidine, idazoxan, and allied drugs. Existence of specific imidazoline binding sites was propose by Bousquet and colleagues (Bousquet et al. 1984; 1981; Bousquet 1995, 1998) to explain central hypotensive effect of clonidine without involving α2-adrenoceptors. Later, the imidazoline binding sites were described to fulfill all the essential criteria of true receptor (binding, physiological function, location, and correlation of affinity with function) (Ernsberger 1999).

The importance of imidazoline receptors in cardiovascular action of centrally acting antihypertensive agent was discussed in the following publication: Head (1995), Head (1999), Head et al. (1998), Bock et al. (1999), and Szabo et al. (1999).

Three distinct subclasses of imidazoline receptors have been identified – I1, I2, and I3; none of them belongs to the family of G protein-coupled receptors and should be correctly called imidazoline binding sites.

The I1 sites are located in rostroventrolateral medulla, have high affinity to clonidine, and partly mediate antihypertensive effect of clonidine-like substance.

I2-Receptor is characterized by high affinity for idazoxan and low affinity for clonidine. Most I2 binding sites are located on the monoamine oxidase A and B enzymes in the outer membrane of mitochondria in the CNS and peripheral tissues, and binding to these sites allosterically modulates the enzymes (Coupry et al. 1987; Tesson et al. 1991, 1995).

The binding site I3 was identified in pancreatic β-cells; the activation of these sites increase insulin secretion (Morgan et al. 1999; Head and Mayorov 2006; Nikolic and Agbaba 2012; Khan et al. 1999; Szabo 2002; Eglen et al. 1998).

A several endogenous clonidine-displacing substance have been identified (Atlas and Burstein 1984; Prell et al. 2004; Li et al. 1994; Parker et al. 1999; Hudson et al. 1999; Robinson et al. 2003; Musgrave and Badoer 2000; Raasch et al. 2001, 2002; Reis et al. 1995; Chan et al. 1997).

From the first suggestion of the existence of imidazoline receptors, there has been the continuing debate about their contribution to antihypertensive actions of clonidine-like substance or ever existence of imidazoline receptors at all (Berdeu et al. 1996; Eglen et al. 1998; Guyenet 1997; Head et al. 1998; Szabo 2002).

Procedure

Tissue Preparation

Whole bovine brains and adrenal glands are obtained from a local slaughterhouse. The lateral medulla oblongata is isolated by a sagittal section through the lateral margin of the pyramids and then bisected. The ventral half is defined as the ventrolateral medulla.

Fresh bovine adrenal glands are perfused retrogradely through the adrenal vein twice with 25 ml ice-cold Krebs–Henseleit bicarbonate buffer. The glands are perfused again with 25 ml ice-cold Krebs–Henseleit buffer containing 0.025 % collagenase (type I, Sigma Chemical), incubated at room temperature for 1 h, then perfused with 25 ml fresh buffer containing collagenase, and incubated for 30 min at 35 °C. The digested glands are split, and the medulla is removed from the cortex. The adrenal medullae are minced and incubated while being stirred for 30 min at 37 °C. The digest is filtered and centrifuged at 200 *g* for 30 min at 20 °C. The cell pellet is resuspended in 30 ml Krebs' solution without collagenase, recentrifuged, flash-frozen, and stored at −70 °C.

Membrane Preparation

Fresh bovine ventrolateral medulla and collagenase-digested rat renal medulla are homogenized with a Polytron (Tekmar Tissumizer, setting 80 for 15 s twice) in 20 vol of ice-chilled HEPES-buffered isotonic sucrose (pH 7.4) containing the protease inhibitors 1,10-phenanthroline (100 μM) and phenylmethylsulfonyl fluoride (50 μM). Bovine adrenomedullary chromaffin cells are homogenized in 15 ml HEPES-buffered isotonic sucrose by 10 strokes in a glass/glass handhold homogenizer. The homogenates are centrifuged at 1,000 *g* for 5 min at 4 °C to remove nuclei and debris. The pellets (P1) are resuspended in 20 ml of homogenization buffer and centrifuged again at 1,000 *g* for 5 min. The supernatants are centrifuged at 48,000 *g* for 18 min at 4 °C, and the resulting pellet (P2) is resuspended in 10–25 vol 50 mM Tris–HCl buffer (pH 7.7) containing 5 mM EDTA. After recentrifugation at 48,000 *g* for 18 min, the resulting membrane pellet is resuspended in Tris–HCl containing 25 mM NaCl, preincubated for 30 min at 25 °C, chilled on ice, centrifuged again, resuspended a final time in Tris–HCl alone, centrifuged, flash-frozen, and stored at −70 °C.

Binding Assays

For the determination of specific binding to I_1-imidazoline sites and α2-adrenergic receptors, radioligand binding assays are performed with [^{3}H]clonidine, [^{3}H]*p*-iodoclonidine, or [^{3}H]moxonidine. Membranes are slowly thawed and resuspended in Tris–HCl or Tris–HEPES buffer (pH 7.7, 25 °C). Assays are conducted in a total volume of 250 μl in polypropylene 96 well plates (Beckman Macrowell). Each well contains 125 μl membrane suspension, 25 μl radioligand, and 100 μl drug or vehicle. Incubations are initiated by the addition of membrane suspension and carried out for 40 min at 25 °C. Nonspecific binding is defined in the presence of either piperoxan or phentolamine (0.1 mM), which are imidazoline adrenergic agents. Specific α2-adrenergic binding is defined by epinephrine (0.1 mM). In experiments with catecholamines, all samples contain ascorbic acid in a final concentration of 0.001 %. Incubations are terminated by vacuum filtration over Reeves Angel or Whatman GF/C fiberglass filters using a cell harvester (Brandel). The filters are washed four times with 5 ml ice-cold Tris–HCl, placed in scintillation vials, covered with 4 ml scintillation cocktail and counted at 50 % efficiency. Protein is assayed by a modified Lowry et al. method (Peterson 1977) using a deoxycholate–trichloroacetic acid protein precipitation technique which provides a rapid quantitative recovery of soluble and membrane proteins from interfering substances even in very dilute solutions. Sodium dodecyl sulfate is added to alleviate possible nonionic and cationic detergent and lipid interferences and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins.

Evaluation

Data are obtained as disintegrations per min and transferred to the Equilibrium Binding Data

Analysis program (Mc Pherson 1985). Then, several experiments are analyzed simultaneously with the LIGAND program for nonlinear curve fitting (Munson and Rodbard 1980). IC_{50} values are estimated from inhibition curves by nonlinear curve fitting (Mutolsky and Ransnas 1987). Protein assay data are also analyzed by nonlinear curve fitting (Mc Pherson 1985).

Modifications of the Method

Tesson et al. (1991) defined the subcellular localization of imidazoline/guanidinium-receptive sites by performing binding studies with the radioligand [^{3}H]idazoxan.

Lanier et al. (1993) visualized multiple imidazoline/guanidinium-receptive sites with the photoaffinity adduct 2-[3-azido-4-[^{125}I]iodophenoxy]methyl imidazoline.

Molderings et al. (1991) characterized imidazoline receptors involved in the modulation of noradrenaline release in the rabbit pulmonary artery preincubated with [^{3}H]noradrenaline.

Molderings and Göthert (1995) determined electrically or K^+-evoked tritium overflow from superfused rabbit aortic strips preincubated with [^{3}H]noradrenaline in order to characterize presynaptic imidazoline receptors which mediate noradrenaline release and compared them with I_1- and I_2-imidazoline radioligand binding sites.

Ernsberger et al. (1995) described optimization of radioligand binding assays for I_1 imidazoline sites.

Munk et al. (1996) reported the synthesis and pharmacological evaluation of a potent imidazoline-1 receptor specific agent.

Piletz et al. (1996) compared the affinities of several ligands for [^{125}I]*p*-iodoclonidine binding at human platelet I_1-imidazoline binding sites.

Several selective ligands for imidazoline I_2 receptors have been identified, such as:

- LSL 60101 (Alemany et al. 1995; Menargues et al. 1995)
- RS-45041-190 (MacKinnon et al. 1995; Brown et al. 1995)
- RX801077 (= 2-BFI = 2-(2-benzofuranoyl)-2-imidazoline and analogs (Jordan et al. 1996; Lione et al. 1996; Alemany et al. 1997; Hosseini et al. 1997; Wiest and Steinberg 1997; Hudson et al. 1997)

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β-Adrenoceptor Binding

Purpose and Rationale

Subtypes of β-adrenoceptor *β*-adrenergic receptors were differentiated from *α*-receptors (Ahlquist 1948) and subsequently divided into two distinct subtypes, *β*1 and *β*2 (Lands et al. 1967), based on differing pharmacology in different tissues. Emorine et al. (1992) cloned human β3-adrenoceptors.

β-Adrenoceptors are widely distributed, found at both central and peripheral sites, and are activated either via norepinephrine released from sympathetic nerve terminals or via epinephrine released from the adrenal medulla. Important physiological consequences of *β*-adrenoceptor activation include stimulation of cardiac rate and force; relaxation of vascular, urogenital, and bronchial smooth muscle; stimulation of renin secretion from the juxtaglomerular apparatus; stimulation of insulin and glucagon secretion from the endocrine pancreas; stimulation of glycogenolysis in the liver and skeletal muscle; and stimulation of lipolysis in the adipocyte (Daly and McGrath 2011).

β-Adrenoceptors belong to the family of G protein-coupled receptors and considered one of the most powerful regulators of cardiac function among the estimated 200 G protein-coupled receptors in the heart (for reviews see Brodde et al. 2006; Rockman et al. 2002; Ferguson 2001; Rajagopal et al. 2010; Vasudevan et al. 2011; Wachter and Gilbert 2012; Woo and Xiao 2012).

In addition to the heart, they are also expressed in the kidney, central nervous system, adipocytes, bronchial and vascular smooth muscle cells, lymphocytes, endothelial cells, and hepatocytes (Daly and McGrath 2011; Brodde 2008). The mammalian heart expresses all three β-adrenoceptor subtypes, with the β1- and β2-adrenoceptors as the main mediators of cardiac response to adrenergic stimulation. The population of β-adrenoceptors in the non-failing human heart is composed of β1- and β2-adrenocepors in a ratio of 8:2; however, in both ageing and failing heart, the proportion of β1 subtypes decreases due to mRNA downregulation, while levels of β2-adrenoceptors remain stable, thus achieving a 1:1 ratio of β1 and β2 subtypes (Bristow et al. 1986, 1990, 1991; Steinberg 1999; Brodde et al. 2006; Wachter and Gilbert 2012; White et al. 1994; Lohse et al. 2003).

Acute stimulation of cardiac β-adrenergic receptors results in positive ionotropic and chronotropic responses, whereas chronic β-adrenoceptor stimulation leads to pathological cardiac remodeling (Hasenfuss et al. 1996; Teerlink et al. 1994; Bristow et al. 1982, 1986, 1989; Buxton et al. 1987; Calderone et al. 1991; Cartagena et al. 1993; Pelá et al. 1990; Steinfath et al. 1991; Pérez-Schindler et al. 2013; Wang and Dhalla 2000).

The use of gene-targeted mouse and isolated cell line models has advanced identification of distinct molecular signaling mechanisms of β-adrenoceptors (for reviews, see Brodde et al. 2006; Woo and Xiao 2012; Wang and Dhalla 2000; Vasudevan et al. 2011; Philipp and Hein 2004; Wachter and Gilbert 2012; Shore and Moore 2003).

Three *β*-adrenoceptor proteins have been cloned, and the characteristics of these recombinant receptors correspond with those of the three well-characterized *β*-adrenoceptors in native tissue, designated as *β*1, *β*2, and *β*3

(Frielle et al. 1987; Kobilka et al. 1987; Emorine et al. 1989; Tate et al. 1991; Machida et al. 1990). The possible roles of β3-adrenoceptors in the cardiovascular system were discussed by Gauthier et al. (1996). An additional β-adrenoceptor modulating cardiac contractility has been designated as the β_4-adrenoceptor (Kaumann 1997; Kaumann et al. 1998; however, recent evidence suggest that putative β4 receptor is more likely a novel functional state of β1 receptor (Granneman 2001; Strosberg 1998; Kaumann et al. 2001; Konkar et al. 2000). It is now established that β-adrenoceptor polymorphism exists (see reviews by Small et al. 2003; Kirstein and Insel 2004; Leineweber et al. 2004; Brodde and Leineweber 2005; Brodde 2008).

Bronchodilation appears to be mediated by the β2-adrenoceptor. The β3-adrenoceptor is responsible for lipolysis in white adipose tissue and thermogenesis in the brown adipose tissue found in rodents. Renin release appears to be mediated by the β1-adrenoceptor (Waitling 2006).

The β-adrenoceptor population of plasma membranes from bovine heart ventricles consists of 75–80 % β1- and 20–25 % β2-adrenoceptors. The use of this tissue allows a parallel investigation of the binding characteristics of drugs at both the β1- and β2-adrenoceptors. Both the β1- and β2-adrenoceptors coexist in rat ventricular myocytes, but stimulation of these receptor subtypes elicits qualitatively different cell responses at the levels of ionic channels, the myofilaments, and sarcoplasmic reticulum (Xiao and Lakatta 1993).

β-Receptors have been labeled in a number of tissues including the heart, lung, erythrocytes, and brain using the β-agonists [^{3}H]-epinephrine (U'Prichard et al. 1978) or [^{3}H]-hydroxybenzylisoproterenol (Lefkowitz and Williams 1977) or the β-receptor antagonists [^{3}H]-alprenolol (Mukherjee et al. 1975), [^{3}H]-dihydroalprenolol (DHA) (U'Prichard et al. 1978; Bylund and Snyder 1976; Rugg et al. 1978), and (^{125}I)-iodohydroxypindolol (Weiland et al. 1980). DHA is a potent β-antagonist (Mukherjee et al. 1975), which labels both β1- and β2-adrenergic receptors.

A constant concentration of the radioligand ^{3}H-dihydroalprenolol (^{3}H-DHA) (4–6 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine heart ventricles. If the test drug exhibits any affinity to β-adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more effective is the test drug.

Procedure

Materials and Solutions

Preparation buffer	
Tris–HCl	5 mM
$MgCl_2 \times 6H_2O$	1 mM
D(+)-sucrose	250 mM
pH 7.4	
310 mOsm sodium phosphate buffer	
pH 7.4	Rinse buffer
Tris–HCl	50 mM
$MgCl_2 \times 6H_2O$	10 mM
pH 7.4	
Incubation buffer	
Tris–HCl	50 mM
$MgCl_2 \times 6H_2O$	10 mM
Ascorbic acid	1.6 mM
Catechol	0.3 mM
pH 7.4	

Radioligand:

(−)^{3}H-dihydroalprenolol × HCl (^{3}H-DHA) specific activity 1.48–2.59 TBq/mmol (40–70 Ci/mmol) (NEN)

For inhibition of ^{3}H-dihydroalprenolol binding in nonspecific binding experiments:

(−)Isoprenaline(+)bitartrate salt (Sigma)

Bovine hearts are obtained fresh from the local slaughter house. The lower part of the left ventricle from five hearts is separated and kept in ice-cold preparation buffer. In the laboratory,

approx. 60 g wet weight from the five ventricle pieces are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/10 ml buffer); the homogenate is filtered through gauze and centrifuged at 500 *g* (4 °C) for 10 min. The pellets are discarded; the supernatant is collected and centrifuged at 40,000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml 310 mOsm sodium phosphate buffer, homogenized by Ultra-Turrax, and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in sodium phosphate buffer corresponding to 1 g ventricle wet weight/2 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at −77 °C. Protein concentration of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 *g* (4 °C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of approx. 2.0 mg/ml. After homogenizing by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

All incubation samples are performed in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

Total binding:

- 50 µl ^{3}H-DHA

  (12 concentrations, 3×10^{-10}–4×10^{-8} M)

- 50 µl incubation buffer

Nonspecific binding:

- 50 µl ^{3}H-DHA

  (4 concentrations, 3×10^{-10}–4×10^{-8} M)

- 50 µl (−)isoprenaline (10^{-5} M)

Competition Experiments

- 50 µl ^{3}H-DHA

(1 constant concentration, 4–6 $\times$ 10^{-9} M)

- 50 µl incubation buffer without or with non-labeled test drug

  (15 concentrations 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (approx. 2 mg protein/ml). The samples are incubated for 60 min in a shaking water bath at 25 °C. The reaction is stopped by rapid vacuum filtration of the total incubation volume over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding
- Nonspecific binding
- Specific binding = total binding–nonspecific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-DHA versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3H \times IC_{50}}{K_D{}^3H \times [^3H]}$$

IC_{50} = concentration of the test drug, which competes with 50 % of specifically bound ^{3}HDHA in the competition experiment.

$[^3H]$ = concentration of ^{3}H-DHA in the competition experiment.

$K_D{}^3H$ = dissociation constant of ^{3}H-DHA, determined from the saturation experiment.

The K_i value of the test drug is the concentration at which 50 % of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data

Propranolol hydrochloride $K_i = 6–8 \times 10^{-9}$ mol/l

Modifications of the Method

Abrahamsson et al. (1988) performed a receptor binding study on the β1- and β2-adrenoceptor affinity of atenolol and metoprolol in tissues from the rat, the guinea pig, and man with various radioligands, such as $[^{125}I](\pm)$hydroxybenzylpindolol, $[^{125}I](-)$pindolol, $[^3H](-)$dihydroalprenolol, and $[^3H](-)$CGP 12177.

Rugg et al. (1978) proposed coexistence of β1- and β2-adrenoceptors in mammalian lung based on the binding characteristics [^{3}H]DHA and selective agonists in rat and rabbit lung tissue.

Fleisher and Pinnas (1985) used specific binding of $(-)[^3H]$dihydroalprenolol to rat lung membranes for in vitro studies on the relative potency of bronchodilator agents.

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β1-Adrenoceptor Binding

Purpose and Rationale

Lesioning studies (Wolfe et al. 1982; Dooley et al. 1986), combined with nonlinear regression analysis of data, have shown that while β-receptors in rat cerebellum are primarily of the β2 subtype, the β1 occurring in rat cerebral cortex are physiologically more significant. The assay can be used to evaluate the direct interaction of drugs with β-receptors labeled by [^{3}H]-dihydroalprenolol.

Procedure

Reagents

Tris buffer, pH 8.0

1. (a) 44.4 g Tris–HCl q.s. to 1 liter
   (0.5 M Tris, pH 8.0) 26.5 g Tris base
   (b) Dilute 1:10 in distilled water.
   (0.05 M Tris, pH 8.0)
2. (−)-[propyl-1,2,3-^{3}H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled H_2O, and 50 µl is added to each tube (this yields a final concentration of 1 nM in the 1 ml assay).
3. (±)-Propranolol HCl is obtained from Ayerst.
   A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution is added to three tubes to determine nonspecific binding

(yields a final concentration of 1 μM in a 1 ml assay).

4. Test compounds.

   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound.

Tissue Preparation

Male rats are decapitated and the brains rapidly removed. The cerebral cortices are dissected free, weighed, and homogenized in 50 ml of ice-cold 0.05 Tris buffer, pH 8.0. This homogenate is centrifuged at 40,000 *g*, the supernatant decanted and the pellet resuspended and recentrifuged at 40,000 *g*. The final pellet is resuspended in the initial volume of fresh 0.05 Tris buffer, pH 8.0. This tissue suspension is then stored on ice. The final tissue concentration in the assay is 10 mg/ml. Specific binding is about 3 % of the total added ligand and 80 % of the total bound ligand.

Assay

380 μl	H_2O
50 μl	0.5 Tris buffer, pH 8.0
20 μl	Vehicle (for total binding) or 50 μM (±) propranolol (for nonspecific binding) or appropriate drug concentration
50 μl	^{3}H-DHA stock solution
500 μl	Tissue suspension

The tissue homogenates are incubated for 15 min at 25 °C with 1 nM ^{3}H-DHA and varying drug concentrations. With each binding assay, triplicate samples are incubated with 1 μM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed three times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

Modifications of the Method

Dooley et al. (1986) recommended CGP 20712A as a useful tool for quantitating β1- and β2-adrenoceptors.

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β2-Adrenoceptor Binding

Purpose and Rationale

Since [3H]-dihydroalprenolol is a nonspecific ligand, it is necessary to select a tissue which is enriched in β2-receptors in order to convey specificity to this assay. Tissues with predominantly β2-receptors include the lung (U'Prichard et al. 1978; Ariens and Simonis 1983; Lefkowitz et al. 1983), cerebellum (Lefkowitz et al. 1983; Minneman et al. 1983), rat and frog erythrocytes (Mukherjee et al. 1975; Lefkowitz et al. 1983), and ciliary process (Nathanson 1985), whereas the forebrain, heart, and avian erythrocytes are relatively enriched in the β1 subtype (Lefkowitz et al. 1983). Due to poor binding characteristics in cerebellum, the rat lung is chosen as the tissue for β2-adrenergic receptors.

A compound with β2 selectivity would be less likely to produce cardiac effects but more likely to produce bronchiolar constriction. The test is used

to determine the affinity of compounds for the β2-adrenergic receptor subtype. A measure of receptor subtype selectivity can be determined when data are compared with those obtained in the β1-adrenergic assay in rat cerebral cortex.

The present nomenclature of β1, β2, and β3 receptors was reviewed by Alexander et al. (2001).

Procedure

Reagents

1. Tris buffers, pH 8.0
   (a) 44.4 g Tris–HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base.
   (b) Dilute 1:10 in distilled water (0.05 M Tris, pH 8.0).
2. (−)-[Propyl-1,2,3-^{3}H] dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled water, and 50 µl is added to each tube (this yields a final concentration of 1 nM in the assay).

2. (±)-Propranolol HCl is obtained from Ayerst.

A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution is added to three tubes to determine nonspecific binding (yielding a final concentration of 1 µM in a 1 ml assay).

3. Test compounds.

For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound to be tested.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation and the lungs removed, weighed, and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 8.0 using a Tekmar homogenizer. The homogenate is passed through a cheese cloth and centrifuged at 40,000 *g* for 15 min. The final membrane pellet is resuspended in the original volume of Tris buffer, pH 8.0, and used in the assay.

Assay

380 µl	H_2O
50 µl	0.5 Tris buffer, pH 8.0
20 µl	Vehicle (for total binding) or 50 µM (±)-propranolol (for nonspecific binding) or appropriate drug concentration
50 µl	^{3}H-DHA stock solution
500 µl	Tissue suspension

The tissue homogenates are incubated for 15 min at 25 °C with 1 nM ^{3}H-DHA and varying drug concentrations. In each binding assay, triplicate samples are incubated with 1 µM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed three times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

Modifications of the Method

Dooley et al. (1986) recommended CGP 20712 as a useful tool for quantitating β1- and β2-adrenoceptors.

McCrea and Hill (1993) described salmeterol as a long-acting β-adrenoceptor agonist mediating cyclic AMP accumulation in the B50 neuroblastoma cell line.

McConnell et al. (1991, 1992; Owicki and Parce 1992) used a special apparatus, the "cytosensor microphysiometer" which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells were transfected with human β2-adrenergic receptors. The β2-adrenergic receptor activates adenylate cyclase resulting in an increase in the cyclic AMP concentration within the cell which can be measured as acidification. Addition of 10 µM isoproterenol, 500 µM 8-bromo cyclic AMP, or 10 µg/ml forskolin induced a reversible acidification.

Hoffmann et al. (2004) compared human β-adrenergic receptor subtypes using characterization of stably transfected receptors in CHO cells.

Procedure

cDNA of Human β-Adrenergic Receptors. cDNAs coding for human β-adrenergic receptors in pcDNA3 expression vectors were verified by sequencing and comparison with the respective GenBank entries. The translated amino acid sequences corresponded to the published sequences for the β1-adrenergic receptor (Frielle et al. 1987), β2-adrenergic receptor (Schofield et al. 1987), and β3-adrenergic receptor (Emorine et al. 1989). With respect to polymorphisms, the β-adrenergic receptors used in this study corresponded to the following variants: β1-receptor 49-Ser and 389-Gly; β2-receptor 16-Arg, 27-Gln, and 164-Thr; and β3-receptor 64-Trp. All of the variants correspond to the sequences originally termed wild type.

Stable Transfection of Cells

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, Md., USA) were transfected with plasmid DNA for stable expression using the calcium phosphate precipitation method (Chen and Okayama 1987) as described for the rat A_1 adenosine receptor (Freund et al. 1994). Positive clones were selected with 600 µg/ml of the neomycin analog G-418, and single clonal lines were isolated by limiting dilution. Expression of the receptor was verified by radioligand binding.

Cell Culture and Membrane Preparation. Chinese hamster ovary cells stably transfected with human β-adrenergic receptor subtypes were grown adherently and maintained in Dulbecco's Modified Eagle's Medium with nutrient mixture F12 (DMEM/F12), containing 10 % fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and Geneticin (G-418, 0.2 mg/ml) at 37 °C in 5 % CO_2/95 % air. Cells were split two or three times weekly at a ratio of between 1:5 and 1:15. In order to harvest cells, the culture medium was removed, cells were washed twice with PBS, and membranes were prepared or cells were frozen on the dishes for later preparation of membranes. Crude membrane fractions were prepared from fresh (measurement of adenylyl cyclase) or frozen cells (radioligand binding). The resulting membrane pellets were resuspended in 50 mM Tris–HCl buffer pH 7.4 to give a final protein concentration of 1–2 mg/ml.

Radioligand Binding Studies and Adenylyl Cyclase Activity

The radioligand binding experiments were performed with membranes prepared as described above. Assays were done in a volume of 200 µl in 50 mM Tris–HCl, pH 7.4 (assay buffer) in the presence of 100 µM GTP to ensure monophasic binding curves for agonists. For saturation binding experiments at human β1- and β2-receptors, up to 400 pM ^{125}I-CYP and for β3-receptors up to 1,500 pM ^{125}I-CYP were used. Nonspecific binding was determined in the presence of 10 µM alprenolol. For competition binding, 50 pM ^{125}I-CYP in the case of β1- and β2-receptors or 80 pM ^{125}I-CYP for β3-receptors were used. For most of the competition binding experiments, membranes with intermediate receptor expression (β1, 367 ± 75 fmol/mg protein; β2, 282 ± 19 fmol/mg protein; β3, 377 ± 82 fmol/mg protein) were

used. For selected compounds, it was demonstrated that higher receptor expression did not affect K_i values (data not shown). Membranes were incubated for 90 min at 30 °C, filtered through Whatman GF/C filters, and washed three times with ice-cold assay buffer. Samples were counted in a γ-counter (Wallac 1480 wizard 3). K_D values for ^{125}I-CYP were calculated by nonlinear curve fitting with the program SCTFIT. Ligand IC_{50} values were calculated using Origin 6.1 (OriginLab Corporation, Northampton, Mass., USA) and were transformed to K_i values according to Cheng and Prusoff (1973).

Adenylyl cyclase activity in cell membranes was determined according to Jakobs et al. (1976). Membrane protein (50 µg) was added to an incubation mixture with final concentrations of 50 mM Tris–HCl pH 7.4, 100 µM cAMP, 0.2 % BSA, 10 µM GTP, 100 µM ATP, 1 mM $MgCl_2$, 100 µM IBMX, 15 mM phosphocreatine, and 300U/ml of creatine kinase. Membranes were incubated with about 200,000 cpm of [α-^{32}P]-ATP for 20 min in the incubation mixture as described (Klotz et al. 1985). Accumulation of [α-^{32}P]-cAMP was linear over at least 20 min under all conditions. The reaction was stopped by addition of 400 µl of 125 mM ZnAc solution and 500 µl of 144 mM Na_2CO_3. Samples were centrifuged for 5 min at 14,000 rpm in a laboratory microcentrifuge. Then, 800 µl of the resulting supernatant was finally applied to alumina WN-6 (Sigma) columns that were eluted twice with 2 ml of 100 mM Tris–HCl pH 7.4. The eluates were counted in a β-counter (Beckmann LS 1801).

Niclauss et al. (2006) compared the ability of three radioligands, [^{125}I]-cyanopindolol, [^{3}H]-CGP 12,177, and [^{3}H]-dihydroalprenolol, to label the three human β-adrenoceptor subtypes. Saturation and competition binding experiments were performed using membrane preparations from Chinese hamster ovary cells stably transfected with the three subtypes. While [^{3}H]-CGP 12,177 had very similar affinity for β1- and β2-adrenoceptors (about 40 pM), [^{125}I]-cyanopindolol and [^{3}H]-dihydroalprenolol had four- to sixfold higher affinity for β2- as compared to β1-adrenoceptors (10 vs. 45 and 187 vs. 1,021 pM, respectively). The affinity of [^{125}I]-cyanopindolol at β3-adrenoceptors was considerably lower (440 pM) than at the other two subtypes. The β3-adrenoceptor affinity of [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol was so low that it could not be estimated within the tested range of radioligand concentrations (up to 4,000 and 30,000 pM for [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol, respectively). All three radioligands were ill-suited to labeling β3-adrenoceptors, particularly in preparations co-expressing multiple subtypes. In the absence of alternatives, [^{125}I]-cyanopindolol appears the least unsuitable for labeling β3-adrenoceptors. At present, there is still a need for high-affinity radioligands that are selective for β3-adrenoceptors.

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Adenosine Receptor

Michael Gralinski, Liomar A. A. Neves, and Olga Tiniakova

Contents

Adenosine Receptors Subtypes

Adenosine, a purine nucleoside catabolite of ATP, regulates numerous effects in mammalian organ systems. The discovery by Drury and Szent-György (1929) that adenosine can affect several bodily functions inspired much research interest. Regulatory functions of adenosine are especially important when cellular energy supply fails to meet the demand. Adenosine is omnipresent; it is released by nearly all cells and is continuously formed intracellularly as well as extracellularly.

The intracellular production is mediated by an intracellular 5′-nucleotidase, which dephosphorylates AMP (Schubert et al. 1979; Zimmermann et al. 1998), or by hydrolysis of *S*-adenosylhomocysteine (Broch and Ueland 1980). It is estimated that the levels of adenosine in interstitial fluid are within the 30–300 nM range (Winn et al. 1981; Delaney et al. 1998; Delaney and Geiger 1998; Zetterström et al. 1982; Porkka-Heiskanen et al. 1997). Adenosine generated intracellularly is transported into extracellular space via specific bidirectional transporters.

Adenosine is generated in the extracellular space through ATP breakdown by series of ectoenzymes (5′-nucleotidase and apyrase) (Zimmermann 2000). When the levels of adenosine in extracellular space are high, adenosine is transported into the cells and then inside the cells is phosphorylated to AMP by adenosine kinase or degraded to inosine by adenosine deaminase (Arch and Newsholme 1978; Lloyd and Fredholm 1995).

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The levels of adenosine increase, up to 100-fold, as a result of oxidative stress and ischemia (Rudolphi et al. 1992a; Latini et al 1999).

Adenosine mediates its effects by activation of family of four G protein-coupled receptors: the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors. All four adenosine receptors have been cloned from different species. There is a close similarity between receptors of the same subtype, at least among mammals, with exception for A_3 receptor for which there is almost 30 % difference at the amino acid level between human and rat (Fredholm et al. 2000, 2001; Klotz 2000; Zhou et al. 1992).

Activation of adenosine A_1 and A_3 receptors results in decreased adenylate cyclase activity through activation of proteins of G_i/G_o family. (VanCalker et al. 1978; Londos et al. 1980; Palmer and Stiles 1995; Borea et al. 2015).

The adenosine A_{2A} and A_{2B} receptors are coupled to Gs-proteins, and activation of both types A_2 receptors increases adenylate cyclase activity (Palmer and Stiles 1995; Brackett and Daly 1994; Peakman and Hill 1994; Fredholm et al. 2001, 2011; Sattin and Rall 1970; VanCalker et al. 1978). Recently, evidence was presented that A_{2A} receptor may be coupled to different G proteins in different areas (Kull et al. 2000a, b), and adenosine A_{2B} receptors may also activate phospholipase C in human mast cells (Feoktistov and Biaggioni 1995).

Besides the "canonical" signaling pathways of adenosine receptors, numbers of "noncanonical" pathways for each receptor's types have been described (Kull et al. 2000a; Schulte and Fredholm 2000; de Lera Ruiz et al. 2014; Chen et al. 2014).

Today transgenic and knockout mouse models have been generated for all four adenosine receptors subtypes, which advanced research of adenosine and adenosine receptors, revealing their new physiological functions (Wei et al. 2011; Yaar et al. 2005; Morrison et al. 2006; Avila et al. 2002; Linden 1994).

For reviews about four subtypes of adenosine receptors, see Fredholm et al. (2001), Borea et al. (2015), Headrick et al. (2011), Chen et al. (2014), Fredholm et al. (2011), Fredholm (2014), Klotz (2000), Jacobson et al. (1992), and Cohen and Downey (2008).

Adenosine A_1 Receptor Binding

General Considerations

Adenosine A_1 receptors are found in great numbers in the brain (cortex, hippocampus, cerebellum), spinal cord, eye, adrenal gland, and atria. A_1 receptors subtypes are expressed on intermediate levels in skeletal muscles, liver, kidney, adipose tissue, salivary glands, esophagus, colon, testis, and on low levels in lungs and pancreas (Fredholm et al. 2001; Klotz 2000; Kimatrai-Salvador et al. 2013).

The adenosine A_1 receptor is responsible for sedative, anticonvulsant, anxiolytic, and locomotor depressant effect induced by adenosine.

Activation of adenosine A_1 receptors modifies cardiac contractility through the modulation of adrenergic responses (Dobson 1983; Romano and Dobson 1990; Fenton and Dobson 2007; Perlini et al. 1998; Fenton et al. 2010; Headrick et al. 2011; Shryock and Belardinelli 1997).

Adenosine and A_1 receptors inhibit release of noradrenaline from cardiac nerves, reducing noradrenaline levels during ischemia and reperfusion (Lorbar et al. 2004; Burgdorf et al. 2001; Morrison et al. 2006; Shryock and Belardinelli 1997; Baxter 2002), which contributes to cardioprotection.

Purpose and Rationale

The purpose of this assay is to measure the affinity of test compounds for adenosine (A_1) receptors. Evidence for an adenosine A_1 receptor in the guinea pig atrium was given by Collis (1983). Adenosine plays a physiological role in many systems, including platelet aggregation, lipolysis, steroidogenesis, and smooth muscle tone (Daly 1982). The vasodilatory and cardiac depressant effects of adenosine are well known. In addition to cardiovascular effects, adenosine has marked effects in the CNS including depression of electrophysiological activity (Siggins and Schubert 1981), anticonvulsant activity, analgesic

properties (Ahlijanian and Takemori 1985), and inhibition of neurotransmitter release (Harms et al. 1979).

The agonist, [^{3}H]cyclohexyladenosine (CHA), has affinity for the A_1 receptor in the nanomolar concentration range and has proven to be a suitable ligand for A_1 receptor assays (Bruns et al. 1980, 1986). Selective A_1 (Schingnitz et al. 1991) and A_2 antagonists (Shimada et al. 1992; Jacobson et al. 1993) have been described. Adenosine and its nucleotides have not only a cardiovascular but predominantly a cerebral activity (Phillis and Wu 1981; Daly 1982; Fredholm et al. 1982).

Procedure

Reagents

1. (a) 0.5 M Tris buffer, pH 7.7
   (b) 0.05 M Tris buffer, pH 7.7
2. Adenosine deaminase is obtained from Sigma Chemical Co.
   Adenosine deaminase is added to 0.05 M Tris–HCl buffer, pH 7.7, for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. Cyclohexyladenosine, N^6-[Adenine-2,8-^{3}H] (specific activity 34 mCi/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations, [^{3}H]CHA is made up to a concentration of 40 nM, and 50 µl is added to each tube. This yields a final concentration of 1 nM in the assay.
4. Theophylline is obtained from Regis Chemical Co. A 100 mM stock solution is made up in deionized water. 20 µl is added to each of three tubes for the determination of nonspecific binding, yielding a 1 mM final concentration in the assay.
5. Test compounds
   For most assays, a 1 mM stock solution is prepared in DMSO and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebellum are removed, weighed, and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer, and centrifuged again as before. The final pellet is resuspended in 0.05 M Tris buffer containing 0.1 U/ml of adenosine deaminase.

Assay

1,000 µl	Tissue suspension
930 µl	H_2O
20 µl	Vehicle
	Or theophylline
	Or appropriate concentration of test compound
50 µl	^{3}H-CHA

The tubes are incubated for 2 h at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed three times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml liquid scintillation cocktail, left to soak overnight, and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 1 mM theophylline. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors (Bruns et al. 1986) precludes the simple calculation of K_i values by the Cheng–Prusoff equation.

Modifications of the Method

Stiles et al. (1985) used ^{125}I-labeled N^6-2-(4-aminophenyl)ethyladenosine as a selective ligand to probe the structure of A_1 receptors.

Lohse et al. (1987) described 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as a high-affinity antagonist radioligand for adenosine A_1 receptors.

Klotz et al. (1989) described 2-chloro-N^6-[^{3}H] cyclopentyladenosine ([^{3}H]CCPA) as a high-affinity agonist radioligand for adenosine A_1 receptors.

Von Lubitz et al. (1995) studied the therapeutic implications of chronic NMDA receptor stimulation on adenosine A_1 receptors.

The partial agonism of theophylline-7-riboside on the adenosine A_1 receptor has been reported by Ijzerman et al. (1994).

Libert et al. (1992) reported the cloning and functional characterization of a human adenosine A_1 receptor.

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Adenosine A_2 Receptor Binding

Purpose and Rationale

Adenosine A_{2A} receptor was cloned from the dog thyroid (Maenhaut et al. 1990), rat brain (Chern et al. 1992), human (Furlong et al. 1992), and guinea pig (Meng et al. 1994), and also the crystal structure of human adenosine A_{2A} receptor was revealed (Doré et al. 2011; Gutiérrez-de-Terán et al. 2013; Jaakola et al. 2008; Lebon et al. 2011; Liu et al. 2012; Xu et al. 2011).

Adenosine A_{2A} receptors are highly expressed in the spleen, thymus, leukocytes, blood platelets, striatopallidal GABAergic neurons, and olfactory bulb and expressed to a lesser extent in the heart, lung, blood vessels, and other brain regions (see reviews by Fredholm et al. (2001, 2003); de Lera Ruiz et al. (2014); Chen et al. (2014)).

Adenosine A_{2A} receptors may form heterodimers with other G protein-coupled receptors such as dopamine D2/A_{2A} and D3/A_{2A}, cannabinoid CB1/A_{2A}, and glutamate mGluR5/A_{2A} (Fuxe et al. 2005; Torvinen et al. 2005; Ferré et al. 2009).

Adenosine A_{2A} receptors are responsible for regulating myocardial blood flow by vasodilating the coronary arteries. Knockout mice, in which adenosine A_{2A} receptors were targeted, showed aggressiveness, decreased sensitivity to pain, and slightly higher blood pressure (Ledent et al. 1997; Chen et al. 1999).

A_{2A} receptor is also expressed in the brain, where it regulates glutamate and dopamine release (Ongini et al. 1997; Torvinen et al. 2005; Ferre et al. 1991, 2008; Dasgupta et al. 1996; Pollack and Fink 1995).

Adenosine A_{2B} receptor was cloned by Stehle and colleagues (1992) and Jacobson and colleagues (1995).

Adenosine A_{2B} receptor is coupled to adenylate cyclase stimulatory G proteins (Brackett and Daly 1994; Peakman and Hill 1994) but has also been reported to activate phospholipase C in human mast cells (Feoktistov and Biaggioni 1995). The receptor is expressed widely, but generally at very low levels, with high expression in the cecum, colon, and bladder and lower level in the lung, blood vessels, eye, mast cells, adipose tissue, adrenal glands, brain, kidney, liver, ovary, and pituitary gland (Fredholm et al. 2001).

It plays a role in mediating vasodilation in some vessels, such as guinea pig aorta (Martin 1992), rat renal artery, and dog coronary artery (Balwierczak et al. 1991), and also in mediating allergic or inflammatory disorders (Feoktistov and Biaggioni 1995; Auchampach et al. 1997; Fredholm and Persson 1982; Linden et al. 1999; Johnston-Cox et al. 2012).

Interestingly, that activation of adenosine A_2 receptors may promote angiogenesis during ischemia/hypoxia, with the new blood vessels helping to maintain tissue oxygenation (Auchampach 2007; Feoktistov et al. 2002, 2003; Takagi et al. 1996; Grant et al. 1999; Rudolphi et al. 1992).

This assay uses ^{3}H-NECA (5′-*N*-ethylcarboxamido[8-3H]adenosine) to label A_2 receptors in rat striatum by the method described by Bruns et al. (1986). The comparison of data from this assay and the A_1 receptor assay provides a measure of selectivity for these two receptors.

Procedure

Reagents

1. (a) 0.5 M Tris buffer, pH7.7
   (b) 0.05 M Tris buffer, pH 7.7
   (c) 0.05 M Tris buffer, pH 7.7, containing 12 mM $CaCl_2$ (final assay concentration: 10 mM)
2. Adenosine deaminase is obtained from Sigma Chemical Co.
   Adenosine deaminase is added to 0.05 M Tris–HCl buffer, pH 7.7, containing 12 mM $CaCl_2$ for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. 5′-*N*-Ethylcarboxamido[8-^{3}H]adenosine (specific activity 23–40 mCi/mmol) is obtained from Amersham.
   For IC_{50} determinations, ^{3}H-NECA is made up to a concentration of 80 nM, and 50 µl is

added to each tube. This yields a final concentration of 4 nM in the assay.

4. Cyclopentyladenosine (CPA) is obtained from Research Biochemicals, Inc.

   A 5 mM stock solution is made up in DMSO. 20 µl is added to each of three tubes for the determination of nonspecific binding, yielding a 100 µM final concentration in the assay.

   Since [^{3}H]NECA is not a specific ligand for A_2 receptors, CPA is added to all other tubes to mask the A_1 receptors at a final concentration of 50 nM.

5. Test compounds

   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed, and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer, and centrifuged again as before. The final pellet is resuspended in 100 volumes of 0.05 M Tris buffer containing 10 mM $CaCl_2$ and 0.1 U/ml of adenosine deaminase.

Assay

830 µl	Tissue suspension
100 µl	CPA
20 µl	Vehicle or CPA or appropriate concentration of test compound
50 µl	^{3}H-NECA

The tubes are incubated at 25 °C for 2 h. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed three times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml liquid scintillation cocktail, left to soak overnight, and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 100 µM CPA. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors precludes the simple calculation of K_i values by the Cheng–Prusoff equation.

Modifications of the Method

Jarvis et al. (1989) reported on [^{3}H]CGS 21 680, a selective adenosine A_2 receptor agonist which directly labels A_2 receptors in rat brain. [^{3}H]CGS 21 680 binding was greatest in striatal membranes with negligible specific binding obtained in rat cortical membranes.

Gurden et al. (1993) described the functional characterization of three adenosine receptor types.

Hutchinson et al. (1990) described 2-(arylalkylamino)adenosin-5′-uronamides as a new class of highly selective adenosine A_2 receptor ligands.

Adenosine A_{2A} receptors from rat striatum and rat pheochromocytoma PC12 cells have been characterized with radioligand binding and by activation of adenylate cyclase (Hide et al. 1992).

Nonaka et al. (1994) reported on KF17837 ((E)-8-(3,4-dimethoxystyryl)-1, 3-dipropyl-7-methylxantine), a potent and selective adenosine A_2 receptor antagonist.

The in vitro pharmacology of ZM 241385, a potent, non-xanthine, selective adenosine A_{2a} receptor antagonist has been reported by Poucher et al. (1955).

Monopoli et al. (1994) described the pharmacology of the selective adenosine A_{2a} receptor agonist 2-hexynyl-5′-*N*-ethylcarboxamidoadenosine.

Jacobson et al. (1993) described structure–activity relationships of 8-styrylxanthines as selective adenosine A_2 antagonists.

Varani et al. (1996) reported pharmacological and biochemical characterization of purified

adenosineA_{2a} receptors in human platelet membranes by [^{3}H]-CGS 21680 binding.

Van der Ploeg et al. (1996) characterized adenosine A_2 receptors in human T-cell leukemia Jurkat cells and rat pheochromocytoma PC12 cells using adenosine receptor agonists.

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Adenosine A_3 Receptor Binding

Purpose and Rationale

The adenosine A_3 receptor has been cloned and characterized (Ali et al. 1990; Meyerhof et al. 1991; Zhou et al. 1992). Adenosine A_3 receptor is widely expressed, its mRNA being revealed in the testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, and eye of rats, sheep, and humans (Zhou et al. 1992; Salvatore et al. 1993;

Linden 1994; Linden et al. 1993; Rivkees 1994; Dixon et al. 1996; Burnett et al. 2010; Borea et al. 2015). There are differences in expression levels within and between species (for extended review, see Borea et al. 2015).

Despite a low adenosine A_3 receptor expression in myocardial tissue, the multiple studies reported cardioprotective effect of A_3 receptor stimulation (Germack and Dickenson 2005; McIntosh and Lasley 2012; Auchampach et al. 1997; Tracey et al. 1997; Thourani et al. 1999a, b; Ge et al. 2006, 2010; Xu et al. 2006; Chanyshev et al. 2012; Cross et al. 2002; Harrison et al. 2002; Shneyvays et al. 1998, 2001; Headrick and Peart 2005; Hussain et al. 2014; Baxter 2002).

Adenosine A_3 receptors also play a role in the modulation of blood vessel function (see review by Burnstock and Ralevic (2013)). Stimulation of the adenosine A_3 receptor facilitates release of allergic mediators in mast cells (Ramkumar et al. 1993) inducing hypotension (Hannon et al. 1995; Tilley et al. 2000; Zhao and Kukreja 2002; Jin et al. 1997).

Adenosine A_3 receptors are present in immune cells and are involved in the regulation of inflammatory and immune processes mediated by adenosine (see reviews by Antonioli et al. 2010; Haskó and Cronstein (2013) Borea et al. 2015).

The design of selective ligands of adenosine A_3 receptors and the therapeutic concepts including effects on locomotor activity, cardiovascular effects, effects in cerebral ischemia (von Lubitz et al. 1994), effects in cardiac preconditioning, and as antagonists in inflammation and asthma has been discussed by Jacobson et al. (1995). A binding site model and structure–activity relationships for the rat adenosine A_3 receptor are described by van Galen et al. (1994).

Procedure

Cell Culture and Membrane Preparation

Chinese hamster ovary (CHO) cells stably expressing the rat adenosine A_3 receptor are grown in F-12 medium containing 10 % fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively) at 37° in a 5 %CO_2 atm. When cells reach confluency, they are washed twice with 10 ml of ice-cold lysis buffer (10 mM EDTA, pH 7.4). After addition of 5 ml of lysis buffer, cells are mechanically scraped and homogenized in an ice-cold Dounce homogenizer. The suspension is centrifuged at 43,000 *g* for 10 min. The pellet is suspended in the minimum volume of ice-cold 50 mM Tris/10 mM MgCl2/1 mM EDTA (pH 8.26 at 5 °C) buffer required for the binding assay and homogenized in a Dounce homogenizer. Aminodeaminase (ADA, Boehringer Mannheim) is added to a final concentration of 3 units/ml, and the suspension is incubated at 37 °C for 15 min; the membrane suspension is subsequently kept on ice until use.

Radioligand Binding Assay

Binding of [^{125}I]APNEA (N^6-2-(4-aminophenyl)-ethyladenosine) to CHO cells stably transfected with the rat adenosine A_3 receptor clone is performed according to Stiles et al. (1985). Assays are performed in 50/10/1 buffer in glass tubes and contain 100 μl of the membrane suspension and 50 μl of inhibitor. Incubations are carried out in duplicate for 1 h at 37 °C and are terminated by rapid filtration over Whatman GF/B filters, using a Brandel cell harvester. Tubes are washed three times with 3 ml of buffer. Radioactivity is determined in a Beckman γ-counter. Nonspecific binding is determined in the presence of 40 μM R-PIA = N^6-[(R)-1-methyl-2-phenylethyl] adenosine.

Evaluation

K_i values are calculated according to Cheng and Prusoff (1973), assuming a K_d for [^{125}I]APNEA of 17 nM.

Modifications of the Method

^{125}I-4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine has been recommended as a high-affinity radioligand for the rat adenosine A_3 receptor (Olah et al. 1994).

Molecular cloning and functional expression of a sheep adenosine A_3 receptor has been reported by Linden et al. (1993).

G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain was reported by Abbracchio et al. (1995).

Molecular cloning and characterization of the human adenosine A_3 receptor was reported by Salvatore et al. (1993).

The differential interaction of the rat adenosine A_3 receptor with multiple G proteins has been described by Palmer et al. (1995).

Baraldi and Borea (2000) described new potent and selective human adenosine A_3 receptor antagonists using radioligand binding studies to the human A_3 receptor.

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Inhibition of Adenosine Uptake in Human Erythrocytes

Purpose and Rationale

Adenosine regulates multiple physiological functions in animals and humans. It plays a potent neuromodulatory role mainly by inhibiting the presynaptic transmitter release, e.g., of glutamate and aspartate. It is released by synaptic stimulation and during hypoxia in the central and peripheral nervous system. Adenosine plays a neuroprotective role in hypoxia and ischemia since it reduces the excessive stimulation of the NMDA receptors. The use of adenosine uptake inhibitors has been proposed as a new therapeutic strategy for hypoxic/ischemic disease. Due to its vasodilatory action, adenosine plays a key role in the regulation of coronary and cerebral blood flow. The rapid cellular uptake of adenosine by erythrocytes is a reason for the short duration of action of adenosine.

Human erythrocytes are used as a cellular model to detect adenosine uptake inhibitors. Erythrocytes are treated with test compound and thereafter incubated with ^{3}H-adenosine. The uptake of ^{3}H-adenosine is evaluated in relation to the untreated control group.

Dipyridamole is a potent inhibitor of adenosine uptake (IC_{50} of 3×10^{-7} M).

Standard compounds:

- Theophylline
- Dipyridamole (Persantin)
- Propentofylline (HWA 285)

Procedure

Materials and Solutions

Isotonic glycylglycine buffer, pH 7.4

KCl	5.0 mM
NaCl	119.5 mM
$MgCl_2$	2.0 mM
Glycylglycine	50 mM
Na_2HPO_4	2.0 mM
2-[^{3}H]-Adenosine (specific activity 0.2 μCi/μmol)	5 μM

Buffer-washed fresh human erythrocytes are depleted of ATP by incubation in an isotonic glycylglycine buffer at 37 °C. Aliquots of the erythrocyte suspensions are incubated for 2 min in fresh glycylglycine buffer solution containing additional 10 mM glucose and tester standard compound. In screening assays, test compounds are added at a concentration of 5×10^{-4} M. Drugs showing an effect in this assays are further tested at a concentration range of 10^{-5}–5×10^{-4} M to determine IC_{50} values (triplicate samples for each concentration).

The suspension is then incubated with 5 μM radioactively labeled 2-[^{3}H]-adenosine for 30 s. The adenosine uptake is stopped by adding cold buffer (4 °C) containing 5 μM adenosine, 10 μM glucose, and 7.4 μM dipyridamole. After centrifugation, the tritium radioactivity is determined in the supernatant.

Evaluation

The percent change of ^{3}H-adenosine uptake relative to the vehicle control group is determined. The ^{3}H-adenosine uptake of the control group is taken as 100 %; subsequent results are expressed as percentages of this.

IC_{50} values are determined by plotting the percent inhibition against test compound concentration; IC_{50} is defined as the dose of drug leading to a 50 % inhibition of adenosine uptake.

Statistical evaluation is performed by means of the Student's *t*-test.

Standard data:

- IC_{50} of dipyridamole 3×10^{-7} M

Modifications of the Method

Marangos et al. (1982) and Verma and Marangos (1985) recommended [^{3}H]nitrobenzylthioinosine binding as a probe for the study of adenosine uptake sites in the brain of various species. The highest density of binding sites was found in the caudate and hypothalamus of human and rat brain.

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Different Channels

Liomar A. A. Neves and Olga Tiniakova

Contents

TRP Channels

General Considerations

The transient receptor potential (TRP1) ion channels are named after the role of the channels in *Drosophila* phototransduction (Montell 2001). The mammalian genes are encoded by at least 28 channel subunit genes (Clapham 2003; Moran et al. 2004; Clapham et al. 2005). Six protein families comprise the mammalian TRP superfamily: the classic TRPs (TRPCs), the vanilloid receptor TRPs (TRPVs), the melastatin or long TRPs (TRPMs), the mucolipins (TRPMLs), the polycystins (TRPPs), and ankyrin transmembrane protein 1 (ANKTM1, TRPA1). The TRP channel primary structures predict six transmembrane (TM) domains with a pore domain between the fifth (S5) and sixth (S6) segments and both C and N termini presumably located intracellularly (Vannier et al. 1998).

The TRP superfamily includes >20 related cation channels that play critical roles in processes ranging from sensory physiology to vasorelaxation and male fertility. Defects in TRP channels have been associated with changes in growth control and one TRP-related protein may be a tumor suppressor. Moreover, mutations in a member of the TRP superfamily are a common cause of polycystic kidney disease, while disruption of another is responsible for mucolipidosis, a neurodegenerative disease. TRP proteins are widely expressed in the nervous system, and, in

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nonexcitable cells, TRP-related channels may be the primary mode of Ca^{2+} entry. TRP proteins are cation channels; however, they vary significantly in their selectivity and mode of activation. Nevertheless, members of the TRP superfamily share significant sequence homology and predicted structural similarities.

Based on primary amino acid homology, the conventional TRP proteins can be classified into three subfamilies: TRPC, TRPV, and TRPM (Birnbaumer et al. 2003). Three additional, more distantly related subfamilies have recently been defined: ANKTM1, a Ca^{2+}-permeant, nonselective cation channel, is the only mammalian member of the TRPA branch of TRP proteins. It is activated by noxious cold temperature, pungent mustard oil compounds and may additionally serve as a mechanosensitive transduction channel in auditory hair cells as well as an ionotropic cannabinoid receptor. The three mucolipins, TRPML1, 2, and 3, appear to be ion channels in intracellular vesicles. Mutations in TRPML1 cause mucolipidosis type IV, a neurodegenerative lysosomal storage disorder, while genetic defects in TRPP2, a member of the TRPP subfamily, are frequently encountered in patients suffering from autosomal dominant polycystic kidney disease.

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TRPC Channels

The TRPC family can be divided into three subgroups by sequence homology as well as functional similarities: C1/C4/C5, C3/C6/C7, and C2.

TRPC1 was the first member of the mammalian TRP family purported to form an ion channel (Zitt et al. 1996). Given the widespread expression of TRPC1 and its ability to coassemble with other TRPC subunits (Xu et al. 1997; Lintschinger et al. 2000; Strübing et al. 2003), TRPC1 might be a component of different heteromeric TRP complexes. The subgroup most closely related to TRPC1 comprises TRPC4 and TRPC5. TRPC4 and TRPC5 are PDZ motif-containing proteins that can form homomeric cation channels that are activated following stimulation of G_q-coupled receptors (Okada et al. 1999) as well as receptor tyrosine kinases (Schaefer et al. 2000).

Xu and Beech (2001) found that TRPC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells.

Vandebrouck et al. (2002) described involvement of TRPC in the abnormal calcium influx observed in dystrophic (*mdx*) mouse skeletal muscle fibers.

Venkatachalam et al. (2003) discussed regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C.

Bergdahl et al. (2003) found that cholestrerol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca^{2+} entry dependent on TRPC1.

Amiri et al. (2003) published FRET-based analysis of TRPC subunit stoichiometry.

Jho et al. (2005) reported that angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^{2+} influx.

Rao and Kaminski (2006) found that induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels.

Takai et al. (2004) distinguished two types of nonselective cation channels by muscarinic stimulation with carbachol in bovine muscle cells.

Kumar et al. (2006) studied upregulated TRPC1 channel in vascular injury in vivo and its role in human neointimal hyperplasia.

Less information is available about **TRPC2**, which shares approximately 30 % sequence identity with the TRPC3/6/7 subfamily. Full-length TRPC2 mRNA and several N-terminal splice variants have been found in mouse and rat tissue, but TRPC2 seems to be a pseudogene in humans (Vannier et al. 1999; Liman 2003). TRPC2 protein was localized to neuronal micovilli in rat vomeronasal organ (Liman 2003) and in the head of mouse sperm (Jungnickel et al. 2001).

TRPC3, **TRPC6**, and **TRPC7** are 75 % identical. When expressed they constitute nonselective cation currents that rectify in both the inward (− voltages) and outward (+ voltages) directions. TRPC3, TRPC6, and TRPC7 are inwardly and outwardly rectifying, have relatively low selectivity for Ca^{2+} over Na^{+}, and are activated by diacylglycerol (DAG) (Hofmann et al. 1999; Okada et al. 1999; Putney et al. 2004). These channels seem to play important roles in vascular and airway smooth muscle (Trebak et al. 2003; Yu et al. 2003; Corteling et al. 2004).

Groschner and Rosker (2005) described TRPC3 as a versatile transducer molecule that serves integration and diversification of cellular signals.

Graziani et al. (2006) investigated the potential role of membrane cholesterol as a regulator of cellular TRPC3 conductance.

Smyth et al. (2006) found a dissociation of regulated trafficking of TRPC3 channels in the plasma membrane from their activation by phospholipase C.

TRPC4 and TRPD5 are receptor-operated Ca^{2+}-permeable nonselective cation channels (Plant and Schaefer 2003).

Odell et al. (2005) found that epidermal growth factor induces tyrosine phosphorylation, membrane insertion and activation of transient receptor potential channel 4.

Saleh et al. (2006) described that angiotensin II activates two cation channels with distinct TRPC1 and TRCP6 channel properties in rabbit mesenteric artery myocytes.

Shimizu et al. (2006) reported that Ca^{2+}-calmodulin-dependent myosin light chain kinase is essential for activation of TRPC5 channels expressed in HEK293 cells.

Shi et al. (2004) described multiple regulation by calcium of murine homologs of transient

receptor potential proteins TRP6 and TRP7 expressed in HEK293 cells.

Xu et al. (2006) identified sphingosine-1-phosphate as an activator of the calcium channel TRPC5, which controls vascular smooth muscle activity.

Estacion et al. (2004) studied activation of human TRPC6 channels by receptor stimulation.

Basora et al. (2003) reported that 20-hydroxyeicosatetraenoic acid (20-HETE) activates mouse TRPC6 channels expressed in HEK293 cells.

Maruyama et al. (2006) found that heteromultimeric TRPC6-TRPC7 channels contribute to arginine vasopressin-induced cation current of A7r5 vascular smooth muscle cells.

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TRPM Channels

The TRPM subfamily has eight members divided into four groups: M1 (melastatin)/M3, M7 (TRPPLIK)/M6, M2/M8, and M4/M5 (Hartzeneck 2005; Fonfrai et al. 2006). **TRPM1** may be regulated through direct interaction with a cytosolic isoform generated by alternative RNA splicing (Xu et al. 2001). **TRPM2** is a 1,503-amino-acid protein that is highly expressed in brain (Nagamine et al. 1998) and present in blood cells.

Perraud et al. (2003) reviwed TRPM2 Ca^{2+}-permeable cation channels.

Hill et al. (2004a, b) reported inhibition of TRPM2 channels by the antifungal agents clotrimazole and econazole as well as by flufenamic acid; Kraft et al. (2006) by *N*-(*p*-amylcinnamoyl) anthranilic acid.

Identified first by sequencing projects, the function of **TRPM3** is poorly understood. The hTRPM3 gene maps to human chromosome 9q-21.12 and encodes a 1,555-amino-acid protein.

TRPM4 and **TRPM5** have similar characteristics. TRPM4b, a splice variant of TRPM4, and TRPM5 are Ca^{2+}-activated, voltage-modulated, monovalent-selective cation channels with 25-pS single-channel conductances (Launay et al. 2002; Hofmann et al. 2003; Nilius et al. 2003). Pérez et al. (2003) studied the ion channel TRPM5 in taste receptor cells.

TRPM6 and **TRPM7** comprise a unique subfamily of TRP proteins with both channel and kinase activities. TRPM7, which has 1,863 amino acid residues, was identified in a yeast two-hybrid screen as a protein interacting with phospholipase C (PLC) (Runnels et al. 2002; Schmitz et al. 2003). It seems to be ubiquitously expressed. Takezawa et al. (2004) described receptor-mediated regulation of the TPRM7 channel through its endogenous protein kinase domain.

TRPM8 is a 1,104-amino-acid protein that does not seem to contain associated enzymatic domains. TRPM8 is a nonselective, voltage-modulated conductance. At colder temperatures (8–28 °C) or in the presence of menthol, TRPM8 current is activated at a more physiological range of voltages (Brauchi et al. 2004; Voets et al. 2004). This channel is expressed in small-diameter primary sensory neurons, where it presumably functions as a thermosensor (McKemy et al. 2002; Peier et al. 2002).

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TRPV Channels

The TRPV subfamily is named after the first mammalian member of the subfamily, vanilloid receptor 1 (Caterina et al. 1997; Benham et al. 2002; Montell et al. 2002; Clapham 2003; Mutai and Heller 2003). TRPV5 and TRPV6 are highly Ca^{2+}-selective TRP channels that mediate transepithelial Ca^{2+} transport in kidney and intestine (Hoenderop et al. 2003).

The TRPV channel subfamily has six members divided into two groups: V1/V2/V3/V4 and V5/V6. The vanilloid receptor, TRPV1, is the best understood ion channel in this class (Caterina et al. 1997; Caterina and Julius 2001).

TRPV channels as temperature sensors were reviewed by Benham et al. (2003).

The expressed **TRPV1** capsaicin receptor is a heat/proton/lipid/voltage-modulated Ca^{2+}-permeant (P_{Ca}/P_{Na} 10) ion channel. A more voltage-gating centric explanation is that at warmer temperatures (>37 °C) or in the presence of capsaicin, TRPV1 current is activated by a more physiological range of voltages (Brauchi et al. 2004). TRPV1 is desensitized by internal Ca^{2+}; it is not activated by store depletion.

TRPV1, V2, and V3 are activated by the synthetic compound 2-aminoethoxydiphenylborate (2-APB) (Chung et al. 2004; Hu et al. 2004). Endogenous cannabinoid receptor ligands, such as anandamide, are potential TRPV1 agonists.

The vanilloid receptor-like channel **TRPV2** is 50 % identical to TRPV1, but is insensitive to capsaicin (Caterina et al. 1999). Like TRPV1 it is more permeable to Ca^{2+} than to Na^{+} ($P_{Ca}/P_{Na} =$ 3:1). It has been proposed to mediate high-threshold noxious heat sensation, perhaps in the lightly myelinated A nociceptors, but its presence in nonsensory tissue suggests other functions as well.

TRPV3 is expressed widely but most strikingly in skin. Increasing temperature from 22 °C to 40 °C in mammalian cells transfected with hTRPV3 elevates intracellular calcium by activating a nonselective cationic conductance (P_{Ca}/P_{Na} 10:1) (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002). As in sensory neurons, the current is steeply dependent on temperature, sensitizes with repeated heating, and displays a striking hysteresis on heating and cooling, but the extent of expression in sensory neurons is controversial. Based on these properties, TRPV3 is thermosensitive in the physiological range of temperatures between TRPM8 and TRPV1 and may play a role in pain.

TRPV4 is 40 % identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na} = 6$), which, like TRPV1, displays a gently outwardly rectifying *I–V* relation. TRPV4 is 40 % identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na} = 6$), which, like TRPV1, displays a gently outwardly rectifying *I–V* relation.

TRPV5 and TRPV6 comprise a separate subfamily of TRPVs with only 30 % identity with TRPV1. The expressed channels strongly inwardly rectify and are the most Ca^{2+}-selective ($P_{Ca}/P_{Na} > 100$) (Nilius et al. 2000; Vennekens et al. 2000; Yue et al. 2001; Den Dekker et al. 2003) of all TRP channels. These properties are consistent with proposed mechanisms for Ca^{2+}-selective channels in which negatively charged glutamic or aspartic acid residues provide a binding site for divalents within the pore. Intra- and extracellular [Ca^{2+}] (Yue et al. 2001; Hirnet et al. 2003; Bödding and Flockerzi 2004) and calmodulin (Lambers et al. 2004) regulate TRPV6 activity. The localization of TRPV5 and TRPV6 to the proximal small intestine and collecting duct of the kidney, along with mouse knockout data, suggests that this family is important in calcium uptake via epithelial cells (Hoenderop et al. 2005).

Lee et al. (2005) reported that PIP_2 activates TRPV5 and releases its inhibition by intracellular Mg^{2+}.

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TRPV1 Receptor Assay

Purpose and Rationale

The vanilloid receptor-1 (TRPV1) is a nonselective cation channel, predominantly expressed by peripherally sensory neurons,

which is known to play a key role in the detection of noxious painful stimuli, such as capsaicin and heat. Smart et al. (2000, 2001) found that the endogenous lipid anandamide is a full agonist at the human vanilloid receptor and characterized human vanilloid VR1 pharmacology using FLIPR. With these methods, Gunthorpe et al. (2004) identified and characterized a potent and selective vanilloid receptor (VR1/TRPV1) antagonist.

Procedure

Cloning and Expression of VR1 Receptors in HEK293 Cells

Human VR1 cDNA was identified using the published rat VR1 sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double-strand sequenced, and stably expressed in HEK293 cells. Rat VR1 cDNA was amplified from rat DRG cDNA and similarly expressed in HEK293 cells.

Cell Culture

hVR1-HEK293 cells were grown as monolayers in minimum essential medium (MEM) supplemented with nonessential amino acids, 10 % fetal calf serum, and 0.2 mM L-glutamine and maintained under 95 %/5 % O_2/CO_2 at 37 °C. Cells were passaged every 3–4 days and the highest passage number used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper et al. (1990).

Electrophysiological Studies

Cells were plated and cultured on glass coverslips at 26,000 cells cm^{-2} and whole-cell voltage clamp recordings were performed at room temperature (20–24 °C), using standard methods. The extracellular solution consisted of (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 30, HEPES-NaOH 25, pH 7.3. For anandamide application, this solution was supplemented with 0.2 % lipid-free bovine serum albumin. Patch pipettes of resistance 2–5 MΩ were fabricated on a Sutter Instruments P-87 electrode puller and were filled with the following solution (mM): CsCl 140, $MgCl_2$ 4, EGTA 10, HEPESCsOH 10, pH 7.3. All recordings were made from single, well-isolated, phase-bright cells. Currents were recorded at a holding potential of −70 mV using an Axopatch 200B amplifier. Data acquisition and analysis were performed using the pClamp7 software suite. Drug applications were affected with an automated fast-switching solution exchange device (Warner Instruments SF-77B; time for solution exchange ≈30 ms).

Measurements of $[Ca^{2+}]_i$ Using the FLIPR

hVR1-HEK293 cells were seeded into black-walled, clear-base 96-well plates (Costar, UK) at a density of 25,000 cells per well inMEM, supplemented as above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator Fluo-3AM (4 μM; Teflabs, Austin, Tex., USA) at 25 °C for 120 min. The cells were washed four times with, and finally cultured in Tyrode's medium containing 0.2 % BSA before being incubated for 30 min at 25 °C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, UK) to monitor cell fluorescence (λ_{EX} = 488 nM, λ_{EM} = 540 nM) (Sullivan et al. 1999) before and after the addition of various agonists.

Evaluation

Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± SEM unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software, Calif., USA). p*KB* values were generated from

IC50 curves for the antagonist vs a fixed EC_{80} concentration of agonist using the Cheng–Prusoff equation.

Modifications of the Method

Rao and Kaminski (2006) reported that induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels.

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Effect of Different Peptides

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Measurement of Contractile Force of Isolated Cardiac Myocytes

Purpose and Rationale

Eschenhagen et al. (1997) developed a method for culturing embryonic cardiomyocytes in a collagen matrix to produce a coherently contracting three-dimensional model heart tissue that allows direct measurement of isometric contractile force.

Procedure

Ventricles from 9- to 11-day incubated chicken embryos (Cavanaugh 1955) are minced in Dulbecco's minimal essential medium (DMEM); washed once with 0.25 % trypsin/0.1 % EDTA in phosphate-buffered saline (PBS), pH 7.45; and then digested in fresh trypsin/EDTA for 15 min at 37 °C. The supernatant is discarded, and the pellet is subjected to digestion with 0.1 % collagenase (144 U/mg) in PBS, pH 7.45, for 30 min at 37 °C. This supernatant is discarded and the pellet digested further with several cycles of collagenase for 10–20 min each until the pellet is completely digested. DNase I (40 µl, 1 mg/ml in PBS) is added between cycles depending on the presence of viscous DNA. The isolated cells are kept in Petri dishes in DMEM supplemented with 15 % heat-inactivated fetal calf serum in the CO_2 incubator. After completion of the digestion, the cells are incubated for another 30–60 min in the CO_2 incubator (preplating). The cell suspension is centrifuged at 250 rpm (12 g). The pellet is resuspended in 10 ml culture medium (DMEM), 10 % inactivated horse serum, 2 % chicken embryo extract (Gibco BRL), 2 mmol/L-glutamine, 10 µg/ml streptomycin, and 100 U/ml penicillin G, recentrifuged at 250 rpm and finally resuspended in culture medium at 2–3 $\times$ 10^6 cells per ml.

For casting cardiomyocyte-populated collagen gels, strips of Velcro are glued with silicone rubber to glass tubes (13 mm length, 3 mm outer diameter, 2 mm inner diameter). Pairs of Velcro-coated tubes, kept at a fixed distance by a stainless steel wire spacer, are placed in rectangular wells (15 × 17 × 4 mm) cut into a layer of silicone rubber in a 100 mm polymethylpentene Petri dish. This assembly is autoclaved before use. For each gel, 1 ml of an ice-cold collagen/cell mixture is poured into each well between the Velcro-coated glass tubes. This mixture has the same composition as the culture medium and contains in addition to 1 mg neutralized collagen I from rat tail (Upstate Biotechnology, Inc.) 1×10^6 cardiomyocytes, the acetic acid in the collagen solution, and the NaOH to neutralize it. The mixture is allowed to gel at 37 °C for 60 min before culture medium is added to the dish. Medium changes are performed after overnight and then every other day.

After 6–11 days in culture, the gels are removed from the culture dish, the spacers are withdrawn, and one of the glass tubes is mounted on a fixed electrode; the other tube is connected by an inelastic silk string to an isometric force transducer attached to a Wekagraph thermal array recorder (Föhr Instruments, Heidelberg, Germany). The preparation is adjusted to its original (spacer) length before it is immersed in a conventional water bath filled with modified Tyrode's solution maintained at 35 °C and continuously gassed with 95 % O_2 and 5 % CO_2.

After a 30–60 min equilibration period without pacing, force and frequency reach a stable value. Gels are then electrically stimulated with rectangular pulses (10 ms, 20–40 V) at a standard frequency of 1.5 Hz. Preload is stepwise adjusted to L_{max}, the length at which the preparation develops maximal force. Cumulative doses of inotropic compounds, e.g., isoprenaline or forskolin, are added. All gels are exposed to a concentration–response curve for calcium (1.8–12.6 mmol/l) and one or two additional inotropic stimuli.

Evaluation

All values are presented as arithmetic means ± SEM. Student's *t*-test for paired observations is used to compare force of contraction, resting tension, or beating frequency before and after other interventions.

Modifications of the Method

Ferrara et al. (1997) studied the role of Gi proteins and β-adrenoceptors in the age-related decline of contraction in guinea pig ventricular myocytes. The isolated myocytes were placed in Krebs–Henseleit solution in a Perspex chamber on the stage of a Zeiss IM inverted microscope and superfused with Krebs–Henseleit solution containing 1 mmol/l Ca^{2+} at 2 ml/min and 32 °C. Cells were selected using the following criteria: rod shaped, without sarcolemmal blebs, no spontaneous contractions, stable baseline contraction to electrical stimulation at 0.5 Hz, and sarcomere length not shorter than 1.67 μm. The image of the cells was displayed on a TV monitor and the length change measured with a video motion detector. Contraction amplitude and velocity were expressed as change in sarcomere length, calculated from the change in length of the myocyte and its original sarcomere length.

Using a similar technique, Harding et al. (1988) studied contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium, and Harding et al. (1992) isolated ventricular myocytes from failing and non-failing human heart.

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Adrenomedullin

General Considerations

Adrenomedullin is a 52-amino-acid peptide originally discovered in human adrenal pheochromocytoma by monitoring the elevating activity of platelet cAMP (Kitamura et al. 1993). Molecular cloning of rat adrenomedullin was reported by Sakata et al. (1993). The genomic structure of human adrenomedullin gene was reported by Ishimitsu et al. (1994). Adrenomedullin and **proadrenomedullin N-terminal 20 peptide (PAMP),** which are both hypotensive and bronchodilating, are derived from **preproadrenomedullin** (Kanazawa et al. 1995; Iwasaki et al. 1996; Shimosawa and Fujita 1996; Hinson et al. 1998; Samson 1998; Autelitano and Tang 1999; Jimenez et al. 1999; Lopez et al. 1999; Tajima et al. 1999).

Adrenomedullin is found ubiquitously in tissues and organs, especially in cardiovascular tissues and in the kidney, lung, brain, and endocrine glands (Wimalawansa 1996; Van Rossum et al. 1997; Eto et al. 1999; Jougasaki and Burnett 2000; Kitamuro et al. 2000). The main biological effect is vasodilatation (Ishiyama et al. 1993; Nikitenko et al. 2002). A hypotensive effect has been found in rats (Khan et al. 1997), rabbits (Fukuhara et al. 1995), and man (Lainchbury et al. 1997). Adrenomedullin belongs to the calcitonin gene-related peptide/calcitonin peptide family as it shares approximately 25 % homology with calcitonin gene-related peptide (Kitamura et al. 1993). Several pharmacological studies are related to the vasodilating effect of adrenomedullin, e.g., in mouse aorta (Ashton et al. 2000), in the mesenteric vascular bed (see section "Effect on Mesenteric Blood Flow in Rats," chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus") (Santiago et al. 1995), in the hind limb vascular bed (see section "Perfused Hindquater Preparation with Sympathetic Nerve Stimulation in Rats," chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus") (Santiago et al. 1994; Champion et al. 1996, 1997), in the pulmonary vascular bed (see section "Effect of Pulmonary Blood Flow," chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus") (DeWitt et al. 1994; Lippton et al. 1994; Heaton et al. 1995; Nossaman et al. 1995), on cerebral blood flow in dogs (Baskaya et al. 1995) and in cats (Takao et al. (1999), on renal hemodynamics in dogs (see section "Effect of Peripheral Blood Flow in Anesthetized Dogs," chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus") (Ebara et al. 1994; Yukawa 1998), or on vasodilation in perfused rat kidneys (Hayakawa et al. 1999). Intravenous infusion of adrenomedullin exerted diuresis and natriuresis without major changes in blood pressure and produced beneficial hemodynamic and renal vasodilator effects in rats with compensated heart failure (Vari et al. 1996; Nagaya et al. 1999). In isolated perfused, paced rat heart preparations, adrenomedullin showed a dose-dependent inotropic effect (Szokodi et al. 1998). Pulmonary vasodilator responses and vasorelaxant effects in isolated pulmonary artery rings were found by Gumusel et al. (1998). Adrenomedullin is a growth-promoting factor for cultured vascular smooth muscle cells (Iwasaki et al. 1998) and fibroblasts (Isumi et al. 1998).

Willenbrock et al. (1999) showed a beneficial effect of adrenomedullin on renal function in rats with aortocaval shunt.

Adrenomedullin inhibits gastric secretion in rats with chronic gastric fistula (see section "Chronic Gastric Fistula in Rats," chapter "▶ Pharmacological Effects on Gastric Function") (Rossowski et al. 1997) and inhibits reserpine-induced gastric lesions in rats (Clementi et al. 1998). Tsuchida et al. (1999) found an inhibition of cholecystokinin-stimulated amylase secretion by adrenomedullin in rat pancreatic acini.

Rademaker et al. (2003) discussed the role of adrenomedullin in the pathophysiology of heart failure.

Lewis et al. (1998) described a specific and sensitive radioimmunoassay for human adrenomedullin.

Ohta et al. (1999) developed a one-step direct assay for adrenomedullin with monoclonal antibodies.

N-terminal fragments of adrenomedullin show vasopressor activities (Watanabe et al. 1996).

Adrenotensin, another adrenomedullin gene product, contracts pulmonary blood vessels in an endothelium-dependent manner (Gumusel et al. 1996).

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Receptor Binding of Adrenomedullin

Purpose and Rationale

Muff et al. (1995) and Poyner (1997) reviewed the binding characteristics of the structurally related hormones calcitonin, calcitonin gene-related

peptide, amylin, and adrenomedullin. Vine et al. (1996) compared in in vitro binding of adrenomedullin, calcitonin gene-related peptide, and amylin.

Specific adrenomedullin binding sites were described in the human brain (Sone et al. 1997), in the rat spinal cord (Owji et al. 1996), in cultured brain cells (Zimmermann et al. 1996), and in cultured rat mesangial cells (Osajima et al. 1996).

Procedure

Human brain is obtained at autopsy. For preparation of membranes, tissues are homogenized in ice-cold 50 mM HEPES buffer, pH 7.6, containing 0.25 M sucrose, 10 μg/ml soybean trypsin inhibitor, 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin, 0.5 μg/ml antipain, 0.1 mg/ml benzamidine, 0.1 mg/ml bacitracin, and 30 μg/ml aprotinin. The homogenates are centrifuged at 1,500 g for 20 min at 4 °C. The pellets are resuspended in 10 vol of the above buffer without sucrose and centrifuged at 100,000 g for 1 h at 4 °C. The final pellets are resuspended to a concentration of 2–10 mg protein/ml, aliquoted, and stored at −80 °C.

For the receptor-binding assay, brain membranes (100 μg protein) are incubated at 4 °C in 0.5 ml binding buffer (20 mM HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM NaCl, 4 mM KCl, 1 mM EDTA, and 0.3 % BSA) containing 0.3 nM ^{125}I-human adrenomedullin in siliconized microcentrifuge tubes. Pellets are washed with 0.5 ml binding buffer at 4 °C and counted in a γ-counter.

Evaluation

Nonspecific binding is determined in the presence of 200 nM unlabeled human adrenomedullin. Specific binding is defined as total binding minus nonspecific binding. Data are calculated as mean ± SEM.

Modifications of the Method

Eguchi et al. (1994) studied the binding of human adrenomedullin and analogs and the adenylate cyclase activity in cultured rat vascular smooth muscle cells.

Zimmermann et al. (1995) showed that adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured human neuroblastoma SK-N-MC cells.

Moody et al. (1997) investigated the binding affinity of adrenomedullin in C6 glioma cells.

Findings of Belloni et al. (1998) suggested the existence of different receptor subtypes for adrenomedullin in the human adrenal cortex.

Mazzocchi et al. (1999) found abundant [^{125}I]-adrenomedullin binding sites in both zona glomerulosa and adrenal medulla in the rat adrenal gland.

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Atrial Natriuretic Factor (ANF)

General Considerations

Purpose and Rationale

The atria of mammalian hearts synthesize and secrete peptides with potent natriuretic and vasoactive properties known as atrial natriuretic factor (ANF) (de Bold et al. 1981). The atrial natriuretic peptide hormonal system consists of a 126-amino-acid prohormone synthesized within myocytes of the heart and stored in storage granules within the heart before release into circulation (Kangawa and Matsuo 1984; Oikawa et al. 1984; Vesely 1992). This hormonal system contains several peptides from the 126-amino-acid prohormone with blood pressure-lowering, natriuretic, diuretic, and/or kaliuretic properties (Martin et al. 1990; Vesely et al. 1994). Thus, peptides consisting of amino acid 1–30 (LANP = long-acting natriuretic peptide), 31–67 (vessel dilator), 79–98 (kaliuretic peptide), and 99–126 (ANF) each have blood pressure-lowering, natriuretic, diuretic, and/or kaliuretic properties both in humans and in animals. Human and rat atria predominantly secrete a peptide of 28-amino-acid residues, ANF (99–126), which represents the C-terminus of a precursor sequence of 126-amino-acid residues. In addition, vessel dilator and LANP circulate as distinct entities after having been proteolytically cleaved from the rest of the amino terminus by proteases (Ackerman et al. 1997).

Plasma immunoreactive ANF (99–126) concentration increases in normal rats after volume expansion, while infusion of the peptide lowers blood pressure in several animal models of hypertension.

An international standard for atrial natriuretic factor was established by an international collaborative study (Poole et al. 1988). Human ANF (99–126) was synthesized, highly purified, and distributed to several laboratories who performed radioimmunoassays, radioreceptor assays, and an in vitro assay using the vasorelaxant activity in precontracted rat aortic strips.

The C-type natriuretic peptide is a 22-amino-acid peptide that was initially identified in the central nervous system (Ogawa et al. 1992; Barr et al. 1996; Amin et al. 1996). The distribution of C-type natriuretic peptide, which has structural homology with atrial and brain natriuretic peptides and also similar activities, is wide and includes the endothelium, myocardium, and gastrointestinal and genitourinary tracts. **Brain natriuretic peptide** has been described as a novel cardiac hormone (Nakao et al. 1991). Yasue et al. (1994) studied the localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. N-terminal pro-brain natriuretic peptide became a diagnostic screening tool to differentiate between patients with normal and left ventricular systolic function (Bay et al. 2003; Gardner et al. 2003).

Jiao and Baertschi (1993) reviewed the neural control of the endocrine rat heart. Stimulation of cardiac sympathetic nerves potently stimulates ANF secretion.

ANF inhibits proliferation in non-myocardial cells and is anti-hypertrophic in cardiomyocytes. Silberbach et al. (1999) reported that activation of an extracellular signal-regulated protein kinase is required for the anti-hypertrophic effect of atrial natriuretic factor in neonatal rat ventricular myocytes.

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Bioassay for ANF

Purpose and Rationale

Matsui et al. (1987) described a rapid bioassay for quantification of atrial natriuretic polypeptides in rats with continuous recording of the conductivity of the urine, urine flow, and blood pressure.

Procedure

Male Sprague Dawley rats weighing 180–240 g are anesthetized with 60 mg/kg i.p. pentobarbital sodium. Anesthesia is maintained by injection of supplemental doses of pentobarbital sodium. After tracheotomy, catheters are placed into the left jugular vein and the right carotid artery for injection of samples or infusion of 10 % mannitol in 0.9 % saline and for blood pressure recording. Through a small suprapubic incision, the bladder is cannulated for collection of urine, and the cannula is connected to a device for continuous measurement of urine conductivity, by which the electrolyte concentration is estimated. Urine flow rate is recorded using a drop counter, and urine samples are collected in a plastic tube.

After completion of surgery, 0.6–0.8 ml of 10 % mannitol in 0.9 % saline is administered into the jugular vein and is continuously infused at a rate of 4.0 ml/h by a syringe pump. Following an equilibration of 45–60 min, the bioassay is started when the urine flow is increased to 50–75 µl/min. All test samples with a volume of 100 µl are directly injected into the jugular vein followed by a wash injection of ~30 µl of saline. The mean arterial blood pressure, urine conductivity, and urine flow rate are simultaneously recorded.

For dose–response curves, serial dilutions of human ANF (α-hANP) and of test substance are prepared. Vehicle and various doses of α-hANP or test substance are injected in a randomized sequence. Immediately after the injection, urine is collected for 10 min. Urine volumes are determined by weighing, and urinary sodium and potassium concentrations are measured by flame photometry.

Evaluation

Linear regression analyses by the method of least squares are used for evaluating dose–response relationship. One-way analysis of variance for repeated measures and the Newman–Keuls test are used to detect statistical differences.

Modifications of the Method

Petersen et al. (1988) determined atrial content and plasma levels of atrial natriuretic peptides in rats with chronic renal failure. The natriuretic activity in the bioassay was estimated as the increase in Na excretion in urine samples from the control period to the maximal natriuretic response.

Allen and Gellai (1987) measured cardioinhibitory effects of atrial peptide in conscious chronically instrumented rats. The hemodynamic and renal excretory responses were measured with and without replacement of urinary fluid losses.

Thibault et al. (1984) characterized the biological activities of atrial natriuretic factor-related peptides in vivo by a natriuretic bioassay and

in vitro by relaxation of contracted intestinal smooth muscle (chick rectum).

Schiller et al. (1986) tested synthetic analogs of atrial natriuretic peptide in the rabbit aorta assay and in a bioassay monitoring suppression of aldosterone secretion from bovine zona glomerulosa cells.

Dlouha and McBroom (1986) measured diuretic and natriuretic activity of atrial extracts of taurine-treated normal and cardiomyopathic hamsters by urine flow and Na^+ excretion in the rat bioassay.

St.-Louis and Schiffrin (1988) measured vasorelaxant effects of different atrial natriuretic peptides on rat aortic and mesenteric artery rings and compared the results with the potency of the same peptides to displace ^{125}I-labeled ANP on membrane preparations of aorta and of mesenteric vein bed.

Kohse et al. (1992) described a bioassay for quantitative determination of natriuretic peptides in human biological samples using bovine aortic and bovine kidney epithelial cultured cells. The amount of cyclic AMP produced by these cells was measured by radioimmunoassay.

Kecskemeti et al. (1996) studied the effects of atrial natriuretic peptide (ANP) on action potential characteristics in various (human, rabbit, guinea pig) atrial and guinea pig ventricular papillary muscles. The data suggested that ANP inhibits the slow inward Ca^{2+} channel activity and facilitates the K^+ channel activity.

Salt-sensitive hypertension was found in ANP knockout mice (Melo et al. 1998).

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Receptor Binding of ANF

Purpose and Rationale

Schiffrin et al. (1985) described receptors for atrial natriuretic factor in the rat.

Procedure

Synthetic ANF (99–126) is iodinated with ^{125}I by a modification (Gutkowska et al. 1984) of the chloramine T method (Greenwood and Hunter 1963). Separation of radiolabeled ANF from free iodine is achieved by immunoaffinity chromatography followed by C-18 reversed phase high-pressure liquid chromatography.

For preparation of membranes, Sprague Dawley rats weighing 300 g are sacrificed by decapitation. The atria, ventricles, renal arteries, mesentery, mesentery vascular bed, and adrenals are processed for binding studies. Adrenal capsules are separated by manual compression. The tissues are immersed in 0.25 M sucrose solution, finely minced with scissors, and homogenized in a Polytron (setting 8, 10 s twice). The homogenate is centrifuged at 1,550 g for 10 min at 4 °C; the supernatant is decanted and recentrifuged. The final supernatant is filtered through a cheesecloth then centrifuged at 10,400 g for 30 min. The pellet is resuspended in a 0.05 M Tris–HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 % bacitracin, and 1 μM aprotinin. Proteins are measured by the Coomassie blue method (Spector 1978). Next, bovine serum is added at a concentration of 0.2 %, and the membranes are diluted to a protein concentration of 0.25–1 mg/ml in the Tris buffer containing 0.2 % albumin.

The ^{125}I-AFN binding assay uses 30–50 pM of labeled ANF and 10^{-13} to 10^{-6} unlabeled ANF in competition experiments. In saturation experiments, increasing concentrations of ^{125}I-AFN (6–200 pM) are used, and nonspecific binding is determined by incubation in the presence of 1 μM unlabeled ANF for each point of the saturation curves. Incubation is done with 25–100 μg of receptor protein per tube, at 4 °C for 60 min. All assays are performed in duplicate. Separation of bound and free radioactivity is achieved by rapid filtration through polyethylenimine-treated Whatman GF/C filters soaked with the assay buffer. The filters are washed twice with 3 ml of 0.9 % NaCl and then are allowed to dry and are counted in a gamma counter.

Evaluation

Binding data are analyzed by computer-assisted nonlinear regression analysis using the LIGAND program (Munson and Rodbard 1980). The inhibition constant K_i is calculated according to the Cheng and Prusoff equation.

Modifications of the Method

Misono (2000) found that the binding of atrial natriuretic factor to its receptor is dependent on chloride concentration.

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ANF Gene Expression

Purpose and Rationale

Production of atrial natriuretic factor and brain natriuretic peptide can be measured by gene expression using total RNA extraction and

Northern blot analysis and the quantitative competitive reverse transcription polymerase chain reaction (Hama et al. 1995; Ogawa et al. 1996, 1997, 1998, 1999).

Procedure

Extraction of Plasma and Tissue Samples

Plasma samples are acidified by adding 100 µl/ml of 1 mol/l HCl and passed through Sep-Pak C_{18} cartridges (Millipore) that are pre-wetted with 5 ml of 80 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) and 10 ml of 0.1 % TFA. The cartridges with the absorbed peptides are washed with 20 ml of 0.1 % TFA and eluted with 3 ml of 60 % acetonitrile in 0.1 % TFA. Tissue samples are homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/l acetic acid, and 1 % NaCl and centrifuged at 10,000 g for 30 min at 4 °C. The supernatants are then extracted with the use of Sep-Pak C_{18} cartridges by elution with 80 % acetonitrile in 0.1 % TFA. The eluates from tissue or plasma are freeze-dried and processed for RIA.

Total RNA Extraction and Northern Blot Analysis

Atrial and ventricular tissue samples from individual rats are extracted using Trizol (Gibco BRL). Total RNA from the atrium (10 µg) and ventricle (20 µg) are electrophoretically separated in an agarose-formaldehyde gel followed by blotting to nylon membranes (Hybond N+, Amersham) overnight. Membranes are prehybridized in 2.5 × Denhardt's solution, 5 × SSC, 50 % formamide, 25 mmol/l KH_2PO_4, pH6.4, 0.2 % SDS, and 0.2 mg/mL herring ssDNA for 3 h at 42 °C for cDNA probes or prehybridized in 5 × Denhardt's solution, 6 × SSC, 50 mmol/l NaH_2PO_4, 0.5 % SDS, and 0.2 mg/ml herring ssDNA for 3 h at 5 °C below the calculated T_m for oligonucleotide probes. Hybridization is then carried out for 16 h at the same temperature and the same solution as the prehybridization condition except for the presence of the radiolabeled probes. Five cDNA probes and two oligonucleotide probes are used. The cDNA probes used are as follows: (1) a 900-bp *Eco*RI/*Hin*dIII fragment containing the full-length rat ANF cDNA, (2) a 595-bp *Sal*I fragment containing full-length rat BNP cDNA, (3) a 5-kb *Eco*RI/*Sal*I fragment of the mouse 28S rRNA cDNA probe, (4) a 2-kb *Bam*HI/*Bgl*II fragment of the mouse PGK gene cDNA, and (5) rat α_1-III collagen cDNA containing 1,300 bp of the 3′ noncoding and coding regions. The two oligonucleotide probes are 39 and 24 base fragments specific for unique regions in the 3′ untranslated regions of the rat α-MHC and β-MHC genes. The α-sequence is 5′GGGATAGCAACAGCGAGGCTCTTTCT-GCTGGACAGGTTA-3′($T_m = 60$ °C), and the β-sequence is 5′-CTCCAGGTCTCAGGGCTT-CACAGG-3′ ($T_m = 52$ °C).

The cDNAs are labeled with 5′-[α^{32}P]dCTP (3,000 Ci/mmol, Amersham) using the Megaprime DNA labeling system (Amersham). The oligonucleotides are labeled with [γ -^{32}P] ATP (3,000 Ci/mmol, Amersham) using a 5′-end-labeling kit (Amersham). At the end of hybridization, the membranes are washed twice at 42 °C with 2 × SSC and 1 % SDS and twice at 55 °C with 1 × SSC and 0.1 % SDS for the cDNA probes or are washed once at 30 °C with 5 × SSC and 0.1 % SDS and twice at the same temperature as the hybridization with 1 × SSC and 0.1 % SDS for the oligonucleotide probes. Before additional probing, bound counts are completely stripped from the membranes by washing twice in 10 mmol/l sodium citrate, pH 6.8, and 0.25 % SDS for 10 min at 100 °C. Autoradiographs are scanned with an Ultrascan XL laser densitometer (LKB Produkter) and LKB 2400 Gelscan XL software package. The scanning values of ANF, BNP, collagen-III, and α-MHC and β-MHC mRNAs are normalized to 28S ribosomal RNA or PGK mRNA as internal controls to correct for differences in the amount of RNA applied and transfer efficiency.

Plasma and cardiac tissue concentrations of immunoreactive ANF and BNP are determined by RIA with anti-rat $ANF_{99–126}$ and anti-rat $BNP_{64–95}$ sera, respectively, from Peninsula Laboratories.

Quantitative Competitive Reverse Transcription Polymerase Chain Reaction

RNA samples are reverse transcribed with Super-Script II RNase H2 Reverse Transcriptase and oligo(dT)12–18 primer with the use of a reverse transcription kit (Gibco BRL). An aliquot of the cDNA product is used for PCR amplification with ANF primers. A dilution series of total RNA (5 mg) aliquots is prepared for each sample. Each dilution is spiked with competitor RNA. After the PCR, aliquots (5 ml) of the PCR product are electrophoresed on a 2 % agarose gel and visualized by ethidium bromide staining. Photographs are taken with Polaroid 55 film, and the negatives are scanned with the use of an Ultrascan XL laser densitometer and Gelscan XL 2000 software package. The ratio of the density of the competitor RNA to the target RNA is plotted against the amount of the competitor RNA added to each reaction.

Evaluation

All results are expressed as mean ± SEM. A level of $P < 0.5$ is considered significant. ANOVA is performed to determine statistical differences among multiple groups. When significance is obtained by ANOVA, Fisher's least squares difference post hoc analysis is used to determine pairwise differences.

Modifications of the Method

Ramirez et al. (1997) reported that the nuclear δ_B isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates atrial natriuretic gene expression in cultured neonatal rat ventricular myocytes.

Thuerauf et al. (1998) found that the p38 mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element and discussed the potential role for the transcription factor ATF6.

Kakita et al. (1999) studied p300 protein as a coactivator of the transcription factor GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene.

Bianciotti and de Bold (2000) investigated the effect of selective ET_A receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension in rats.

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Radioimmunoassay for ANF

Purpose and Rationale

Gutkowska et al. (1984) developed a direct radioimmunoassay of atrial natriuretic factor (ANF). The method uses a synthetic 26-amino-acid fragment (8–33 ANF) of the native peptide.

Procedure

Because 8–33 ANF is a small molecule, it is necessary to covalently conjugate the peptide to a larger protein (bovine thyroglobulin) for immunization. To 50 mg thyroglobulin dissolved in 2 ml distilled water, pH 7.4, 30 mg CDI [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl] is added in 1 ml distilled water, pH 7.4. Then 5 mg 8–33 ANF in water is added dropwise while stirring. The solution is kept overnight at 4 °C, then another 30 mg CDI is added and the mixture is kept for 2 h at room temperature with constant stirring. The cloudy mixture is dialyzed for 24 h at 4 °C against 0.9 % saline. The dialyzed material is then fractionated and stored at −70 °C.

For immunization, 100 μg of the ANF–thyroglobulin complex is suspended in 1 ml saline, thoroughly mixed with 1 ml complete Freund's adjuvant and injected into the shaved backs of New Zealand white rabbits. Each animal receives also 0.5 ml *Bordetella pertussis* vaccine subcutaneously with the primary immunization. The animals are reimmunized at monthly intervals with 100 μg of antigen in incomplete Freund's adjuvant and bled by ear artery 10 days after the booster injection.

For iodination, 5 μg ANF in 5 μl 0.01 M ammonium acetate, pH 5.0, is introduced in a 1.5 ml Eppendorf vial followed by the addition of 1 mCi Na ^{125}I in a volume of 25 μl. Chloramine T 10 μg/10 μl is added to the reaction vial, and 30 s later, sodium metabisulfite (20 μg/10 μl) is added. Each addition is followed by mixing. Purification of the iodinated tracer is achieved by HPLC on a μBondapax C_{18} column, eluted with a linear gradient of 20–50 % acetonitrile with 0.1 % trifluoroacetic acid with a slope of 0.5 %/min and a flow rate of 1 ml/min.

The radioimmunoassay procedure is performed in polystyrene tubes at 4 °C by mixing 100 μl of standard or sample, 100 μl of antiserum diluted 1:4,000, 100 μl of ^{125}I-ANF, and 300 μl of the same buffer containing 1 % BSA. After incubation for 24 h at 4 °C, the free from antigen-bound ^{125}I-ANF is separated by dextran-coated charcoal. One ml of dextran-charcoal suspension is added to each tube. After 5 s agitation, the tubes are centrifuged at 4,000 rpm at 4 °C for 10 min. The supernatant is decanted and the radioactivity counted in a gamma counter.

Evaluation

Dose–response curves are prepared and Scatchard analysis is performed.

Modifications of the Method

Radioimmunoassays were also developed for long-acting natriuretic peptide and vessel dilator (Vesely et al. 1994; Winters et al. 1989).

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Urotensin II

General Considerations

Purpose and Rationale

Urotensin II is a cyclic peptide originally isolated from the urophysis, the hormone storage-secretion organ of the caudal neurosecretory system of teleost fishes, such as *Gillichthys mirabilis* (Pearson et al. 1980; Maguire and Davenport 2002).

Several structural forms of urotensin II have been reported in different species (Grieco et al. 2004). These peptides show smooth muscle contracting activity. Itoh et al. (1987) reported contraction of major artery segments of rat, especially of the thoracic aorta, by urotensin II.

Human urotensin II is an 11-amino-acid peptide that retains the cyclic portion typical of fish urotensin II. It has been found in vascular and cardiac tissues and is a very potent constrictor of certain human isolated arteries and veins as well as of several vessels of other species (Douglas et al. 2000; Douglas 2003).

The potency of urotensin II as a vasoconstrictor is an order of magnitude greater than that of endethelin-1, making human urotensin II the most potent vasoconstrictor identified so far.

Human urotensin II is also a potent endothelium-dependent relaxant in rat precontracted arteries (Katano et al. 2000) and in human small pulmonary and systemic abdominal resistance arteries (Stirrat et al. 2001).

Bottrill et al. (2000) found that human urotensin II contracted endothelium-intact rat isolated left anterior descending coronary arteries. The contractile response was significantly enhanced by removal of the endothelium. However, human urotensin II caused concentration-dependent relaxation of 5-HT-precontracted arteries, which was abolished by *N*-nitro-L-arginine methyl ester (L-NAME) or removal of the endothelium.

Using merino ewes as experimental animals, Watson et al. (2003) found that urotensin II acts centrally to increase epinephrine and adrenocorticotropic hormone (ACTH) release and causes potent inotropic and chronotropic actions.

Coy et al. (2002) investigated structural requirements at the N-terminus of urotensin II octapeptides.

Carotenuto et al. (2004) investigated the active conformation of urotensin II by CD spectroscopy and NMR analysis in SDS micelles.

Watson and May (2004) reviewed the role of urotensin II in central and peripheral cardiovascular control.

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Rat Thoracic Aorta Bioassay for Urotensin II

Purpose and Rationale

Most studies on urotensin analogs, agonists, and antagonists used the isolated thoracic aorta of rats as a pharmacological model (Itoh et al. 1988; Nothacker et al. 1999; Sauzeau et al. 2001; Camarda et al. 2002a; Grieco et al. 2002a, b; Rossowski et al. 2002). Several urotensin II antagonists have been tested in this model (Behm et al. 2002; Herold et al. 2003).

Patacchini et al. (2003) tested urantide, an ultrapotent urotensin II antagonist peptide, in the rat aorta.

Procedure

Male albino rats (Wistar strain, 275–350 g) are decapitated under ether anesthesia. The thoracic aorta is cleared of surrounding tissue and excised from the aortic arch to the diaphragm. From each vessel, a helically cut strip is prepared, and then it is cut into two parallel strips. The endothelium is removed by gently rubbing the vessel intimal surface with a cotton-tip applicator; the effectiveness of this maneuver is assessed by the loss of relaxation response to acetylcholine (1 μM) in preparations precontracted with noradrenaline (1 μM). All preparations are placed in 5 ml organ baths filled with oxygenated normal Krebs–Henseleit solution. Motor activity of the strips is recorded isotonically (load 5 mN). A cumulative concentration–response curve to hU-II is constructed on one of the two strips, which serves as control. The other strip receives the antagonist peptide under examination and, after a 30-min incubation period, hU-II is administered cumulatively. Maximal contractile responses of preparations to hU-II are obtained by administration of KCl (80 mM) at the end of the cumulative curves.

Evaluation

Antagonist activity is expressed in terms of pK_B (negative logarithm of the antagonist dissociation constant) and, assuming a slope of –1.0, is estimated as the mean of the individual values obtained with the equation: $pK_B = \log[\text{dose ratio}–1]–\log[\text{antagonist concentration}]$ (Kenakin 1997). Competitive antagonism is checked by the Schild plot method: a plot with linear regression line and slope not significantly different from

unity is considered as proof of simple reversible competition (Kenakin 1997).

Modifications of the Method

Gibson et al. (1988) studied the influence of urotensin II on calcium flux in rat aorta. Urotensin II caused an increase in uptake of ^{45}Ca by segments of rat aorta. This increase was abolished by calcium channel-blocking drugs.

Douglas et al. (2000) found differential vasoconstrictor activity of human urotensin II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset, and cynomolgus monkey, depending on species and anatomical localization.

Camarda et al. (2002b) studied the effects of human urotensin II in isolated vessels (aorta, large arteries, veins) of various species (rats, guinea pigs, rabbits, pigs, human) and compared them with other vasoactive agents (noradrenaline, angiotensin II, endothelin I).

Watanabe et al. (2001) found a synergistic effect of urotensin II with serotonin on rabbit vascular smooth muscle cell proliferation.

Tamura et al. (2003) examined the effects of urotensin II on activation of extracellular signal-regulated kinase and focal adhesion kinase in cultured vascular smooth muscle cells.

Tzanidis et al. (2003) studied direct actions of urotensin II on the heart in a rat model of heart failure after myocardial infarction and the implications for cardiac fibrosis and hypertrophy.

Matsushita et al. (2003) showed that urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line LLCPK1.

Behm et al. (2004) investigated the role of urotensin II in the etiology of essential hypertension. Intravenous injection in anesthetized cats induced an increase in systemic blood pressure and peripheral vascular resistance.

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Intracellular Calcium Mobilization Assay

Purpose and Rationale

Human urotensin II induces concentration-dependent increases in intracellular calcium in HEK293 cells expressing human GPR14 (Ames et al. 1999). Herold et al. (2003) used this assay to test a synthetic antagonist at human and rat urotensin II receptors.

Procedure

Cell Culture

HEK293 cells stably expressing the hUT or rUT receptors are generated and propagated as described previously (Ames et al. 1999).

Intracellular Calcium (Ca^{2+}_i) Mobilization Assay

hUT-HEK293 cells or rUT-HEK293 cells are seeded in blackwalled, clear-bottomed 96-well BioCoat plates (Becton-Dickinson, Bedford, Mass., USA, Herold et al. 2003) at a density of 45,000 cells/well, grown in the incubator at 37 °C for 18–24 h and prepared for Ca^{2+} i measurements (Ames et al. 1999). Plates are placed into the Fluorometric Imaging Plate Reader (Molecular Devices, Sunnyvale, Calif., USA) where cells, loaded with Fluo-3 (Molecular Probes, Eugene, Ore., USA), are exposed to excitation (488 nm) from a 6-W argon laser. Fluorescence is monitored at 566 nm emission for all 96 wells simultaneously, and data are read every 1 s for 1 min and then every 3 s thereafter. An agonist is added after 10 s, and concentration–response curves are obtained by calculating the maximal fluorescence counts above background after the addition of each concentration of agonist. For antagonist studies, BIM-23127 (Bachem, King of Prussia, Pa., USA) is added 10 min prior to the addition of hU-II (California Peptide Research, Napa, Calif., USA).

Evaluation

Concentration–response curves are analyzed by nonlinear regression using GraphPad Prism 3.0 software (GraphPad, San Diego, Calif., USA).

Modifications of the Method

Flohr et al. (2002) performed structure–activity relationship studies on urotensin II by investigating peptide analogs and their ability to mobilize calcium in GPR14-transfected CHO cells.

Camarda et al. (2002) evaluated a new ligand for the urotensin II receptor ([Orn^8]U-II) in calcium functional assays performed on HEK293 cells expressing the recombinant rat and human UT receptor.

Croston et al. (2002) used a functional mammalian cell-based R-SAT assay (ACADIA Pharmaceuticals, San Diego, Calif., USA) to identify non-peptide agonists of the CRP14/urotensin II receptor in high-throughput screening. According to Shapiro et al. (2002), NIH-3 T3 cells were grown in 96-well tissue culture plates to 70–80 % confluence in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum and 1 % penicillin/streptomycin/Gln. Cells were transfected for 12–16 h with plasmid DNAs using SuperFect Reagent (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocols. RSATs were performed with 0.5–50 ng/well receptor and 20 ng/well β-galactosidase plasmid DNA. After overnight transfection, the medium was replaced with serum-free Dulbecco's modified Eagle's medium containing 2 % cyto-sf3 (Kemp Biotechnologies, Frederick, Md., USA) and 1 % penicillin/streptomycin/Gln and varying concentrations of drug. Cells were grown in a humidified atmosphere with 5 % ambient CO_2 for 4–6 days. The medium was removed from the plates, and β-galactosidase activity was measured by the addition of *o*-nitrophenyl β-D-galactopyranoside (in phosphate-buffered saline with 5 % Nonidet P-40 detergent). The resulting colorimetric reaction was measured using a spectrophotometric plate reader (Titertek, Huntsville, Ala., USA) at 420 nM. For HTS, NIH-3 T3 cells transiently transfected with the urotensin II receptor expression vector and plasmid were frozen and for the assay, thawed, plated, and exposed to drug.

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Receptor Binding of Urotensin II

Purpose and Rationale

The G-protein-coupled receptor GPR14/SENR was described by Marchese et al. (1995) and Tal et al. (1995). Liu et al. (1999) and Mori et al. (1999) identified urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. It was renamed as urotensin II (UT) receptor by UPHAR (Douglas and Ohlstein 2000). Human, rat, mouse, and monkey receptors have been cloned. The amino-acid sequence identity of the monkey UT receptor is 97 % and 77 % identical to the human and rat sequences, respectively, while the mouse UT receptor is 76 % and 93 % identical to the human and rat sequences, respectively.

Brkovic et al. (2003) performed functional and binding characterizations of urotensin-II-related peptides in human and rat urotensin II receptor assays.

Procedure

Reagents and Solvents

The following fluorenylmethyloxycarbonyl-protected amino acids were purchased from Chem-Impex International (Wood Dale, Ill., USA): Ala, Cys(Trt), His(Trt), Phe, Trp, Lys-(Boc), Tyr(tBu), Asp(OtBu), Glu(OtBu), Pro, Gln(Trt), Thr(tBu), Arg(Pbf), Orn(Boc), HomoCys(Trt), Cys(Acm), and D-Trp. Biograde TFA was obtained from Halocarbon (River Edge, N.J., USA). Diisopropylethylamine was from Aldrich (Milwaukee, Wis., USA). Wang resin and benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate were purchased from Albatross (Montreal, QC).

Basal Iscove's medium and fetal calf serum (FCS) were from Biochrom (Berlin, Germany). The GC-melt PCR kit and the human and rat genomic DNA were purchased from Clontech (Palo Alto, Calif., USA). The pEAK8 mammalian episomal expression vector and the selection marker puromycin were from Edge Biosystems (Gaithersburg, Md., USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, HEPES, Lipofectamine reagent, and penicillin–streptomycin were from Invitrogen (Carlsbad, Calif., USA). The pCDNA3.1(+) mammalian expression vector was from Invitrogen. The calcium-sensitive fluorescence dye Fluo-4 and Pluronic F-127 were obtained from Molecular Probes (Eugene, Ore., USA). FlashPlates PLUS and monoiodinated human [^{125}I-Tyr9] urotensin II for radioligand binding assays were from Perkin-Elmer Life Sciences (Boston, Mass., USA). Gentamicin, the transfection reagent FuGENE 6, and cOmplete protease inhibitor were purchased from Roche (Basel, Switzerland). Bacitracin, EDTA disodium salt, probenecid, $MgCl_2$, NaCl, and sucrose were obtained from Sigma-Aldrich (St. Louis, Mo., USA).

Cloning of Human and Rat Urotensin II Receptor

As the putative human urotensin II receptor sequence is intronless, this protein was cloned from human genomi DNA via PCR. PCR conditions, established to amplify the human GPR14 sequence, were 94 °C, 10 min, followed by 35 cycles of 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 2 min, using the GC-melt kit. Primers designed to amplify the coding sequence contained a *Bam*HI site in the forward and a *Xba*I site in the reverse primer, respectively. The urotensin receptor coding region, flanked by *Bam*HI/*Xba*I sites, was cloned into the pCDNA3.1(+) mammalian expression vector and sequenced in both directions. For generation of stable cell lines, the human U-II receptor coding sequence flanked by a 5′*Eco*RI site and a 3′*Eco*RV site was cloned into the mammalian episomal expression vector pEAK8.

The rat U-II receptor coding sequence flanked by a 5′ *Eco*RI and 3′ *Not*I site was amplified via

PCR from rat kidney cDNA and cloned into the mammalian pEAK8 expression vector. Sequences of all urotensin-II-receptor-expressing plasmids were verified by dideoxy sequencing in both directions.

Cell Culture and Transfection

CHO-K1 cells were grown in basal Iscove's medium supplemented with 10 % FCS, 2 mM L-glutamine, penicillin–streptomycin (10,000 IU/ml–10,000 μg/ml), and 25 mg/ml gentamicin at 37 °C in a humidified 5 % CO_2 incubator. Cells were transiently transfected with the U-II receptor cDNAs using the Lipofectamine reagent according to the manufacturer's protocol. After 18–24 h following the transfection, cells were split into blackwalled 96-well plates at a density of 50,000 cells/well and cultured for an additional 18–24-h period before being used in the functional fluorescence imaging plate reader (FLIPR) assay (described below in detail) measuring intracellular Ca^{2+} release upon receptor activation.

FLIPR Assay

Cells were loaded in 96-well plates for 1 h (37 °C, 5 % CO_2) with 100 μl of PBS (without Ca^{2+}, Mg^{2+}, and $NaHCO_3$) containing 4 μM of the fluorescent calcium indicator Fluo-4, 0.22 % Pluronic F-127 in dimethyl sulfoxide, 2.5 mM probenecid, 1 mM EGTA, and 1 % FCS. Cells were then washed three times with PBS (without Ca^{2+}, Mg^{2+}, and $NaHCO_3$) containing 1 mM EDTA, 0.5 mM $MgCl_2$, and 2.5 mM probenecid. After the final wash, a 100-μl residual volume remained on the cells. Peptides were aliquoted as 2 × solutions in 96-well plates and transferred by the instrument from the ligand plate to the cell plate. Fluorescence was recorded with the fluorometric imaging plate reader FLIPR (Molecular Devices, Sunnyvale, Calif., USA) over a period of 3 min. Fluorescence was recorded simultaneously in all wells at 3-s intervals during the first minute and at 10-s intervals during the last 2 min. Fluorescence data were generated in duplicate and repeated at least three times.

Generation of Stable Human and Rat U-II Receptor Expressing Cell Lines

For the generation of stable cell lines expressing the human and rat urotensin II receptor, HEK293 cells were transfected with human- and rat-pEAK8 constructs using the FuGENE 6 transfection reagent according to the supplier's protocol. Two days after transfection, cells were selected in DMEM, supplemented with 10 % FCS, 20 mM HEPES, penicillin–streptomycin (10,000 IU/ml–10,000 μg/ml), and 1 μg/ml puromycin for a period of 4 weeks (37 °C, 5 % CO_2, 95 % relative humidity). Functional activity of the urotensin-II-receptor-expressing cell population was verified with a FLIPR assay recording urotensin-II-mediated intracellular Ca^{2+} release, as described above.

Membrane Preparation and Radioligand Binding Assays

HEK293 cells stably expressing human or rat urotensin II receptors were cultured up to 80 % confluency in DMEM, supplemented with 10 % FCS, 20 Mm HEPES, penicillin–streptomycin (10,000 IU/ml–10,000 μg/ml), and 1 μg/ml puromycin (37 °C, 5 % CO_2, 95 % relative humidity). Cells were washed once with ice-cold PBS and a second time with PBS containing the protease inhibitor cocktail (cOmplete). Cells were scraped off and centrifuged gently. The pellet was resuspended in a buffer containing 5 mM HEPES, 1 mM EDTA disodium salt, and the cocktail of protease inhibitor cOmplete and then incubated on ice for 15 min. Cells were pelleted again and resuspended with a homogenizer (Unit F8B, Constant cell disruption systems; Honiley, Warwickshire, UK). The supernatant and resuspended pellets were combined and centrifuged at 50,000 g (Beckman Avanti J251). The cell membrane pellet was resuspended in a buffer consisting of 20 mM HEPES, 1 mM EDTA disodium salt, 150 mM NaCl, and 10 % sucrose. Membrane aliquots were stored at −80 °C. One day before the binding assay, membranes were thawed, pelleted, and resuspended in the assay buffer consisting of 20 mM HEPES, 150 mM NaCl, 1 mM EDTA disodium salt, 160 μg/ml bacitracin, and cOmplete protease inhibitor (two tablets per 100 ml).

Membranes were distributed into 96-well wheat germ agglutinin FlashPlates PLUS and incubated overnight for adsorption, and then the FlashPlates PLUS were washed twice with the assay buffer. For equilibrium binding assays, 0.2 nM human [^{125}I-Tyr9] urotensin II (initial specific activity 2,200 Ci/mmol or 81,400 GBq/mmol) was incubated with the indicated amounts of unlabeled competitors for 4 h and radioactivity counted in a 1450 Microbeta Wallac Jet (Wallac, Turku, Finland).

Evaluation

Data of the FLIPR assay were analyzed by nonlinear curve fitting using GraphPad Prism version 3.0 (GraphPad Software, San Diego, Calif., USA).

Nonspecific binding was determined in the radioligand binding assays in the presence of 10 μM human U-II and corresponded to 31 ± 2 % ($n = 5$, mean ± SEM) of the total binding for membranes expressing the human urotensin II receptor and to 8 ± 1 % ($n = 5$, mean ± SEM) of the total binding for membranes expressing the rat urotensin II receptor. Data analysis was performed with GraphPad Prism 3.0.

Modifications of the Method

Flohr et al. (2002) identified non-peptide urotensin II receptor antagonists by virtual screening on a pharmacophore model derived from structure–activity relationships and nuclear magnetic resonance studies on urotensin II.

Actelion's palosuran, a urotensin II receptor antagonist, was used in two human studies of diabetic nephropathy; the lack of measurable effects on blood pressure, renal function, or albuminuria resulted in the cessation the trials (Sidharta et al. 2006, 2009, Vogt et al. 2010). Recently, a non-peptide antagonist SB-611812 has delivered positive findings in the rat models of cardiac remodelling and atherosclerosis (Bousette et al. 2006, Papadopoulos et al. 2009). Another non-peptide antagonist UTS2R, with a higher selectivity and exhibiting prolonged pharmacodynamic activity (Dalrymple et al. 2008).

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Urotensin II Gene Expression

Purpose and Rationale

The UT receptor is expressed in vascular tissue (Ames et al. 1999; Maguire et al. 2000). A direct link between U-II, the UT receptor, and vasoconstriction has to be assumed (Ames et al. 1999; Douglas and Ohlstein 2000). Behm et al. (2003) used homologous recombination in embryonic stem (ES) cells to generate mice lacking the UT receptor coding region to determine the effect(s) of this receptor on vascular reactivity both in vivo and in vitro and to verify that U-II exerts its effects on vascular smooth muscle tone via an interaction with UT.

Procedure

Targeting the UT Gene and Generation of Mutant Mice

Gene targeting was performed in murine E14.1 ES cells, replacing the single coding exon of the UT receptor locus with a positive selection cassette containing the neomycin phosphotransferase gene (Neo) driven by the phosphoglycerate kinase I (PGK) promoter. 5′- and 3′-homology arms, both of ∼4.0 kb, were cloned from a 129SVJ mouse genomic bacterial artificial chromosome (BAC) library and placed on either side of the positive selection cassette. Homologous recombination in neomycin-resistant ES cells was confirmed by Southern blot of *Bam*HI-digested genomic DNA using an ∼800-bp *Bam*HI/*Sma*I restriction fragment as the 5′ external probe (which detects 6.5- and 6.0-kb bands at the wild-type and targeted

locus, respectively). Approximately 1 in 80 G418-resistant clones had undergone homologous recombination. Homologous recombination at the 3′ end was confirmed in these ES cell clones by Southern blot of *Hind*III-digested genomic DNA using a ~700-bp *Xmn*I/*Hind*III restriction fragment as the 3′-external probe (which detects 5.5- and 5.0-kb bands at the wild-type and targeted locus, respectively). Three targeted clones were injected into C57B16/J-derived blastocysts. Male chimeras were crossed with C57B16/J females to give N1F0 offspring, which were subsequently intercrossed to generate N1F1 offspring. In addition, N1F0 offspring were successively backcrossed to C57B16/J females to generate N5F0 mice. These were intercrossed to create an N5F1 population.

Genotyping of Study Populations

N1F1 and N5F1 study populations were genotyped by polymerase chain reaction (PCR) and Southern blot of genomic DNA isolated from the hearts of animals used in the studies. Hearts of wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice were cut into small pieces (~1 mm) and placed into polypropylene tubes. Extraction buffer (2 ml; 10 mM Tris–HCl pH 8.0, 50 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 0.5 % SDS, 20 μg ml^{-1} RNase, 100 μg ml^{-1} proteinase K) was added and samples were incubated at 50–55 °C for 4 h until completely lysed. The mixture was then extracted two times each with phenol, phenol/chloroform, and chloroform (Maniatis et al. 1989). Genomic DNA was precipitated by adding 2.5 vols of cold ethanol, washed with 70 % v/v ethanol (−20 °C) and dissolved in 200 μl of 10 mM Tris–HCl, 1 mM EDTA TE, pH 8.0. Once dissolved, the purity and concentration of the DNA were measured by spectrophotometry (absorbency at 260 and 280 nm wavelength).

PCR amplification was performed using 50-ml aliquots (50mM KCl, 10mM Tris–HCl, pH8.3, 17mM MgCl2, 200nM 2′-deoxynucleotide 5′-triphosphates (dNTPs), 10 % v/v dimethyl sulfoxide and 1.25U *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA)) using 200ng of genomic DNA as the template and PCR primers specific to the neomycin resistance gene present at the targeted locus (5′-TGA ACA AGA TGG ATT GCA CGC AGG TTC TCC GGC-3′ and 5′-GCC AAG CTC TTC AGC AAT ATC ACG GGT AGC-3′, yielding an 700-bp product) and mouse UT gene-specific primers (5′-CTG GCT GAC CTG CTG TAT CTG CT-3′ and 5′-CAG GGT CAC ACA AAG CAC TCT CA-3′, yielding an 900-bp product). A 500-bp mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon was used as the internal control (5′-TGG CCA AGG TCA TCC ATG AC-3′ and 5′-GTC CAC CAC CCT GTT GCT GTA G-3′, yielding an ~500-bp product). Amplification was performed for 30 cycles at 60 °C annealing for 30 s, 72 °C extension for 90 s, and 94 °C denaturing for 30 s. Amplification of a 500-bp (GAPDH)/700-bp doublet alone corresponded to a $UT^{(-/-)}$ genotype, and a 500-bp (GAPDH)/900-bp doublet alone corresponded to a $UT^{(+/+)}$ genotype.

Genomic DNA (20 μg) was digested with *Bam*HI and run on an agarose gel (1 %). The agarose gel was then treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, followed by neutralization with 0.5 M Tris–HCl (pH7.5) and 1.5 M NaCl for 30–40 min. The DNA was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Mass., USA) and probed with cDNA corresponding to the full-length mouse UT receptor open reading frame (ORF). cDNA fragments were labeled with [α-^{32}P] 2′-deoxycytidine 5′-triphosphate (dCTP) using standard random primed methods (T7 Quick-Prime; Pharmacia Biotech, Piscataway, N.J., USA). Membranes were prehybridized for 2 h at 42 °C and incubated overnight at 42 °C with 1×10^9 cpm μg^{-1} denatured radiolabeled probe in standard buffer (50 % deionized formamide, 6 × sodium chloride, sodium citrate (SSC), 5 × Denhardt's reagent, 0.5 % sodium dodecyl sulfate (SDS), 100 μg ml^{-1} denatured, fragmented salmon sperm DNA). Membranes were washed under conditions of low stringency (three 15-min washes in 1 × SSC, 0.1 % SDS at 28 °C) followed by a high-stringency wash in 0.1 × SSC/0.1 % SDS

for 30 min at 55 °C. Hybridization signals were detected by conventional X-ray autoradiography (Hyper film, Amersham Life Science, UK) and phosphor imaging (Storm 860, Molecular Dynamics, Sunnyvale, Calif., USA).

Hemodynamics and Echocardiography

Male wild-type ($UT^{(+/+)}$) and homozygous UT receptor knockout ($UT^{(-/-)}$) mice, anesthetized with 1.5 % isoflurane, underwent transthoracic echocardiographic determination of left ventricular end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), cardiac output (CO), and ejection fraction (EF). Further to this, mice were re-anesthetized the following day for hemodynamic evaluation where a fluid-filled catheter was inserted into the left carotid artery for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The catheter was then advanced into the left ventricle (LV) to obtain measurements of left ventricular end-systolic (LVESP) and end-diastolic pressure (LVEDP). At the end of the study, selected organs (right and left kidney, heart, right and left ventricle, and lungs) were isolated and wet weights were measured.

Preparation and Utilization of Mouse Isolated Aortae and Mesenteric Arteries

Male (4 months; 27 g) wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice were anesthetized with inhaled isoflurane (5 % in O_2) and killed by cervical dislocation. Proximal descending thoracic aortae were isolated and cleaned of adherent tissue. Vessels approximately 3 mm in length were suspended in 10-ml organ baths containing Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, and dextrose 11.0. Krebs solution was maintained at 37 ± 1 °C and aerated with 95 % O_2 and 5 % CO_2 (pH 7.4). For contraction studies, vessels were denuded of endothelium by rubbing with fine forceps, and indomethacin (10 mM) was added to the buffer. Changes in isometric force were measured under 0.5 g optimal resting tension using FT03 force-displacement transducers (Grass Instruments, Quincy, Mass., USA) coupled to Model 7D polygraphs.

The Halpern–Mulvany wire myograph (Model 610 M; Danish Myo Technology, Denmark) was used for measurement of isometric force development of endothelium-intact superior mesenteric arteries (optimal resting tension of 0.5 g), and data were recorded using a Grass 7400 direct thermal recorder.

Following a 60-min equilibration period, vessels were treated with standard concentrations of KCl (60 mM) and phenylephrine (1 mM) to which subsequent agonist-induced responses were normalized. Once the contractile response to phenylephrine had plateaued, carbachol (10 μM) was added to the vessels in order to evaluate endothelial integrity.

Cumulative concentration–response curves to phenylephrine (0.1 nM to 10 μM), angiotensin II (0.1 nM to 10 μM), endothelin-1 (0.1 nM to 1 mM), and hU-II (0.01 nM to 3 μM) were obtained for each vessel by adding the spasmogen to the tissue bath in half-log increments. During relaxation studies, vessels were preconstricted with an EC_{80} concentration of phenylephrine, and contractile tone was reversed by adding cumulative amounts of carbachol (1 nM to 30 μM) or sodium nitroprusside (0.1 nM to 1 μM). Each response was allowed to plateau before the addition of subsequent agonist concentrations. Vessels were allowed to recover for at least 30 min between subsequent agonist–response curves and were not exposed to subsequent agonists after treatment with either endothelin-1 or hU-II.

Evaluation

All values are expressed as mean ± SEM, and n represents the total number of animals from which the vessels were isolated. Statistical comparisons were made using an unpaired, two-tailed t-test or Fisher's exact tests, and differences were considered significant when $P < 0.05$. Concentration–response curves were fitted to a logistic equation as previously described (Douglas et al. 1995).

Modifications of the Method

Coulouarn et al. (1998) reported cloning of the cDNA encoding the urotensin II precursor (Prepro-UII cDNA) in frog and human. Intense expression of the urotensin gene in motoneurons of the spinal cord was found indicating important physiological functions of urotensin II.

Elshourbagy et al. (2002) reported molecular and pharmacological characterization of genes encoding urotensin II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. Monkey and mouse preproU-II genes were identified to encode 123 and 125 amino acids. Monkey and mouse UT receptors were 389 and 386 amino acids, respectively. Expression of mouse and monkey U-II/UT receptor mRNA was found also in extravascular tissues, including the lung, pancreas, skeletal muscle, kidney, and liver.

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Apelin

General Considerations

Purpose and Rationale

The peptide apelin, consisting of 36 amino acids, is found increasingly to play a role in biology and medicine (Kleinz and Davenport 2005; Masri et al. 2005). The family of apelin peptides is derived from a single gene and activates the 7-transmembrane G-protein-coupled receptor APJ. In the search for an endogenous ligand of the orphan G-protein-coupled receptor APJ, Tatemoto et al. (1998) isolated and characterized

endogenous peptide ligands, designated apelin, from bovine stomach extracts. Apelin-13, consisting of 13 amino acids, was more active than apelin-36. APJ was first identified in a human gene by O'Dowd et al. (1993), sharing close identity to the angiotensin receptor. In rats, the greatest expression of APJ mRNA was detected in the lung, suggesting that APJ and its ligand play an important role in the pulmonary system (Hosoya et al. 2000). Cloning, pharmacological characterization, and brain distribution of the rat apelin receptor were reported by De Mota et al. (2000) and the physiological role of apelin and its receptor in rat brain by Reaux et al. (2001). The hypothalamic and hypophyseal distribution of the receptor suggested an involvement of apelin in the control of neurohypophyseal and adenohypophyseal hormone release, whereas the presence in the pineal gland and in discrete higher brain structures pointed to possible roles in the regulation of circadian rhythms and of water and food intake behavior. Studying the distribution of apelin-synthesizing neurons in the adult rat brain, Reaux et al. (2002) suggested multiple roles of apelin especially in the central control of ingestive behaviors, pituitary hormone release, and circadian rhythms. Studying apelin immunoreactivity in the rat hypothalamus and pituitary, Brailoiu et al. (2002) concluded that apelin may be a signaling peptide released from the hypothalamic–hypophyseal axis. De Mota et al. (2004) found that apelin is a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. Masri et al. (2002) reported that apelin activates extracellular signal-regulated kinases via a system sensitive to pertussis toxin (PTX). Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin were reviewed by Medhurst et al. (2003). Jászberényi et al. (2004) studied the behavioral, neuroendocrine, and thermoregulatory actions of apelin-13. Wang et al. (2004) investigated the localization of apelin in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Boucher et al. (2005) found in isolated mouse and human adipocytes that apelin is upregulated by insulin and obesity.

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Cardiovascular Actions of Apelin

Purpose and Rationale

Most reports concentrate on the functional role of apelin in the cardiovascular system (Tatemoto et al. 2001; Katugampola et al. 2002; Szokodi et al. 2002; Katugampola and Davenport 2003; Chen et al. 2003; Berry et al. 2004; Kleinz and Davenport 2004; Kagiyama et al. 2005; Losano 2005).

Cheng et al. (2003) studied the venous dilator effect of apelin in conscious rats using a method for measurement of body venous tone published by Pang (2000).

Ashley et al. (2005) found that apelin improves cardiac contractility and reduces cardiac loading in vivo in mice.

Procedure

Male C57Bl/6 mice were used. The following studies were performed: magnetic resonance imaging of the heart, pressure–volume hemodynamics, effects of chronic apelin infusion, assessment of ventricular hypertrophy after sacrifice, and expression of the APJ receptor.

Pressure–volume hemodynamics were assessed using the Aria system (Millar Instruments, Houston, Tex., USA). This measurement platform, specifically designed for small rodents, comprises an ultraminiature 1.4 F (0.47 mm outer diameter) catheter, which incorporates pressure and conductance sensors, possessing hardware including analog–digital conversion and analysis software. Pressure is measured directly in mmHg, while the conductivity of blood is used to estimate volume and allow construction of pressure–volume relationships in real time.

Male mice aged 8–16 weeks were anesthetized with 1–2 % isoflurane in oxygen. The internal jugular vein was cannulated with PE tubing and a 10 % albumin solution infused at 5 µl/min following a bolus of 150 µl over 5 min. After tracheostomy, a 19-gauge cannula was inserted into the trachea, and the animal was ventilated at a tidal volume of 200 µl at 100 breaths per minute. Following an incision just dorsal to the xyphoid cartilage, the diaphragm was visualized from below, and after diaphragmatic incision, the left ventricular apex was visualized. The pressure–volume catheter was inserted along the long axis of the left ventricle, from where it was adjusted to obtain rectangular-shaped pressure–volume loops. Baseline loops were

recorded following volume replacement, at which point the inferior vena cava was visualized within the chest and occlusion parameters were recorded during and after a 5-s manual occlusion of the vessel. Next, the albumin solution was replaced by one containing 100 nM apelin, which was infused at 5 μl/min for 20 min, following which baseline and occlusion loops were recorded once again.

Signals from the catheter were digitized using the PowerLab system and stored for offline analysis using the PVAN software. This allows analyses of pressure (e.g., end-systolic pressure, end-diastolic pressure) and derivation of pressure–time and volume–time parameters at steady state.

For **magnetic resonance imaging**, male C57Bl/6 mice aged 16 weeks were scanned twice on subsequent days. The animals underwent general anesthesia while breathing spontaneously via a nose cone fitted carefully to minimize escape of anesthetic into the environment. Two percent isoflurane was administered with an oxygen flow rate of 1–2 l/min. Platinum needle ECG leads were inserted subcutaneously. Respiration was monitored by means of a pneumatic pillow sensor positioned against the abdomen. Mouse body temperature was maintained during scanning at 37 °C by a flow of heated air thermostatically controlled by a rectal temperature probe. Magnetic resonance images were acquired on a 4.7 T Oxford magnet controlled by a Varian Inova console (Varian, Palo Alto, Calif., USA) using a transmit–receive, quadrature, volume coil with an inner diameter of 3.5 cm. Image acquisition was gated to respiration and to the ECG R wave (SA Instruments, Stony Brook, N.Y., USA). Coronal and sagittal scout images led to the acquisition of multiple contiguous 1-mm-thick, short-axis slices orthogonal to the interventricular septum. Nine cine frames were taken at each slice level with the following sequence parameters: TE = 2.8 ms, NEX = 12, FOV = 3 × 3 cm, matrix = 128 × 128, and flip angle = 60°. Cine frames were spaced 16 ms apart and acquired through slightly more than one cardiac cycle guaranteeing acquisition of systole and diastole. On the second day of scanning, mice received 300 μg/kg body weight of apelin as an intraperitoneal injection 1 h prior to scanning. A pilot study had previously identified 1 h as an appropriate time within which to identify apelin effects resulting from peritoneal absorption. Planimetry measurements of end-diastolic and end-systolic dimensions were derived offline from short-axis views of the left ventricle at the level of the papillary muscles using ImageJ software (National Institutes of Health, Bethesda, Md., USA). Ejection fraction was calculated as [LVEDA-LVESA]/LVEDA, where LVEDA is left ventricular end-diastolic area and LVESA is left ventricular end-systolic area.

Evaluation

Data were analyzed using Student's *t* statistic (paired) or repeated measures analysis of variance using the post hoc comparison of Fisher.

Modifications of the Method

Katugampola et al. (2001, 2002) described [^{125}I](Pyr^{1})apelin as a radioligand for localizing the APJ orphan receptor in human and rat tissues.

Kasai et al. (2004) found that apelin worked as an angiogenic factor in the retinal endothelial cell line RF/6A.

Chun et al. (2008) reported that APJ dimerize with the angiotensin receptor 1 resulting in inhibition of apelin on Ang II signaling. Further studies has established that APJ heterodimerize with the k-opioid receptor (Li et al. 2012).

Azizi et al. (2013) showed that administration of (Pyr^{1})-Apelin-13 to a rat model of myocardial infarction reduces infarct size and increases HR with long-term antioxidant cardioprotective action.

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Na^+/H^+ Exchange

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Inhibition of Na^+/H^+ Exchange

Purpose and Rationale

Na^+/H^+ exchange was first described by Murer et al. (1976) in a study of intestinal and renal brush-border vesicles. The plasma membrane Na^+/H^+ exchanger is an ubiquitous pH-regulating cellular ion transport system. It is driven by the Na^+ gradient and extrudes protons from the cytosol in exchange for extracellular Na^+ ions (Aronson 1985; Frelin et al. 1988; Fliegel and Dyck 1995; Orlowski and Grinstein 1997; Wakabayshi et al. 1997; Dibrov and Fliegel 1998). Six mammalian Na^+/H^+ exchangers, NHE1, NHE2, NHE3, NHE4, NHE5 (Attaphitaya et al. 1999; Szabo et al. 2000), and NHE6, have been described (Tse et al. 1994; Orlowski 1999; Counillon and Pouysségur 2000).

In cardiac tissue the exchanger has a major role in the control of intracellular pH. At the onset of cardiac ischemia and during reperfusion, Na^+/H^+ exchange is excessively activated by low intracellular pH. Since the deleterious Na^+ influx in this condition was found to originate mainly from Na^+/H^+ exchange (Frelin et al. 1984; Schömig et al. 1988), the exchanger seems to be responsible for an increase of cytosolic sodium in ischemic cells. The accumulation of intracellular Na^+ causes an activation of Na^+/K^+ ATPase (Frelin et al. 1984; Rasmussen et al. 1989) which in turn increases ATP consumption.

During ischemia the aerobic metabolism of glucose terminates in lactic acid. A vicious circle leads to a further decrease of intracellular pH and to a further activation of Na^+/H^+ exchange, resulting in energy depletion, cellular Na^+ overload, and, finally due to the coupling of Na^+ and Ca^{2+} transport via Na^+/Ca^{2+} exchange, cellular Ca^{2+} overload (Lazdunski et al. 1985; Tani and Neely 1990; Scholz and Albus 1993). Especially in ischemic cardiac tissue, where Na^+/H^+ exchange is the predominant pH-regulating ion transport system (Weissberg et al. 1989), these pathological events can lead to increased excitability and precipitation of cellular death. Therefore, it is desirable to find potent and well-tolerated inhibitors of Na^+/H^+ exchange which should be able to interrupt this vicious cycle, to conserve cellular energy stores and to diminish cellular excitability and necrosis during cardiac ischemia. Such effects have been found with relatively weak inhibitors of Na^+/H^+ exchange at high toxic doses, such as amiloride and ethyl isopropyl amiloride (Scholz et al. 1992).

The myocardial Na^+/H^+ exchanger is regarded as a therapeutic target for the prevention of myocardial ischemic and reperfusion injury and attenuation of postinfarction heart failure (Karmazyn et al. 2001).

More potent Na^+/H^+ exchange inhibitors showed beneficial effects on ischemia/reperfusion injury (see sections "Coronary Artery Ligation, Reperfusion Arrhythmia and Infarct Size in Rats" and "Ventricular Arrhythmia After Coronary Occlusion," chapter "▶ Cardiovascular Analysis In Vivo") in rats (Aye et al. 1997; Myers et al. 1998; Aihara et al. 2000), dogs (Gumina et al. 1998, 2000), and pigs (Portman et al. 2001). Heart hypertrophy and heart failure after myocardial infarction are reduced (Yoshida and Karmazyn 2000; Kusumoto et al. 2001). Ischemia-induced apoptosis in isolated rat hearts is attenuated by sodium–hydrogen exchange inhibitors (Chakrabarti et al. 1997).

Linz and Busch (2003) demonstrated the effects of NHE1 inhibition from protection during acute ischemia/reperfusion to prevention of myocardial remodeling.

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Inhibition of Na^+/H^+ Exchange in Thrombocytes

Purpose and Rationale

The inhibition of Na^+/H^+ exchange has been studied in platelets by measuring the optical density after osmotic cell swelling (Rosskopf et al. 1991).

Procedure

About 5 ml blood is withdrawn by venipuncture from human donors or from the vena jugularis externa of Beagle dogs or from the aorta of anesthetized Wistar rats (weighing 250–350 g). Coagulation is inhibited by 0.8 ml citrate acid dextrose (65 mM citric acid, 11 mM glucose, 85 mM trisodium citrate). Platelet-rich plasma (PRP) is obtained by centrifugation of whole blood at 90 g for 10 min at room temperature. Platelet count is measured, e.g., with a Casey 1 multichannelyser (Schärfe System, Reutlingen, Germany).

Each of the experiments is performed with 10–50 µl PRP containing 20×10^6 platelets in a volume of 100 µl with saline. To activate Na^+/H^+ exchange in the platelets by intracellular acidification, 500 µl propionate buffer (135 mM Na propionate, 1 mM HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 6.7, 22 °C) is added to the PRP/NaCl solution. Swelling of the platelets results in a decrease of optical density which can be measured with an aggregometer, e.g., with a Turbitimer (Behringwerke, Marburg, Germany). The system is activated photometrically by the addition of the propionate buffer to the cuvette. The experiments are performed with and without the addition of the Na^+/H^+ exchange inhibitor to be tested. The inhibitors are added in concentrations between 10^{-4} and 10^{-8} mol/l. 5-(*N*-Ethyl-isopropyl)amiloride (EIPA) is used as standard. During the experiments all solutions are kept at 22 °C in a temperature-controlled water bath.

Evaluation

Results are given as means ± SD. Student's *t*-test is employed for statistical evaluation. IC_{50} values are calculated from dose–response curves.

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Inhibition of Na^+/H^+ Exchange in Cholesterol-Activated Rabbit Erythrocytes

Purpose and Rationale

The inhibition of Na^+/H^+ exchange has been studied in cholesterol-activated rabbit erythrocytes by flame photometry of sodium (Scholz et al. 1992, 1993).

Procedure

White rabbits (New Zealand strain, Ivanovas) are fed with a rabbit standard chow with 2 % cholesterol for 6 weeks to increase the Na^+/H^+ exchange (Scholz et al. 1990) and to make the erythrocytes suitable for measurement of sodium influx via Na^+/H^+ exchange by flame photometry. Blood is drawn from the ear artery of the rabbits and coagulation prevented with 25 IU/ml potassium heparin. The hematocrit of the samples is determined in duplicate by centrifugation. Aliquots of 100 µl are taken to measure the initial sodium content of the erythrocytes.

To determine the amiloride-sensitive sodium influx into erythrocytes, 100 µl of each blood sample is added to 5 ml of buffer made hyperosmolar by sucrose (140 mM NaCl, 3 mM KCl, 150 mM sucrose, 0.1 mM ouabain, 20 mM tris(hydroxymethyl)aminomethane, pH 7.4) and incubated for 60 min at 37 °C. Subsequently, the erythrocytes are washed three times in ice-cold $MgCl_2$–ouabain solution (112 mM $MgCl_2$, 0.1 mM ouabain).

For determination of intracellular sodium content, the cells are hemolyzed in distilled water, the cell membranes are centrifuged, and the sodium concentration of the hemolysate is measured by flame photometry. Net influx of sodium into the erythrocytes is calculated from the difference between the initial sodium content and the sodium content after incubation. Amiloride-sensitive sodium influx is calculated from the difference between sodium content of erythrocytes incubated with and without amiloride (3×10^{-4} M). Each experiment is done with the erythrocytes from six different animals. In each case, the comparison of Na^+ contents is based on erythrocytes from the same animal. Doses between 10^{-4} and 10^{-7} M of the inhibitor are tested.

Evaluation

Statistical analysis of the data obtained is performed with Student's *t*-test for paired groups. IC_{50} values are calculated from dose–response curves.

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Sodium Influx into Cultured Cardiac Myocytes

Purpose and Rationale

The inhibition of Na^+/H^+ exchange has been studied in cultured cardiac myocytes (Scholz et al. 1992).

Procedure

Rat myocardial cells are isolated from hearts of neonatal rats by trypsin digestion. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's minimum essential medium (DMEM, GIBCO) in an atmosphere containing 10 % CO_2.

After confluence, the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs–Ringer solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (choline chloride 130 mM, $CaCl_2$ 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37 °C in the same buffer with added 0.1 % bovine serum albumin (BSA) and 10 mM/l glucose. The culture dishes are then incubated for another 10 min with Na^+ propionate for cytosolic acidification and stimulation of Na^+/H^+ exchange. The compounds are dissolved in 500 µl/dish KRB in which 50 % of the sodium chloride has been replaced by choline chloride containing additionally 2 µCi/ml $^{22}Na^+$ bicarbonate and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). After the stimulation period, sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lysed with 250 µl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 3×10^{-4} and 10^{-8} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

Evaluation

Mean values ± SD are compared with Student's *t*-test. IC_{50} values are calculated from dose–response curves.

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Inhibition of Na^+/H^+ Exchange into Cultured Aortic Endothelial Cells

Purpose and Rationale

The inhibition of Na^+/H^+ exchange has been studied in endothelial cells (Scholz and Albus 1993) by measuring the $^{22}Na^+$ influx.

Procedure

Bovine aortic endothelial cells (BAEC) are isolated by dispase digestion form bovine aorta obtained from animals killed at the local slaughter house. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's minimum essential medium (DMEM, GIBCO) in an atmosphere with 10 % CO_2. Three days after confluence, the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs–Ringer solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (choline chloride 130 mM, CaCl2 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37 °C in the same buffer with added 0.1 % bovine serum albumin (BSA) and 10 mM glucose. To stimulate Na^+/H^+ exchange, the culture dishes are incubated for another 10 min with 500 µl/dish KBR in which all sodium chloride has been replaced by 65 mM each of choline chloride and Na^+ propionate or with KBR in which 50 % of the sodium chloride has been replaced by choline chloride for unstimulated controls. In addition, the buffer contains 2 µCi/ml $^{22}Na^+$ and the test compounds or the standard. After the stimulation period, the sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lysed with 250 µl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 10^{-5} and 10^{-7} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

Evaluation

Mean values ± SD are compared with Student's *t*-test. IC_{50} values are calculated from dose–response curves.

Modifications of the Method

Ewart et al. (1997) studied lipoprotein lipase activity in cultured rat cardiomyocytes in the presence of insulin and dexamethasone.

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NHE Activity Measured by Intracellular pH in Isolated Ventricular Myocytes

Purpose and Rationale

Changes of the intracellular pH of cultured bovine endothelial cells have been fluorometrically monitored using the pH-dye 2′,7′-*bis*(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by Kitazono et al. (1988). This method has been used to study the activity of inhibitors of Na^+/H^+ exchange (Scholz et al. 1995).

Procedure

For preparation of isolated rat ventricular muscular cells (Yazawa et al. 1990), hearts of male Wistar rats are dissected, mounted on a Langendorff apparatus and perfused first at 37 °C for 3 min with Tyrode solution adjusted to pH 7.4, second for 5–7 min with nominally calcium-free Tyrode solution, and finally with calcium-free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma type I). After 15–20 min collagenase treatment, the heart is washed with storage solution (composition in mmol/L: KOH 70, L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 20, HEPES 10, and EGTA 0.5, pH 7.4). The ventricles are cut into small pieces, and myocytes are dispersed by gently shaking and finally by filtration through a nylon mesh (365 µm). Thereafter, the cells are washed twice by centrifugation at 600–1,000 rpm for 5 min and kept at 4 °C until use. For the pH recovery experiment, the cells are loaded with the membrane-permeable acetoxymethyl ester (AM) form of the fluorescent indicator 2′,7′-*bis*(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). BCECF-AM is dissolved in DMSO and diluted to a 1.25 µM storage solution. Cardiomyocytes are loaded in this solution for 30 min at room temperature and are then centrifuged and resuspended in storage solution. The measurements are performed in bicarbonate-free NaCl solution (NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1, glucose 10, and HEPES 10 mM/L, pH 7.4) at 34 °C using an apparatus according to Nitschke et al. (1991). The pH-dependent signal of BCECF is obtained by illuminating at 490 and 437 nm and dividing the emitted light signals (520–560 nm). The background signal, determined by closing the shutter, is subtracted from the total signal. The autofluorescence determined by illuminating unloaded cells can be ignored. In order to investigate the function of the Na^+/H^+ exchange system, the intracellular pH (pH_i) of the cells is decreased by the NH_4Cl prepulse technique, and the rate of return to resting pH_i is determined. Test compounds are dissolved in the incubation medium. For each test concentration, the recovery of pH_i is first recorded in control NaCl solution.

Evaluation

Data are analyzed by fitting a straight line to the initial (5 min) data points of the pH

recovery curve. For statistical presentation, the slopes of the linear curves are demonstrated. All reported data are presented as means ± SEM. Statistical comparisons are made using either a paired or unpaired *t*-test.

Modifications of the Method

The pH-sensitive fluorescence dye C-SNARF-1 (= carboxy-seminaphthorhodafluor-1) was used by Yasutake et al. (1996), Shipolini et al. (1997), and Yokoyama et al. (1998).

Fischer et al. (1999) tested new drugs for the Na^+/H^+ exchanger in Chinese hamster ovary cells which are enriched with the NHE1 isoform of the Na^+/H^+ antiporter. The Na^+/H^+ exchanger was stimulated with NaCl, and the rate of extracellular acidification was quantified with the cytosensor.

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NHE Subtype Specificity

Purpose and Rationale

Molecular identification of mammalian Na^+/H^+ exchanger subtypes has been pioneered by Pouysségour and coworkers (Sardet et al. 1989) who used genetic complementation of fibroblast cell lines that lack all endogenous NHEs. Schwark et al. (1998) studied an inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types.

Procedure

cDNAs for the NHE subtypes human NHE1, rabbit NHE2, rat NHE3 (Pouysségour) or cloned by reverse transcription-polymerase chain reaction from human kidney mRNA are used. These cDNAs are cloned into the mammalian expression vector pMAMneo and transferred into the NHE-deficient mouse fibroblast cell line LAP1. Cells expressing the NHE subtypes are selected by the acid load survival method (Sardet et al. 1989). Clonal cell lines for each subtype are used for intracellular pH (pH_i) recovery after acid load. For studies of pH_i recovery (Faber et al. 1996), cells are scraped off the culture dishes, washed, and incubated with 5 μmol/l

BCECF-AM [2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester] for 20 min at 37 °C in a buffer containing 20 mM NH_4Cl. The cells are then washed to remove extracellular dye and resuspended in the loading buffer without BCECF-AM. Intracellular acidification is induced by addition of 975 µl NH_4Cl-free and HCO_3^--free solution (the so-called recovery medium: HCO_3^--free to inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger of LAP1 cells) to a 25 µl aliquot of cells (≅25,000 cells). The pH_i recovery is recorded with a dual-grating DeltaScan single-photon counting fluorometer (Photon Technology International, South Brunswick, NJ, USA) with excitation wavelength of 505 and 440 nm and an emission wavelength of 535 nm. The measurement time varies between subtypes (120 s for NHE1, 300 s for NHE2, 180 s for NHE3). The inhibitors are first dissolved in DMSO, diluted in recovery medium, and added in a volume of 975 µl to this medium.

A cloned opossum kidney cell line (Helmle-Kolb et al. 1990) is used additionally. Cells are grown as a monolayer in growth medium (1:1 mixture of nutrient mixture Ham F12 and Dulbecco's modified medium Eagle with 10 % fetal calf serum). For subcultivation and pH-recovery experiments, the cells are detached from the surface of the culture vessels with trypsin–EDTA solution (2.5 g trypsin + 0.2 g EDTA per liter in Dulbecco's phosphate-buffered saline) and suspended in growth medium. Measurement time in pH_i recovery experiments is 400 s.

Porcine renal brush-border membrane vesicles (BBMV) prepared by a Mg^{2+} precipitation technique are loaded with 150 mmol/l NaCl, 5 mmol/l HEPES/Tris, pH 7.0, and preincubated for 10 min at 37 °C with various concentrations of NHE inhibitors. Intravesicular acidification through Na^+/H^+ exchange is started by diluting BBMV into Na^+-free buffer (150 mmol/l tetramethylammonium chloride, 5 mmol HEPES/Tris, pH 7.0) containing the appropriate concentrations of the NHE inhibitors and the fluorescent ΔpH indicator acridine orange (12 µmol/l). The fluorescence changes of acridine orange are recorded continuously by a Hitachi F-2000 spectrofluorometer at 495 nm excitation and 525 nm emission wavelength. The initial acridine orange fluorescence quenching in controls (no inhibitor) is set to 100 %.

Evaluation

Values are presented as means ± SD (four measurement per concentration). The IC_{50} values and Hill coefficients are calculated using the SigmaPlot software. Statistical significance is calculated by means of the distribution-independent *H*-test and nonparametric *U*-test. $P < 0.05$ is considered as significant.

Modifications of the Method

Counillon et al. (1993) and Scholz et al. (1995) determined the NHE subtype specificity of Na^+/H^+ antiporters by their ability to inhibit initial rates of amiloride-sensitive $^{22}Na^+$ uptake in fibroblast cell lines separately expressing the NHE1, NHE2, and NHE3 isoforms.

Ko et al. (2004) determined the inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships.

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NO Activity and Rho Kinase Activity

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Nitric Oxide

General Considerations on Nitric Oxide

Purpose and Rationale

The endothelium releases a labile, diffusible, vasorelaxing substance that has been termed endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor (Palmer et al. 1987; Vanhoutte 1999).

Nitric oxide plays a role in a wide range of physiological processes including regulation of blood flow and arterial pressure via endothelium-dependent relaxation of blood vessels (Rees et al. 1989; Moncada et al. 1991; Umans and Levi 1995; Huraux et al. 1999; McIntyre et al. 1999; Zanzinger 1999; Hropot et al. 2003), ischemia/reperfusion injury (Gao et al. 2002; Schulz et al. 2004), peripheral nitrergic transmission at smooth muscle (Rand and Li 1995), intracellular communication in the CNS with activation of guanylyl cyclase in target neurons (Southam and Garthwaite 1993), experimental stroke (Willmot et al. 2005), learning and memory (Susswein et al. 2004), neurogenic inflammation (Kajekar et al. 1995), regulation of leukocyte recruitment (Hickey 2001), and macrophage defense mechanisms following exposure to bacterial products (Förstermann et al. 1992; Förstermann and Kleinert 1995; Knowles and Moncada 1994). Fiorucci et al. (2002) discussed the effects of nitric oxide-releasing NSAIDs.

NO-donor drugs, such as sodium nitrite, sodium nitroprusside, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), and 3-morpholino-sydnonimine (SIN-1), are used as vasodilators (Schrör et al. 1989; Megson 2000). N^G-Nitro-L-arginine was described as an antagonist of endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release (Moore et al. 1990; Lamontagne et al. 1991). Ribero et al. (1992) proposed inhibition of nitric oxide synthesis by long-term treatment of rats with nitro-L-arginine as a new model of arterial hypertension.

Excessive production of NO damages DNA and activates poly(ADP-ribose)polymerase (PARP) (Pieper et al. 1999). In cases of massive NO production, neurons enter the PARP-suicide pathway. NO damages DNA via two major pathways: the first involves nitrosation of primary or secondary amines and nucleic acid bases, whereas the second involves the combination of NO with superoxide to form peroxynitrite (Szabó 1996, Szabó et al. 1997). The most likely reactive oxidant intermediate responsible for DNA breakage is peroxynitrous acid which rapidly oxidizes sulfhydryl groups and also nitrates and hydroxylates aromatic compounds including tyrosine, tryptophan, and guanosine (Halliwell 1997). Downstream DNA damage that follows excessive NO production results in significant activation of poly(ADP-ribose)polymerase which leads to rapid energy depletion and cell death (Feihl et al. 2001).

Davis et al. (2001) reviewed the non-3′,5′-cyclic guanosine-monophosphate-mediated effects of NO including modifications of proteins, lipids, and nucleic acids.

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Bioassay of EDRF Release

Purpose and Rationale

EDRF release from arterial endothelium can be studied by a sandwich technique using donor tissue with intact endothelium facing with its intimal side, the intimal side of a detector tissue.

Procedure

Rabbits are subjected to various kinds of treatment, e.g., atherogenic diet or drug treatment for the prevention of arteriosclerosis. Aorta segments, about 2 cm in length, are prepared, cut open along their longitudinal axis, and pinned to a tissue suspender without damaging the endothelium. These segments serve as donor tissue for EDRF. Circumferential aorta strips from the abdominal aorta of untreated control rabbits are de-endothelialized by gently blotting their luminal surfaces on wet filter paper. These denuded abdominal aorta strips are pinned opposite to the donor segments (intimal surface facing intimal surface) and function as detector for luminally released EDRF. Each sandwich preparation is suspended in a 40 ml organ bath, filled with oxygenated Krebs–Ringer buffer at 37 °C containing 10 mM indomethacin. After connecting the detector strip to a force transducer, the angle between the detector strip and the donor segment is minimized and the distance between donor and detector tissue standardized. After 1 h stabilization, the strips are brought to their optimum length–tension relationship by repeated exposure to 80 mM KCl. When a stable contractile response is established, the strips are precontracted with phenylephrine to 80–100 % of their KCl-induced contraction. After stabilization of plateau phase, cumulative doses of acetylcholine (0.01–10 mM) are added to induce EDRF release from donor tissues.

Evaluation

Relaxations of the detector strip induced by EDRF release from treated donor rabbit aortas are compared with aortas from control rabbits.

Modifications of the Method

Another bioassay for measuring function of cultured endothelial cells using a computer system for the acquisition and analysis of vascular contractility has been published by Winn et al. (1992).

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Isolated Arteries With and Without Endothelium

Purpose and Rationale

Endothelial cells are able to synthesize and release potent vasoconstrictive agents, such as endothelin and angiotensin as well as vasodilating agents, such as EDRF. In isolated arterial segments, the endothelial surface can be functionally destroyed allowing a differentiation between a direct action of drugs on the smooth muscle cells and an indirect effect via the endothelium. Isolated rings of rabbit or rat aorta are useful models to study the effects of endothelium-derived factors such as EDRF or endothelins and their antagonists (Linz et al. 1986; Tracey et al. 1990; Fujimoto et al. 1992; Fukuroda et al. 1992; Wiemer et al. 1992). A survey of the history and on techniques leading to the discovery of endothelium-dependent relaxation was given by Furchgott (1993).

Procedure

The descending thoracic aorta from rabbits of either sex (weighing 2.5–3.5 kg) is excised and dissected free from connective tissue. Care is taken to avoid damage of the endothelium. The aorta is divided into 2 mm wide rings and cut off in small strips. From some strips, the endothelium is removed by gently rubbing the intimal surfaces between the fingers for approximately 30 s. The strips are suspended in a 25 ml organ bath containing Krebs-bicarbonate solution at 37 °C being gassed continuously with 5 % CO_2/95 % O_2. Contractions of the strips are recorded isometrically with a load of 2 g on the tissues. After an equilibration period of 2 h, a stable baseline tone is reached.

To study the vasodilating effects of a compound, the strips are contracted with norepinephrine (10^{-8} M), or angiotensin II (10^{-7} M), or potassium chloride (20 mM). When a stable contraction plateau has been reached, the vasodilating agent is added in various concentrations. In these concentrations, norepinephrine, angiotensin II, and KCl evoke a response of 60–80 % of maximal contraction in intact rings of rabbit aorta. Rings without endothelium exhibit a response which is significantly enhanced in comparison with the response of the intact preparation after norepinephrine and angiotensin II precontraction.

To indicate the functional removal of the endothelium, the responsiveness of each preparation is tested with the known endothelium-dependent dilator, acetylcholine. In endothelium-intact rings, acetylcholine relaxes contractions induced by norepinephrine or angiotensin II. In precontracted rings devoid of endothelium, acetylcholine does not show any relaxing effect or causes contractions by itself at higher concentrations (Furchgott and Zawadzki 1980). As an example, atriopeptin III causes a similar concentration-dependent relaxation of all precontracted preparations with intact and with functionally destroyed endothelium indicating a direct effect on the smooth muscle cells. The relaxation is accompanied by an increase of cGMP.

Evaluation

Statistical analyses are performed by regression analysis of dose–response curves to determine EC_{50} values. Data are given as means ± standard deviation.

Critical Assessment of the Method

The isolated aortic ring of rabbits with and without functionally intact endothelium is a useful tool to differentiate direct effects on the arterial smooth musculature from effects mediated by the endothelium.

Modifications of the Method

Fujimoto et al. (1992) used the thoracic aorta from rats to study the effects between endothelin and an endothelin receptor antagonist. In transverse strips from **rat** thoracic aorta, 2 mm wide and 4–5 mm long, the endothelium was removed by gently rubbing the interior surface of the aorta. Concentration–response curves of contractions after ET-1 in the presence and the absence of the inhibitor were compared.

Pellisier et al. (1992) perfused the isolated mesenteric vascular bed of the **rat** with Tyrode solution and measured the perfusion pressure after injection of graded doses of norepinephrine and the dose-dependent relaxation due to acetylcholine in the vascular bed precontracted by norepinephrine infusion. In order to destroy the endothelial layer, the perfusate was changed to a hypotonic Tyrode solution containing all of the constituents present in normal Tyrode solution but one-tenth of the concentration resulting in disruption of more than 95% of the endothelial cells. The effect of norepinephrine was enhanced, whereas the effect of acetylcholine was abolished.

Legan and Sisson (1990) described a method to denude **rat** aortic endothelium in vitro with saponin.

Bohn and Schönafinger (1989) used helical strips of pulmonary arteries of **guinea pigs** in which the endothelium has been removed for biological detection of NO.

Fukuroda et al. (1992) used spiral strips from **porcine** coronary artery and vein and from intrapulmonary artery and vein removing the intimal surface by lightly rubbing with wet filter paper. Concentration–contraction curves for ET-1 and ET-3 were obtained with and without an endothelin antagonist.

Hayashi et al. (1988) described functional and anatomical recovery of endothelium after balloon denudation of the left circumflex coronary artery in **dogs**.

Endothelial denudation of the left circumflex coronary artery was used by Chu and Cobb (1987) to study the vasoactive effects of serotonin on proximal coronary arteries in awake **dogs**.

Experiments in isolated rings of the left circumflex or left anterior descending coronary artery of **dogs** with and without endothelium were performed by Desta et al. (1995).

Terrón (1996) analyzed the effects of 5-HT_1 receptor antagonists on 5-HT and sumatriptan-induced isometric contractions in endothelium-denuded segments of **canine** coronary arteries.

Ren et al. (1993) isolated coronary arteries from Japanese **monkeys** (*Macaca fuscata*) with and without endothelium to study muscarinic receptor subtypes mediating vasodilatation and vasoconstriction.

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Nitric Oxide Formation by Cultured Endothelial Cells

Purpose and Rationale

Endothelial cells are able to synthesize and to release not only potent vasoconstrictor peptides such as angiotensin and endothelin but also potent dilators such as nitric oxide (NO), ATP, substance P, and bradykinin.

NO formation can be assessed by determination of intracellular cyclic GMP in cultured endothelial cells, whereas release of NO from these cells can be measured by the stimulatory effect of NO on the activity of soluble guanylyl cyclase (Lückhoff et al. 1988; Wiemer et al. 1991; Linz et al. 1992; Bogle et al. 1992; review by Moncada et al. 1991).

Procedure

Endothelial Cell Culture

Bovine or porcine aorta is obtained from local slaughter houses. Endothelial cells are isolated by digestion with dispase (Lückhoff et al. 1988). The cells are seeded on 6- or 24-well plates (e.g., Nunc Intermed, Wiesbaden, Germany) and

grown to confluence. Dulbecco's modified Eagle's/Ham's F-12 medium containing 20 % fetal calf serum is supplemented with penicillin (10 U/ml), streptomycin (10 µg/ml), L-glutamate (1 mM/l), glutathione (5 mg/ml), and L(+) ascorbic acid (5 mg/ml) (Biotect protection medium).

Measurement of Cyclic GMP

Primary cultures of endothelial cells are used. After the removal of the culture medium by aspiration, the monolayer is washed twice with 2 ml HEPES–Tyrode's solution (37 °C). Thereafter, the cells are preincubated for 15 min at 37 °C with 3-isobutyl-1-methylxanthine (IBMX) (10^{-4} M/l). After this time, drugs or solvents are added. After predetermined periods, the incubation medium is quickly removed. The cells are then immediately extracted with 0.6 ml 6 % trichloroacetic acid and scraped off with a rubber scraper. The cell suspension is sonicated for 10 s before being centrifuged for 5 min at 4,000 *g*. The supernatants are extracted with four volumes of water-saturated diethyl ether, and the samples frozen (−20 °C) until analysis. The protein contents of the samples are measured according to Lowry et al. (1951). Cyclic GMP can be determined in the acetylated samples by various methods (Heath et al. 1992), e.g., using a commercially available radioimmunoassay (New England Nuclear). Cyclic GMP content is expressed as picomoles GMP per milligram protein.

Measurement of NO Release

The release of NO from endothelial cells is assayed on the basis of the stimulatory effect of NO on the activity of soluble guanylyl cyclase (purified from bovine lung) (Gerzer et al. 1981). The activity of the enzyme is determined in terms of the formation of cyclic [^{32}P]GMP from α-[^{32}P] GTP. Reactions are carried out in a reaction mixture containing 30 mM triethanolamine HCl (pH 7.4), 1 mM reduced glutathione, 4 mM $MgCl_2$, 1 mM cGMP, and 0.1 mg/ml bovine γ-globulin (total volume of 0.18 ml) at 37 °C in the presence of α-[^{32}P]GTP (0.03 mM; 0.2 µCi) and soluble guanylyl cyclase (4 µg). Ten µl samples are quickly transferred to the reaction mixture. Enzymatic formation of cGMP is allowed to proceed for 60 s and then stopped by the addition of 450 µl zinc acetate (120 mM) and 500 µl sodium carbonate (120 mM). A complete inhibition of cGMP formation can be achieved by preincubation of the monolayers for 30 min with the stereospecific inhibitor of NO synthase, N^G-nitro-L-arginine.

Evaluation

Time–response curves and dose–response curves after the addition of various activators or inhibitors of NO synthase are established. Data are reported as mean values ± SEM of cGMP (pmol/mg protein) or guanylyl cyclase activity (nmol/mg/min). Statistical evaluation is performed with Student's *t*-test.

Modifications of the Method

The clinical pharmacology of L-arginine has been reviewed by Böger and Bode-Böger (2001).

Isolation of porcine cerebral capillary endothelial cells has been described by Wiemer et al. (1994).

Feelisch and Noack (1987) and Nakazawa et al. (1992) used chemiluminescence techniques for the determination of NO.

A method for online detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy was described by Mordvintcev et al. (1991). Similar methods were used by Ichimori et al. (1992), Lancester et al. (1992), and Steel-Goodwin et al. (1992).

Hecker et al. (1995) used a cascade superfusion bioassay to characterize a stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells.

Electrochemical microprobes for direct measurement of NO in tissues have been developed (Shibuki 1990; Ishida et al. 1996; Smits and Lefebvre 1997).

Malinski and Taha (1992), Malinski and Huk (2001) and Linz et al. (1999) measured nitric oxide release by a porphyrinic-based microsensor with a detection limit of 10 nmol/l. The amperometric signal at a constant potential of 0.67 V was measured with a voltametric analyzer (PAR model 273, Princeton Applied Research) interfaced with an IBM80486 computer with data acquisition and software.

Gabriel et al. (1997) developed a method for the detection of intracellular nitric oxide generation in dissociated cerebellar granule cells using dichlorofluorescein diacetate and flow cytometry.

Sumpio et al. (1987) found that cyclic mechanical stress stimulates cultured bovine aortic endothelial cells to proliferate.

Using this method, Rosales et al. (1997) found that exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools.

Malinski (2015) describes the use of electrochemical nanosensors to simultaneously detect NO and ONOO− in near real time from a single cell.

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Expression of Nitric Oxide Synthase

Purpose and Rationale

Properties of various forms of nitric oxide synthase (NOS) have been described by Mayer et al. (1992), Leone et al. (1992), Hevel et al. (1992), Förstermann et al. (1992), Förstermann and Kleinert (1995), Salter et al. (1992), Pollock et al. (1992), Schmidt et al. (1992), and Mungrue et al. (2003), among them type I which is constitutively expressed in neurons; the inducible type II which is found in macrophages and hepatocytes but also in the brain (Moro et al. 1998), where it may contribute to NO-mediated neurotoxicity; and type III which is constitutively expressed in endothelial cells (Knowles and Moncada 1994). NOS can be inhibited by several routes, e.g., competition with L-arginine, NADPH, flavin, or tetrahydrobiopterin, interaction of the heme group of NOS, interference with Ca^{2+} availability, or calmodulin binding to the enzyme (Fukuto and Chaudhuri 1995). A widely used inhibitor is L-NAME (Vargas et al. 1991). Selective inhibition of constitutive NOS can be achieved by 7-nitroindazole (Moore et al. 1993) and of the inducible NOS by aminoguanidine hydrochloride (Griffiths et al. 1993) and by 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (=AMT) (Nakane et al. 1995). Linz et al. (1999) determined NOS in the left cardiac ventricle of hypertensive rats.

Procedure

Tissues are ground at the temperature of liquid nitrogen using a Mikro-Dismembranator (Braun). The powders are extracted for 1 h on ice with 10 mmol/l Tris–HCl, pH 7.4, containing 1 % SDS and protease inhibitors (complete, Boehringer Mannheim). Debris is removed by a 30 min centrifugation at 4 °C (~100,000 *g*). 100 μg of the total of the protein extracts are subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The eNOS protein is detected by the use of a specific antibody (monoclonal anti-NOS III, Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). As a secondary antibody, an anti-mouse IgG antibody coupled to alkaline phosphatase is used (Jackson ImmunoResearch Laboratories). Chemifluorescence is analyzed and quantified by scanning with a FluorImager 595 system (Molecular Dynamics).

Evaluation

The data are given as means ± SEM. ANOVA is used followed by Tuckey's test for post-ANOVA multiple pair comparisons.

Modifications of the Method

Linz et al. (1997) measured expression of ecNOS in the carotid artery of hypertensive rats by

Western blot analysis. Frozen (−70 °C) vessels were thawed and extracted with guanidium isothiocyanate/phenol/chloroform (Chomczynski and Sacchi 1987). Crude protein fractions were obtained by alcohol precipitation of the phenol phase. A total of 100 μg of the protein extracts was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Ponceau staining was performed to verify the quality of the transfer and the equipartition of protein in each lane. EcNOS protein was detected with a specific antibody (mouse NOS III, Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). The autoradiographs were analyzed by scanning densitometry.

McCall et al. (1991) identified *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase.

Bauersachs et al. (1998, 1999) measured vascular reactivity in isolated rat aortic rings mounted in an organ bath (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurement and determined superoxide anion production by lucigenin-enhanced chemiluminescence and endothelial nitric oxide synthase and soluble guanylyl cyclase expression by reverse transcription-polymerase chain reaction.

Von der Leyen et al. (1995) reported gene therapy inhibiting neointimal vascular lesions in rats. After denudation of the endothelium of carotid arteries by balloon injury, endothelial cell nitric oxidase expression in the vessel wall was restored by using the Sendai virus/liposome in vivo gene transfer technique.

Lund et al. (2000) found that gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits.

Mungrue et al. (2002) discussed lessons from murine genetic models on the role of NOS in heart failure.

Constitutive NOS (cNOS) and inducible NOS (iNOS) activity was determined in tissue and red blood cell suspension (Baynosa et al. 2013, Martins et al. 2014). Homogenized tissue or cells are incubated with the 3H-L-arginine and measured 3H-L-citrulline formed by the biochemical conversion of 3H-L-arginine by NOS. NOS activity is quantified in the eluate by counting in a liquid scintillation counter and expressed it as counts per minute (cpm). A commercial available kit Cayman Chemical (Ann Arbor, MI) can be used for determining NOS activity from tissue or cultured cells.

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Inhibition of Rho Kinase

Purpose and Rationale

Rho is a member of the Ras-related family of small molecular weight GTP-binding proteins, and Rho works as a molecular switch by shuttling between the GDP-bound inactive form and the GTP-bound active form. Rho is involved in cell motility, cell adhesion, cytokinesis, Ras-induced transformation, transcriptional activation, and cell cycle progression. These actions, through Rho signaling, are mediated by downstream Rho effectors, such as the ROCK family of Rho-associated serine–threonine protein kinases. Studies with ROCK-specific inhibitors indicate that the ROCK pathway works in the contraction of vascular smooth muscle. Several studies indicate that Rho kinase may be a novel therapeutic target in the treatment of cardiovascular disease (Kobayashi et al. 2002; Shimokawa 2002; Ito et al. 2003, 2004; Nakakuki et al. 2005; Budzyn et al. 2006; Winaver et al. 2006).

Ishizaki et al. (2000) described the pharmacological properties of a specific inhibitor of Rho-associated kinases.

Procedure

Kinase Assay

Recombinant ROCK-I, ROCK-II, PKN, or citron kinase was expressed in HeLa cells as a Myc-tagged protein by transfection using Lipofectamine and was precipitated from the cell lysates by the use of 9E10 monoclonal anti-Myc antibody coupled to G protein Sepharose (Ishizaki et al. 1997). Recovered immunocomplexes were incubated with various concentrations of [^{32}P]ATP and 10 μg of histone type 2 as substrates, in the absence or presence of various concentrations of test compounds at 30 °C for 30 min in a total volume of 30 μl of the kinase buffer containing 50 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.02 % Brij 35, and 2 mM dithiothreitol. PKCα was incubated with 5 μM [^{32}P]ATP and 200 μg/ml histone type 2 as substrates in the absence or presence of various concentrations of test compounds at 30 °C for 10 min in a kinase buffer containing 50 mM Tris–HCl, pH 7.5, 0.5 mM $CaCl_2$, 5 mM magnesium acetate, 25 μg/ml phosphatidyl serine, 50 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate, and 0.001 % leupeptin in a total volume of 30 μl. Incubation was terminated by the addition of 10 μl of 4 × Laemmli sample buffer. After boiling for 5 min, the mixture was subjected to SDS-polyacrylamide gel electrophoresis on a 16 % gel. The gel was stained with Coomassie Brilliant Blue and then dried. The bands corresponding to histone type 2 were excised, and the radioactivity was measured.

Evaluation

K_i values were either determined by the double reciprocal plot or calculated from the equation $K_i = IC_{50}/(1 + S/K_m)$, where S and K_m are the concentration of ATP and the K_m value for ATP, respectively.

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PAF Binding and Endothelins Activity

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PAF-Binding Assay

Principle and Rationale

Injection of platelet-activating factor (PAF) induces a wide range of potent and specific effects on target cells, including aggregation of platelets and shock symptoms like systemic hypotension, pulmonary hypertension, increased vascular permeability, neutropenia, and thrombocytopenia. Inhalation of PAF causes immediate bronchoconstriction followed by inflammation of the airways (for further information, see section "Effect of Arachidonic Acid or PAF on Respiratory Function In Vivo," chapter "▶ Effects of Drugs on Air Ways").

Hikiji et al. (2004) showed in PAF-deficient mice that the absence of platelet-activating factor receptor protects mice from osteoporosis following ovariectomy, a model of postmenopausal osteoporosis.

PAF is also implicated in estrogen-induced angiogenesis via nuclear factor-κB activation (Seo et al. 2004) and in delaying corneal wound healing (Bazan 2005).

The PAF receptor belongs to the superfamily of G protein-coupled receptors (Chao and Olson 1993; Izumi and Shimizu 1995). Cloning studies have indicated a single human PAF receptor gene containing an intron at the 5′ flanking region, providing alternative sequences (Ishii and Shimizu 2000).

PAF antagonists have been reviewed extensively by Koltai et al. (1994) and Summers and Albert (1995).

The following procedure is used to detect compounds that inhibit the binding of ^{3}H-PAF (platelet-activating factor) in rabbit platelets (PAF receptor).

Procedure

Crude rabbit platelets are incubated in plastic tubes for 15 min at 25 °C in a buffer solution (0.54 g/l KH_2PO_4, 0.6 g/l Na_2HPO_4, 5.8 g/l NaCl, 1.0 g/l BSA, pH 7.1) with 1 nM synthetic ^{3}H-labeled PAF (1-*O*-[1,2-$^{3}H_2$]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and various concentrations of test compound. Nonspecific binding is determined in the presence of 10 μM CV 3988. Bound ligand is separated from the incubation medium by rapid filtration through Whatman GF/C glass fiber filters. Following rinsing with ice-cold buffer (3 × 5 ml), the filters are placed in 10 ml scintillation cocktail for radioactivity determination.

Evaluation

The following parameters are calculated:

- Total binding of ^{3}H-PAF
- Nonspecific binding in the presence of 10 μM CV 3988
- Specific binding = total binding–nonspecific binding
- % inhibition: 100–specific binding as percentage of the control value

Compounds are first tested at a single high concentration (5,000 nM) in triplicate. For those showing more than 50 % inhibition, a displacement curve is constructed using seven different concentrations of test compound. Binding potency of compounds is expressed either as a "relative binding affinity" (RBA) with respect to the standard compound (CV 3988) which is tested in parallel or as an IC_{50}:

$$\mathrm{RBA} = \frac{\mathrm{IC_{50}\ standard\ compound}}{\mathrm{IC_{50}\ compound}} \times 100\%$$

Standard data:

- CV 3988 IC_{50}: 276 nM ± 24 (n = 20)

Modifications of the Method

Several authors (Casals-Stenzel et al. 1987; Dent et al. 1989a, b; Ring et al. 1992; Ukena et al. 1988) used the specific platelet-activating factor receptor antagonist [^{3}H]WEB-2086 or [^{3}H]apafant to

identify and characterize the PAF receptors expressed on the cell surface of platelets, macrophages, and eosinophils.

Balsa et al. (1996) characterized [^{3}H]apafant binding to the PAF receptor on rabbit platelet membranes and compared a microplate filtration system with the standard method.

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Endothelin

General Considerations

Endothelin is an endothelium-derived peptide family consisting of three peptides (ET-1, ET-2, and ET-3) with very potent and long-lasting vasoconstrictive activity (Yanagisawa et al. 1988a, b; King et al. 1989; Miller et al. 1989; Yanagisawa

and Masaki 1989; Inoue et al. 1989; Shinmi et al. 1989; Vanhoutte et al. 1992; Davenport 2002; Masaki 2004).

Each ET isoform is synthesized as a large pre-pro-endothelin(pre-pro-ET) that is cleaved to pro-ET (big ET-1, ET-2, and ET-3) and then further processed by ET-converting enzymes 1 and 2 to yield mature ET (Kedzierski and Yanagisawa 2001).

Subtypes of endothelin receptors have been described (Takayanagi et al. 1991; Miyazaki et al. 1992). Molecular characterization of the ET_A and ET_B receptors was reported by Miyazaki et al. (1992)and Sakuarai et al. (1992). In addition, the existence of a third type, ET_C, was found in *Xenopus laevis* (Karne et al. 1993).

The comparison of recombinant endothelin receptors shows different affinity rank orders to the three endothelins (Masaki et al. 1994).

Grant et al. (1997) reported the in vitro expression of endothelin-1 and the ET_A and ET_B receptors by prostatic epithelium and stroma.

The ET peptides not only elicit potent and long-lasting contractions of isolated strips of various blood vessels in vitro but also increase blood pressure in vivo suggesting that this peptide family may be involved in the pathogenesis of cardiovascular diseases (Simonson and Dunn 1990; Masaki et al. 1991; Doherty 1992; Goto et al. 1996; Gray and Webb 1996; Douglas and Ohlstein 1997). Sarafotoxin S6c, originally isolated from snake venom, is an agonist which distinguishes between endothelin subtypes (Williams et al. 1991).

Two ET-1 receptor antagonists have been approved for the treatment of pulmonary hypertension, ambrisentan (ETA antagonist), and bosentan (dual ETA/ETB antagonist) (Liang et al. 2012). The potential of ET receptor antagonism for the treatment of hypertension and pulmonary hypertension was reviewed by Miyagawa and Emoto (2014).

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Evaluation of Endothelin Activity

Purpose and Rationale

Most investigators used isolated arteries to evaluate the activity of endothelins and derivatives. Rodman et al. (1989) compared the potency and efficacy of porcine and rat endothelin in rat aortic and pulmonary rings.

Procedures

Arterial rings are obtained from male Sprague–Dawley rats weighing from 300 to

400 g. Rats are anesthetized with 50 mg/kg i.p. pentobarbital, the chest is opened, 100 units heparin sulfate is injected into the right ventricle, and the rats are exsanguinated. Rings are then isolated from either the descending thoracic aorta or the right main pulmonary artery, cleaned of adventitia, and suspended from Grass FT03 force-displacement transducers in muscle baths containing 10 ml of physiologic salt solution of the following composition ($\times 10^{-3}$ M): $CaCl_2$ 1.80, $MgSO_4$ 0.83, KCl 5.3.6, NaCl 116.34, NaH_2PO_4 0.40, D-glucose 5.50, and $NaHCO_3$ 10.04. The solution is maintained at 37 °C and bubbled with 21 % O_2 and 5 % CO_2. Endothelium-denuded rings are prepared by gently rubbing the intima with a roughened steel rod. Denudation is confirmed by the absence of relaxation to 10^{-5} M acetylcholine in rings precontracted with 10^{-7} M norepinephrine. Resting force is adjusted to the optimum resting tension of 0.75 g for pulmonary artery rings and 1.0 g for aortic rings. Maximum contraction to 8×10^{-2} M KCl is determined, and subsequent responses to endothelin are expressed as a percentage of maximum KCl contraction for the determination of maximum effectiveness or as a percentage of maximum endothelin contraction for the determination of potency.

Evaluation

Concentration–response curves are compared using the method of Carpenter (1986). Data are expressed as means ±SEM, and statistical comparisons are performed using Student's *t*-test, with $P < 0.05$ considered significant.

Modifications of the Method

Lembeck et al. (1989) studied the effects of endothelin on the cardiovascular system and on smooth muscle preparations in different species.

Reynolds and Mok (1990) studied the role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of **rat aorta** to endothelin.

Pang et al. (1990) studied the cellular mechanisms of action of endothelin in **isolated canine coronary arteries**.

Lüscher et al. (1992) used **perfused and pressurized mesenteric resistance arteries of rats** and **human internal mammary arteries** to study the interaction between endothelin- and endothelium-derived relaxing factors.

Michel et al. (2003) studied the endothelin system in various animal models of pulmonary hypertension.

Advenier et al. (1990) studied the contractile activity of three endothelins (ET-1, ET-2, and ET-3) on the **human isolated bronchus**.

Wallace et al. (1989) compared the effects of endothelin-1 and endothelin-3 on the **rat stomach**.

Aldosterone secretion in cultured calf zona glomerulosa cells was stimulated by ET-1 and sarafotoxin S6b to a similar degree, but less than by angiotensin II (Gomez-Sanchez et al. 1990).

Brock and Danthuluri (1992) used **cultured vascular smooth muscle cells** to study the cellular actions of endothelin.

Pigment dispersion in cultured dermal melanophores from *Xenopus laevis* was used as an indicator of ET_C receptor-mediated responses (Karne et al. 1993).

ET-1 content in human serum was previously determined using sandwich ELISA (QuantiGlo Chemiluminescent Immunoassay; R&D Systems, Minneapolis, MN) with a sensitivity of 0.064 pg/ml (Tulppo et al. 2014). Immunoassay was also used to determine ET-1 content in carotid bodies and conditioned media (Jacono et al. 2005; León et al. 2014).

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Endothelin Receptor Antagonism In Vitro

Purpose and Rationale

Competitive endothelin antagonists are of therapeutic interest (Ihara et al. 1991; Fujimoto et al. 1992; Fukuroda et al. 1991; Urade et al. 1992; Breu et al. 1993; Mihara and Fujimoto 1993; Sogabe et al. 1993; Warner 1994; Opgenorth 1995; Brunner 1998).

A sensitive sandwich enzyme immunoassay for human endothelin has been established by Suzuki et al. (1989).

Procedure

The ventricles of rat hearts are minced with scissors and homogenized in 7 vol of ice-cold 20 mM $NaHCO_3$ containing 0.1 mM PMSF (phenylmethylsulfonyl fluoride), pH 7.4, with a Polytron homogenizer (Brinkman Instruments Inc., Westberg, NY). The homogenates are centrifuged at 1,000 *g* for 10 min and then the pellet discarded. The supernatant is centrifuged at 30,000 g for 30 min. The pellet is washed once and resuspended in Tris buffer (50 mM, pH 7.4 at 25 °C) containing 0.1 mM PMSF and stored at −80 °C until use.

For binding studies (Gu et al. 1989), cardiac membranes (0.21 mg/ml as protein) are incubated

with 25 pM [^{125}I]ET-1 or [^{125}I]ET-3 (New England Nuclear) in a final assay volume of 0.1 ml in borosilicated glass tubes, containing 50 mM Tris–HCl, 0.1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 250 µg/ml bacitracin, and 10 µg/ml soybean trypsin inhibitor (pH 7.4). Binding is performed for 60 min at 37 °C. The binding reaction is terminated by the addition of 2.5 ml of ice-cold 50 mM Tris–HCl (pH 7.4), followed by a rapid filtration through a Whatman GF/C glass fiber filter (presoaked in 1 % polyethyleneimine) under reduced pressure. The filters are then quickly washed four times with 2.5 ml of the buffer. Radioactivity retained on the filter is counted.

Evaluation

Nonspecific binding is defined in the presence of ET-1. Specific binding is the difference between total and nonspecific binding. K_i values and Scatchard plots are calculated.

Modifications of the Method

The nomenclature of endothelin receptors has been reviewed by Alexander et al. (2001).

Cain et al. (1991) described an endothelin-1 receptor-binding assay for high-throughput chemical screening using the clonal cell line A10 of smooth muscle cells, derived from embryonic rat thoracic aorta.

Functional endothelin/sarafotoxin receptors were described in rat heart myocytes (Galron et al. 1989) and in the rat uterus (Bousso-Mittler et al. 1989).

Mihara and Fujimoto (1993) cultured rat aortic smooth muscle A7r5 cells expressing ET_A receptors (Takuwa et al. 1990) and human Girardi heart cells expressing ET_B receptors (Mihara and Fujimoto 1992). Receptor specificity could be demonstrated.

Mihara et al. (1994) characterized the nonpeptide endothelin receptor antagonist 97–139, both in vitro (rat aortic smooth muscle cells and Girardi heart cells) and in vivo (ET-1antagonism in pithed rats) and compared it with another endothelin receptor antagonist (BQ-123). Discrepancies between in vitro and in vivo data were explained by a different plasma binding.

Aramori et al. (1993) studied the receptor-binding properties and the antagonistic activities of an endothelin antagonist in transfected Chinese ovary hamster cells permanently expressing the two ET receptor subtypes (ET_A and ET_B).

De Juan et al. (1993) characterized an endothelin receptor subtype B in the retina of rats.

Clozel et al. (1994) performed binding assay on cells or membranes from baculovirus-infected insect cells that expressed recombinant ET_A or ET_B receptor, CHO cells that expressed recombinant ET_A or ET_B receptor, cultured human vascular smooth muscle cells from umbilical veins, rat mesangial cells (for ET_A), and microsomal membranes from human placenta and from porcine cerebellum (for ET_{B1}) and from porcine trachea (for ET_{B2}, using BQ-3020 or sarafotoxin S6C as ligand).

Williams et al. (1965) used CHO cells expressing cloned ET_A or ET_B receptors directly in binding and functional assays without preparing membranes from them.

Reynolds et al. (1995) used CHO-K1 cells expressing recombinant human ET_B receptor, Ltk^-cells expressing human ET_A receptor, and rabbit renal artery vascular smooth muscle cells expressing rabbit ET_A receptor for evaluation of an ET_A receptor antagonist.

Rat or bovine cerebella were used for differentiation of receptor subtypes (Williams et al. 1991).

Peter and Davenport (1995) proposed a selective ligand for ET_A receptors.

Ihara et al. (1992) and Watakabe et al. (1992) described radioligands for endothelin (ET_B) receptors.

Vigne et al. (1996) described the properties of an endothelin-3-sensitive Eta-like endothelin receptor in brain capillary endothelial cells.

The human type B endothelin receptor was cloned from human lung poly A + RNA and

expressed in CHO cells by Chiou et al. (1997). Dissociation characteristics of endothelin receptor agonists and antagonists were determined.

Stables et al. (1997) described a bioluminescent assay for agonist activity at G protein-coupled receptors, such as the endothelin ET_A receptor. Transient expression of apoaequorin in CHO cells and reconstitution with the cofactor coelenterazine resulted in a large, concentration-dependent agonist-mediated luminescent response following cotransfection with the endothelin ET_A, angiotensin AT_{II}, and TRH and neurokinin NK_1 receptors, all of which interact predominantly with the $G\alpha_q$-like phosphoinositidase-linked G-proteins.

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Endothelin Receptor Antagonism In Vivo

Purpose and Rationale

Various pharmacological models have been used for the characterization of endothelins and endothelin antagonists, such as the isolated porcine coronary artery (Hickey et al. 1985; Yanagisawa et al. 1988, Yanagisawa and Masaki 1989; Inoue et al. 1989; Kimura et al. 1989; Ihara et al. 1991; Fukuroda et al. 1991).

Since the smooth musculature is considered to contain mainly ET_A receptors, the preparation is used to test ET_A antagonists.

Procedure

Left anterior descending coronary arteries are isolated from fresh porcine hearts. Connective tissues and adherent fat are removed. For the removal of vascular endothelium, the intimal surface of spiral strips is rubbed gently with filter paper. The endothelium-denuded arteries are cut into spiral strips about 10 mm long and 1 mm wide. Each strip is suspended in an organ bath containing Krebs–Henseleit solution bubbled with 95 % O_2/5 % CO_2 at 37 °C. After equilibration, reference contraction is isometrically obtained with 50 mM KCl. Concentration–response curves for ET-1 are obtained by cumulative additions of ET-1. Antagonists are added 20 min before the cumulative additions of ET-1.

Evaluation

The pA_2 values and slopes are obtained by the analysis of Schild plots.

Modifications of the Method

Opgenorth et al. (1996) characterized an orally active and highly potent ET_A-selective receptor antagonist by in vitro and in vivo methods.

Calo et al. (1996) investigated three **rabbit vessels, the carotid, the pulmonary artery, and the jugular vein,** to identify vascular monoreceptor systems, either ET(A) or ET(B), for structure–activity studies of endothelins and their antagonists.

Vedernikov et al. (1993) used **rings of the left circumflex coronary artery** from **dogs** which were denuded of endothelium and exposed to anoxic periods. August et al. (1989) and Urade et al. (1992) used **rat aortic smooth muscle denuded of the epithelium** and Sogabe et al. (1993) **spirally cut strips of rabbit aorta**.

Williams et al. (1995) used **rat aorta, rabbit iliac and pulmonary artery** for contractile assays, and **anesthetized ferrets and conscious normotensive dogs** as in vivo models to characterize a nonpeptidyl endothelin antagonist.

Itoh et al. (1993) studied the preventive effect of an ET_A receptor antagonist on the **experimental cerebral vasospasm in dogs** using a two-hemorrhage model of subarachnoid hemorrhage. Clozel et al. (1993) performed similar experiments in rats.

The **vasodilating** effect in the **isolated perfused rat mesentery** which is found after the infusion of rat endothelin (Warner et al. 1989) and after the selective ET_B receptor agonist sarafotoxin S6c (Williams et al. 1991) can be antagonized by an endothelin receptor antagonist (Clozel et al. 1993).

Ercan et al. (1996) found an increase of digoxin-induced ectopic ventricular complexes by endothelin peptides in **isolated guinea pig hearts**, which could be antagonized by an endothelin-A receptor antagonist.

The **endothelin-induced sustained increase of blood pressure** in anesthetized rats was studied by Yanagisawa et al. (1988), Inoue et al. (1989) and Ihara et al. (1991). Intravenous bolus injection of endothelin causes a biphasic blood pressure response: a transient decrease, probably mediated from the release of vasodilator mediators (prostacyclin and EDRF), and a sustained increase (Rubanyi and Bothelho 1991).

Nishikibe et al. (1993) examined the **antihypertensive effect** of an endothelin antagonist in a genetic hypertensive model (**stroke-prone spontaneously hypertensive rats**).

Watanabe et al. (1995) characterized the pharmacological profile of a nonselective endothelin receptor antagonist and studied the **inhibition of myocardial infarct size** in rats.

The contractile activity of the **isolated guinea pig trachea without epithelium** and of the guinea pig longitudinal muscle was used by Urade et al. (1992) for the determination of **ET_B receptor**-mediated responses.

Spinella et al. (1991) assessed the bioactivity of a specific endothelin-1 antagonist in an **isolated perfused guinea pig lung** preparation in which pulmonary artery pressure was monitored.

Gosselin et al. (2002) demonstrated the effects of a selective ET_A receptor antagonist in murine models of allergic asthma.

Tabrizchi and Ford (2003) studied the hemodynamic effects of the endothelin receptor antagonist tezosentan in anesthetized rats treated with tumor necrosis factor-alpha.

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Quantitative Autoradiographic Localization of Endothelin-1 Receptor

Purpose and Rationale

The endothelin-1 (ET-1) receptor can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized in vitro autoradiography (Kohzuki et al. 1996)

Procedure

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see section "Myocardial Infarction After Coronary Ligation in Rodents," chapter "▶ Coronary Drugs"). After various time intervals (1–8 months), the animals are decapitated; the hearts rapidly removed and snap-frozen in isopentane at −40 °C. Frozen section (20 μm) is cut in a cryostat at −20 °C. The sections are thaw mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4 °C, and then stored at −80 °C.

Quantitative Autoradiography

Radioligand: Endothelin-1 is iodinated with ^{125}iodine using Iodogen (Pierce Chemical Co, IL, USA).

^{125}I-ET-1 Binding: The sections are preincubated for 15 min at 20 °C in 20 mmol/L Hepes buffer, pH 7.4, containing 135 mmol/L NaCl, 2 mmol/L CaCl2, 0.2 % BSA, and 0.01 % bacitracin. The sections are then incubated with 11.1 KBq/ml ^{125}I-ET-1 in the same buffer for 60 min at 20 °C. Nonspecific binding is determined in the presence of 10^{-6} mol/L ET-1. Binding isotherms are determined using a set of serial sections incubated with 10^{-12} to 10^{-6} mol/L unlabeled ET-1 for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and

stained with hematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

Evaluation

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle, and the non-infarcted area in the left ventricle are estimated by an iterative nonlinear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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Inhibition of Endothelin-Converting Enzyme

Purpose and Rationale

Endothelin-converting enzyme inhibitors that suppress the biosynthesis of endothelin are therefore potential antihypertensive drugs (De Lombaert et al. 1994; Trapani et al. 1995; Morita et al. 1994; Bihovsky et al. 1995; Claing et al. 1995; Descombes et al. 1995; Chackalamannil et al. 1996; Jeng 1997; Jeng and DeLombaert 1997; Brunner 1998).

Purification of rat and porcine endothelin-converting enzyme (ECE) was reported by Ohnaka et al. (1993) and Takahashi et al. (1993). Molecular cloning and characterization of the enzyme ECE-1 was performed in rat (Shimada et al. 1994), bovine (Ikura et al. 1994; Schmidt et al. 1994; Xu et al. 1994), and human tissue (Schmidt et al. 1994; Shimada et al. 1995; Yorimitsu et al. 1995).

A second enzyme, termed ECE-2, was cloned (Emoto and Yanagisawa 1995).

Walkden and Turner (1995) described the expression of endothelin-converting enzyme and related membrane peptidases, e.g., the endopeptidase E-24.11, in the human endothelial cell line EA.hy926.

In Vitro Assay

A rapid and selective in vitro assay for endothelin-converting enzyme was described by Fawzi et al. (1994). The assay is based on the quantitative determination of [^{125}I]endothelin-1 released from (3-[^{125}I]iodotyrosyl^{13}) big endothelin-1 by binding to the membrane-bound endothelin receptor.

Procedure

For the **preparation of lung membranes**, frozen guinea pig lungs are weighed and homogenized in 10 times gram tissue weight of solution A (50 mM

Tris–HCl, pH7.4, 025 M sucrose, and 2 mM EDTA) using a Polytron tissue homogenizer. Homogenization is repeated four times with 5–8 min intervals between homogenization. Homogenates are spun for 30 min at 2,000 g. Supernatants containing membranes are carefully decanted and saved. Pellets are rehomogenized in solution A and homogenates are spun at 2,000 g for 30 min. Supernatants are removed, mixed with supernatants from the first spin, and spun at 100,000 g for 60 min. Pellets containing membranes are suspended in solution B (10 mM Tris–HCl, pH7.4, and 0.125 M sucrose) using a Dounce homogenizer. Samples are divided into 1 ml fractions, rapidly frozen in a dry ice methanol bath, and stored at −80 °C.

Rat liver membranes are prepared with the same method and further purified over a sucrose step gradient (10 mM Tris–HCl, pH 7.4) containing 44 % sucrose at a protein concentration of 2 mg/ml. Samples of 25 ml are placed in ultraclear centrifuge tubes for the Beckman SW28 rotor, overlaid with 10 ml solution C containing 42.3 % sucrose, and spun for 2 h at 27,000 rpm (100,000 g). Top layers containing membrane are collected and diluted with solution C to obtain an 8 % sucrose concentration. Samples are spun in a 45 Ti rotor (100,000 g) for 1 h. Supernatants are discarded. Pellets containing membrane are suspended in solution B, divided into 1 ml samples, rapidly frozen in dry ice-methanol bath, and stored at −80 °C.

For the **endothelin (ET)-binding assay**, membrane preparations are incubated with selected concentrations of [^{125}I]endothelin-1 (final reaction volume = 500 µl) in a solution D containing 60 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 6 mg/ml BSA for 90 min at 37 °C. Reactions are terminated by the addition of 4 ml of solution E containing 10 mM Tris–HCl, pH 7.4, and 150 mMNaCl at 4 °C followed by rapid filtration on Whatman GF/B glass microfiber filters. Filters are presoaked for 1 h at 4 °C in a solution containing 50 mM Tris–HCl, pH 7.4, 10 mg/ml BSA, and 0.1 % sodium azide. Test tubes and filters are washed four times with 4 ml of solution E at 4 °C, and radioactivity retained on the filters is counted in a gamma counter. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture.

For the **endothelin-converting enzyme assay**, samples containing 10 µg of protein are incubated in a solution containing 50 mM Tris–HCl, pH7.0, 100 mM NaCl, and 5 mg/ml BSA in a final volume of 100 µl. Conversion reactions are initiated by addition of [^{125}I]big endothelin-1 to obtain a final concentration of 500 pM. Samples are incubated for 2 h at 37 °C. To measure [^{125}I]endothelin-1 released from [^{125}I]big endothelin-1 conversion, 50 µg of purified rat liver membranes (as a source of ET receptors) is added to the reaction mixture, and reaction volume is adjusted to 500 µl and solution composition is adjusted to that of solution D of the endothelin-binding assay. Following a 90 min incubation at 37 °C to reach equilibrium in binding, reactions are terminated by addition of a solution E at 4 °C followed by rapid filtration on Whatman GF/B glass microfiber filters. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture. Specific ET-1 binding is used as an index of endothelin-converting enzyme activity.

To test the effect of endothelin-converting enzyme inhibitors, endothelin-converting enzyme assays are carried out in the presence of desired concentrations of the compounds.

Evaluation

Endothelin-converting enzyme activity in the presence of compounds is expressed as a percentage of control endothelin-converting enzyme activity in the membrane preparation which is determined simultaneously. The concentration of compounds producing a 50 % inhibition of endothelin-converting enzyme activity (IC_{50} values) is determined from a plot of the percentage of control endothelin-converting enzyme activity versus log concentration of compounds.

In Vivo Assay

Procedure

Male Sprague–Dawley rats weighing 300–400 g are anesthetized with ether, spinalized, and placed

under artificial respiration. The vagus nerves are cut and the carotid arteries ligated. A catheter is placed in one of the carotid arteries to allow measurement of arterial blood pressure. The second catheter is placed into the penile vein to allow infusion or injection of drugs. After stabilization, the animals receive a first injection of either ET-1, big ET-1, norepinephrine, angiotensin I, or AT II. The pressor responses are recorded, and after the return to the baseline, a second injection of the agonist is given either in the presence or the absence of the inhibitor.

Evaluation

Data are calculated as mean ±SEM. Student's *t*-test for paired and unpaired observations is used to analyze the results.

Modifications of the Method

Little et al. (1994) developed a two-step protocol for high-throughput assays of endothelin-converting enzyme activity. Human umbilical vein and human aorta endothelial cells were found to preferentially convert the big endothelin-1 isopeptide through a membrane-bound, thiorphan-insensitive, and phosphoramidon-sensitive zinc metalloendopeptidase. Endothelins are quantified by a separate step using either enzyme immunoassays or radioreceptor assays in 96-well formats. The method can be used to either characterize ECE from different tissues or screen for inhibitors of a specific ECE activity.

McMahon et al. (1993) tested the effects of endothelin-converting enzyme inhibitors and endothelin receptor subtype A antagonists on blood pressure in spontaneously and renal hypertensive rats.

Changes of vascular resistance in isolated perfused kidneys were used by Descombes et al. (1995) to characterize a selective inhibitor of big ET-1 responses. The studies were performed on kidneys taken from adult male Wistar rats (300–400 g). The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the left kidney was prepared for infusion with Tyrode solution. The changes in renal vascular resistance were recorded as changes in perfusion pressure monitored at constant flow (6 ml/min). After stabilization, a bolus injection of ET-1 or big ET-1 was administered, and the resulting pressure responses were recorded. On return to baseline levels, a second injection of the endothelins was given either under control conditions or in presence of the putative enzyme inhibitor.

Because increasing evidence implicates that endothelin plays a role in the pathophysiology of cerebral insults, Kwan et al. (1997) studied the prevention and reversal of cerebral vasospasm in an experimental model of subarachnoid hemorrhage. Three ml of arterial blood was withdrawn from the ear artery of rabbits and injected into the cisterna magna under anesthesia. Drugs were administered either before or 24 h after this procedure. Forty-eight hours later, the animals were anesthetized again, and perfusion fixation was performed with Hank's balanced salt solution followed by a mixture with 2 % paraformaldehyde and 2.5 % glutaraldehyde. Cross sections of the basilar arteries were analyzed by computer-assisted morphometry.

A review on the knowledge of molecular pharmacology of endothelin-converting enzymes was given by Turner and Murphy (1996).

Johnson and Ahn (2000) developed an internally quenched fluorescent substrate selective for endothelin-converting enzyme-1.

Luciani et al. (2001) described highly sensitive and selective fluorescence assays for rapid screening of endothelin-converting enzyme inhibitors.

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Patch-Clamp and Voltage-Clamp Techniques

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Patch-Clamp Technique

Purpose and Rationale

The introduction of the patch-clamp technique (Neher and Sakmann 1976) revolutionized the study of cellular physiology by providing a high-resolution method of observing the function of individual ionic channels in a variety of normal and pathological cell types. By the use of variations of the basic recording methodology, cellular function and regulation can be studied at a molecular level by observing currents through individual ionic channels (Liem et al. 1995; Sakmann and Neher 1995).

The most intriguing method is called the "on-cell" or "cell-attached" configuration, because ion channels can be recorded on an intact cell (Jackson 1993). This mode is well suited for investigation of ion channels that are activated by hormonal stimulation and triggered by intracellular second messengers.

Another versatile mode is the "cell-excised" configuration (Hamill 1993). It is obtained by suddenly removing the patch pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows the investigator to expose the channel proteins to drugs by changing the bath solution. The single-channel currents are recorded on a videotape and are analyzed off-line by a computer system. Various parameters are evaluated, such as the single-channel conductance, the open and closed times of the channel, and the open-state probability, which is the percentage of time the channel stays in its open state.

In addition to these modes, which enable the recording of single-channel currents, it is also possible to measure the current flowing through the entire cell. This "whole-cell mode" is obtained by rupturing the membrane patch in the cell-attached mode (Hamill et al. 1981; Dietzel et al. 1993). This is achieved by applying suction to the interior of the patch pipette. The "whole-cell mode" allows not only the recording of electrical current but also the measurement of cell potential. Moreover, the cell interior is dialyzed by the electrolyte solution contained in the patch pipette.

The fabrication of patch-clamp pipettes has been described by Sakmann and Neher (1995) and Cavalié et al. (1993).

Variations of the patch-clamp technique have been used to study neurotransmitter transduction mechanisms (Smith 1995).

High-throughput methods are required when developing drugs that work on ion-channel function (Mathes 2003; Bennett and Guthrie 2003). Patch clamping suffers from low throughput, which is not acceptable for drug screening.

Fertig et al. (2002) and Brueggemann et al. (2004, 2006) presented nanopatch-clamp technology, which is based on a planar, microstructured glass chip, which enables automatic whole-cell patch-clamp experiments. Planar glass substrates containing a single microaperture produced by ion track etching are used to record currents through ion channels in living mammalian cells.

Falconer et al. (2002) reported high-throughput screening for ion-channel modulators setting up a Beckman/Sagian core system to fully automate functional fluorescence-based assays that measure ion-channel function. Voltage-sensitive fluorescent probes were applied and the activity of channels was measured using Aurora's Voltage/Ion Probe Reader (VIPR). The system provides a platform for fully automated high-throughput screening as well as pharmacological characterization of ion-channel modulators.

Schroeder et al. (2003) described a high-throughput electrophysiology measurement platform consisting of computer-controlled fluid handling, recording electronics, and processing tools capable of whole-cell voltage-clamp recordings from thousands of individual cells per day. The system uses a planar, multiwell substrate (a PatchPlate). The system positions one cell into a hole separating two fluid compartments in each well of the substrate. Voltage control and current recordings from the cell membrane are made subsequent to gaining access to the cell interior by applying a permeabilizing agent to the intracellular side.

Willumsen's group recommended ion-channel screening with QPatch (Asmild et al. 2003; Kutchinsky et al. 2003; Krzywkowski et al. 2004). This system claims to allow fast and

accurate electrophysiological characterization of ion channels, e.g., for determination of IC_{50} values for ion-channel blockers. The system comprises 16 parallel patch-clamp sites, each based on a silicon chip with a micro-etched patch-clamp hole. Intra- and extracellular fluids are administered by laminar flow through integrated miniature flow channels.

Spencer et al. (2012) described a novel microfluidic automated patch-clamp device; the IonFlux™ system utilizes microfluidic channels molded into a polymeric substrate that eliminates the necessity of internal robotic liquid handling.

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Patch-Clamp Technique in Isolated Cardiac Myocytes

Purpose and Rationale

The generation of an action potential in heart muscle cells depends on the opening and closing of ion-selective channels in the plasma membrane. The patch-clamp technique enables the investigation of drug interactions with ion-channel-forming proteins at the molecular level.

Procedure

Isolated cells from ventricular muscle of rat and guinea pig are prepared as described by Yazawa et al. (1990). Animals are sacrificed by cervical dislocation. Hearts are dissected and mounted on a Langendorff-type apparatus and perfused first with Tyrode solution (in mM: 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 5 HEPES, pH adjusted to 7.4 with NaOH) at 37 °C for 3 min at a hydrostatic pressure of 60–70 cmH_2O, then with nominally Ca^{2+}-free Tyrode solution (no Ca^{2+} is added) for 5–7 min, and finally, with nominally Ca^{2+}-free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma, type I). After 15–20 min of collagenase treatment, the heart is now soft and is washed with storage solution (in mM: 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 10 HEPES, 0.5 EGTA, pH adjusted to 7.4 with KOH). The ventricles are cut into pieces (about 5 mm × 5 mm) and poured into a beaker. The myocytes are dispersed by gently shaking the beaker and filtration through a nylon mesh (365 μm). Then, the myocytes are washed twice by centrifugation at 600–1,000 rpm (about 90 *g*) for 5 min and kept at room temperature. The rod shape of the cell and the clear striations of sarcomeres are important criteria for selecting viable cells for the assay. Experiments are performed at 35 °C–37 °C.

For investigation with the patch-clamp technique (Neher and Sakmann 1976; Hamill et al. 1981), the isolated cells are placed into a thermostat-controlled chamber, mounted on the stage of an inverted microscope equipped with differential interference contrast optics. Under optical control (magnification 400×), a glass micropipette, having a tip opening of about 1 μm, is placed onto the cell. The patch pipettes are fabricated from borosilicate glass tubes (outer diameter 1.5 mm, inner diameter 0.9 mm) by means of an electrically heated puller. In order to prevent damage of the cell membrane, the tip of the micropipette is fire polished, by moving a heated platinum wire close to the tip. The patch pipette is filled with either high-NaCl or KCl solution and is mounted on a micromanipulator. A silver chloride wire connects the pipette solution to the head stage of an electronic amplifier. A second silver chloride wire is inserted into the bath and serves a ground electrode.

After establishing contact with the cell membrane, a slight negative pressure is applied to the inside of the patch pipette by means of a syringe. Consequently, a small patch of membrane is slightly pulled into the opening of the micropipette, and close contact between the glass and membrane is formed, leading to an increase of the electrical input resistance into the giga-ohm range (about 10^{10} Ω). This high input resistance enables the recording of small electrical currents in the range of picosiemens (10^{-12} S), which flow through channel-forming proteins situated in the membrane patch. The electrical current is driven by applying an electrical potential across the membrane patch and/or by establishing an appropriated chemical gradient for the respective ion species.

The patch-clamp method allows one to investigate the interaction of drugs with all ion channels involved in the functioning of the heart muscle cell (K^+, Na^+, Ca^{2+}, and eventually Cl^- channels). Moreover, the different types of K^+ channels existing in cardiomyocytes can be distinguished by their different single-channel characteristics or by appropriate voltage-pulse protocols in the whole-cell mode.

Evaluation

Concentration–response curves of drugs which inhibit or activate ion channels can be recorded either at the single-channel level or by measuring the whole-cell current. IC_{50} and EC_{50} values (50 % inhibition or activation, respectively) can be obtained with both methods.

Modifications of the Method

The patch-clamp technique has been used for evaluation of antiarrhythmic agents (Bennett et al. 1987; Anno and Hondeghem 1990; Gwilt et al. 1991).

Gögelein et al. (1998) used isolated ventricular myocytes from guinea pigs to study a cardioselective inhibitor of the ATP-sensitive potassium channel.

Multiple types of calcium channels have been identified by patch-clamp experiments (Tsien et al. 1988).

The effects of potassium channel openers have been measured (Terzic et al. 1994).

Ryttsén et al. (2000) characterized electroporation of single NG108–15 cells with carbon-fiber microelectrodes by patch-clamp recordings and fluorescence microscopy.

Monyer and Lambolez (1995) reviewed the molecular biology and physiology at the single-cell level, discussing the value of the polymerase chain reaction at the single-cell level and the use of patch pipettes for collecting the contents of a single cell on which the reverse transcription is performed.

The patch-clamp technique was found to be very versatile in the investigation of ion channels in atrial myocytes, especially from dogs or humans. Cells were obtained from atria either in sinus rhythm or in atrial fibrillation (reviewed in Bosch et al. 1999).

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Voltage-Clamp Studies on Sodium Channels

Purpose and Rationale

The epithelial Na^+ channel plays an important role in epithelial Na^+ absorption in the distal colon, urinary bladder, salivary and sweat ducts, respiratory tract, and, most importantly, distal tubules of the kidney (Catterall 1986; Palmer 1992). Regulation of this epithelial Na^+ channel has a major impact on Na^+ balance, blood volume, and blood pressure. Inhibition of epithelial Na^+ channel expression is used for the treatment of hypertension (Endou and Hosoyamada 1995). Busch et al. (1995) studied the blockade of epithelial Na^+ channels by triamterenes using two-microelectrode voltage-clamp experiments in *Xenopus* oocytes expressing the three homologous subunits (α, β, and γ) of the rat epithelial Na^+ channel (rENaC).

Procedure

Xenopus laevis oocytes are injected with the appropriate cRNA encoding for the α-, β-, and γ-subunits Canessa et al. (1994) of the rat epithelial Na^+ channel (rENaC). The cRNA for the wild-type α-subunit and its deletion mutant Δ278–273 is always coinjected with an equal amount of β- and γ-subunit cRNA (10 ng/oocyte).

Then, 2–8 days after cRNA injection, the two-microelectrode voltage-clamp method is used to record currents from *Xenopus* oocytes. Recordings are performed at 22 °C using a Geneclamp amplifier (Axon Instruments, Foster City, CA, USA) and MacLab D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The ND 96 solution (control) contains (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and HEPES 5, pH 7.0. In some experiments, Na^+ is replaced by *N*-methyl-D-glucamine (NMDG) solution. The microelectrodes are filled with 3 M KCl solution and have resistances in the range 0.5–0.9 MΩ. Chemicals (e.g., triamterene as standard) are added at concentrations between 0.2 and 100 µM. The amplitude of the induced currents varies considerably, depending on the day of channel expression and the batch of oocytes. The mutant channel induces considerably smaller currents than the wild-type channel. The total Na^+ current amplitude is determined at least once for each experimental day by superfusion with NMDG solution or with 3 µM or 5 µM amiloride solution at the beginning and at the end of each set of experiments.

Evaluation

Data are presented as means ±SEM. A paired Student's *t*-test is used. The level of statistical significance is set at $P < 0.05$.

Modifications of the Method

Nawada et al. (1995) studied the effects of a sodium, calcium, and potassium antagonistic agent on the sodium current by the whole-cell voltage-clamp technique (tip resistance = 5 MΩ $[Na]_i$ and $[Na]_o$ 10 mmol/l at 20 °C) in isolated guinea pig ventricular cells.

Sunami and Hiraoka (1996) studied the mechanism of cardiac Na^+ channel block by a charged class I antiarrhythmic agent, in guinea pig ventricular myocytes using patch-clamp techniques in the whole-cell, cell-attached, and inside-out configurations.

Erdõ et al. (1996) compared the effects of *Vinca* derivatives on voltage-gated Na^+ channels in cultured cells from rat embryonic cerebral cortex. Effects on Na^+ currents were measured by applying voltage steps (20 ms duration) to −10 mV from a holding potential of −70 mV every 20 s. Steady-state inactivation curves were obtained by clamping the membrane at one of a series of 15-s prepulse potentials, followed 1 ms later by a 20-ms test pulse to −10 mV.

Ragsdal et al. (1993) examined the actions of a Na^+ channel blocker in whole-cell voltage-clamp recordings from Chinese hamster ovary cells transfected with a cDNA encoding the rat brain type IIA Na^+ channel and from dissociated rat brain neurons.

Taglialatela et al. (1996) studied cloned voltage-dependent Na^+ currents expressed in *Xenopus* oocytes upon injection of the cRNA encoding α-subunits from human and rat brain.

Wang et al. (1997) investigated pharmacological targeting of long QT mutant sodium channels.

Eller et al. (2000) measured the effects of a calcium antagonist on inward Na^+ currents (I_{Na}) in GH3 cells with the whole-cell configuration of the patch-clamp technique. I_{Na} was recorded after depolarization from a holding potential of −80 mV to a test potential of +5 mV. Initial "tonic" block (resting state-dependent block) was defined as peak I_{Na} inhibition during the first pulse 2 min after drug application as compared with I_{Na} in the absence of drug. "Use (frequency)-dependent" block of I_{Na} was measured during trains of 5- or 50-ms test pulses (3 Hz) applied from −80 mV to a test potential of +5 mV after a 2-min equilibrium period in the drug-containing solution. Use-dependent block was expressed as the percentage decrease of peak I_{Na} during the last pulse of the train as compared with I_{Na} during the first pulse.

Khalifa et al. (1999) characterized the effects of an antidepressant agent on the fast inward current (I_{Na}) in isolated guinea pig ventricular myocytes. Currents were recorded in the whole-cell configuration of the patch-clamp technique in the presence of Ca^{2+} and K^+ channel blockers.

Haeseler et al. (1999) measured the effects of 4-chloro-*m*-cresol, a preservative added to a wide variety of drugs, on heterologously expressed wild-type paramyotonia congenita (R1448H) and hyperkalemic periodic paralysis (M1360V) mutant α-subunits of human muscle sodium channels using whole-cell and inside-out voltage-clamp experiments.

Song et al. (2000) studied the effects of *N*-ethylmaleimide, an alkylating agent to protein sulfhydryl groups, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root neurons using the whole-cell configuration of the patch-clamp technique. Rats at the age of 2–6 days were anesthetized with isoflurane, and the spinal cord was removed and cut longitudinally. Dorsal root ganglia were plucked from the area between the vertebrae of the spinal column and incubated in phosphate-buffered saline solution containing 2.5 mg/ml trypsin at 37 °C for 30 min. After enzyme treatment, ganglia were rinsed with Dulbecco's Modified Eagle Medium supplemented with 10 % horse serum. Single cells were mechanically dissociated by trituration with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips. Cells attached to the coverslips were transferred into a recording chamber on the stage of an inverted microscope. Ionic currents were recorded under voltage-clamp conditions by the whole-cell patch-clamp technique. The solution in the pipette contained (in mM) CsCl 125, NaF 20, HEPES 5, and EGTA 5. The pH was adjusted to 7.2 with CsOH and the osmolarity was 279 mosmol/l on average. The external solution contained (in mM) NaCl 50, choline chloride 90, tetramethylammonium chloride 20, D-glucose 5, HEPES 5, $MgCl_2$ 1, and $CaCl_2$ 1. Lanthanum ($LaCl_3$, 10 μM) was used to block calcium channel current. The solution was adjusted to pH 7.4 with tetramethylammonium hydroxide and the osmolarity was 304 mosmol/l on average. An Ag–AgCl pellet/3 M KCl-agar bridge was used for the reference electrode. Membrane currents were recorded using an Axopatch-1D amplifier. Signals were digitized by a 12-bit analogue-to-digital interface, filtered with a low-pass Bessel filter at 5 kHz, and sampled at 50 kHz using pCLAMP6 software (Axon Instruments) on an IBM-compatible PC. Series resistance was

compensated 60–70 %. Capacitive and leakage currents were subtracted by using a P + P/4 procedure (Bezanilla and Armstrong 1977). The liquid junction potential between internal and external solutions was on average −1.7 mV. TTX (100 nM) was used to separate TTX-R sodium currents from TTX-S sodium currents. For the study of TTX-S sodium channels, cells that expressed only TTX-S sodium channels were used. TTX-S sodium channels were completely inactivated within 2 ms when currents were evoked by depolarizing steps to 0 mV, while TTX-R sodium channels persisted for more than 20 ms. The difference in kinetics was used to identify the type of sodium current.

Abriel et al. (2000) described the molecular pharmacology of the sodium channel mutation DI790G linked to long QT syndrome.

Makielski et al. (2003) showed that a ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5AS heart sodium channels.

Viswanathan et al. (2001) studied gating mechanisms for flecainide action in *SNCN5A*-linked arrhythmia syndromes.

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Voltage-Clamp Studies on Potassium Channels

Purpose and Rationale

Potassium channels represent a very large and diverse collection of membrane proteins which participate in important cellular functions regulating neuronal and cardiac electrical patterns, release of neurotransmitters, muscle contractility, hormone secretion, secretion of fluids, and modulation of signal transduction pathways. The main categories of potassium channels are gated by voltage or an increase of intracellular calcium concentration (Escande and Henry 1993; Kaczorowski and Garcia 1999; Alexander et al. 2001). For ATP-sensitive potassium channels, see section "Interaction with β-Cell Plasma Membranes and K_{ATP} Channels," chapter "▶ Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms".

The delayed outward potassium current in heart muscle cells of several species is made up of a **rapidly** (I_{Kr}) and a **slowly** (I_{Ks}) activating component (Sanguinetti and Jurkiewicz 1990; Wang et al. 1994; Gintant 1996; Lei and Brown 1996; Carmeliet and Mubagawa 1998). Several potent and selective blockers of the I_{Kr} channel have been shown to prolong the effective refractory period but have a reverse rate-dependent activity with both normal and elevated extracellular potassium concentrations (Colatsky et al. 1990). Inhibitors of the slow component I_{Ks} were developed in order to circumvent the negative rate dependence of I_{Kr} channel blockers in the effective refractory period (Busch et al. 1996; Suessbrich et al. 1996, 1997; Bosch et al. 1998). Gögelein et al. (2000) studied the effects of a potent inhibitor of I_{Ks} channels in *Xenopus* oocytes and guinea pig ventricular myocytes.

Procedure

Studies in *Xenopus* oocytes are performed with the two-microelectrode voltage-clamp method. For isolation of the oocytes, the toads are anesthetized using a 1 g/l solution of 3-aminobenzoic acid ethyl ester and placed on ice. A small incision is made to retrieve sacs of oocytes and is subsequently closed with absorbable surgical suture. On waking up, the toads are placed back into the aquarium. The ovaries are cut up into small pieces, and the oocytes are washed in Ca^{2+}-free Or-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) and subsequently digested in Or-2 containing collagenase A (1 mg/ml, Worthington, type II) until follicles are not longer detectable on the oocyte's surface. The oocytes are stored at 18 °C in recording solution ND-96 (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.4) with added sodium pyruvate (275 mg/l), theophylline (90 mg/l), and gentamicin (50 mg/l).

For electrophysiological recordings, the two-microelectrode voltage-clamp configuration is used to record ion currents from *Xenopus* oocytes. Injection of cRNA is performed according to Methfessel et al. (1986) and Golding (1992). Oocytes are injected individually with cRNA encoding for the human protein minK, guinea pig Kir2.1, human H*erg*, human Kv1.5, mouse Kv1.3, or human HNC2. In the case of minK, the functional potassium channel is a heteromultimer composed of the endogenous

(*Xenopus*) KvLQT1 and the injected human minK. This heteromultimeric potassium current is then called I_{Ks} (Barhanin et al. 1996; Sanguinetti et al. 1996).

The electrophysiological recordings are performed at room temperature, using a Geneclamp amplifier (Axon Instruments) and MacLab D/A converter. The amplitudes of the recorded currents are measured at the end of the test voltage steps. To amplify the inward potassium current through Kir2.1 and HNC2, the external potassium concentration is raised to 10 mM KCl and the NaCl concentration lowered to 88 mM (ND-88). The microelectrodes are filled with 3 M KCl and have a resistance between 0.5 MΩ and 1 MΩ. During the recordings the oocytes are continuously perfused with ND-96 (or ND-88 in the case of Kir2.1 and HCN2). The test compounds are dissolved in dimethyl sulfoxide (DMSO) and added to the buffer ND-96 or ND-88. The current amplitude is determined after 5 min of wash-in time.

For the isolation of *ventricular myocytes*, guinea pigs (weight about 400 g) or Sprague–Dawley rats of either sex are sacrificed by cervical dislocation. The hearts are dissected and perfused retrogradely via the aorta at 37 °C: first, with nominally Ca^{2+}-free Tyrode solution (in mmol/l: 143 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 10 glucose, 5 HEPES, pH 7.2) and then with Tyrode solution containing 20 mmol/l Ca^{2+} and 3 mg/ml collagenase type CLS II (Biochrom, Berlin, Germany). After 5–10 min collagenase treatment, the ventricles are cut up into small pieces in the storage solution (in mmol/l: 50 L-glutamic acid monopotassium salt, 40 KCl, 20 taurine, 20 KH_2PO_4, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, pH 7.2). The myocytes are then dispersed by gentle shaking followed by filtration through a nylon mesh (365 μm). The cells are finally washed twice by centrifugation at 90 *g* for 5 min and kept in the storage solution at room temperature.

Whole-cell currents are recorded in the tight-seal whole-cell mode of the patch-clamp technique, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes are pulled from borosilicate glass capillaries (wall thickness 0.3 mm, outer diameter 1.5 mm) and their tips are fire polished. Series resistance is in the range of 1–10 MΩ and 50 % compensated by means of the EPC's compensation circuit.

The I_{Ks}, I_{Kr}, and I_{K1} currents in guinea pig ventricular myocytes are investigated. The voltage pulses for recording the current components are as follows: I_{Ks} current, holding potential −80 mV to −50 mV (200 ms) to +60 mV (3 s) to −40 mV (2 s) to −80 mV; I_{Kr} current, holding potential −80 mV to −50 mV (200 ms) to −10 mV (3 s) to −40 mV (2 s) to −80 mV (I_{Kr} is evaluated as the tail current evoked by a voltage pulse from −10 mV to −40 mV); and I_{K1} current, holding potential −80 mV to −120 mV (200 ms) to −80 mV. In order to suppress the L-type Ca^{2+} current, 5 mmol/l nifedipine is added to the bath solution.

Evaluation

All average data are presented as means ± SEM. Student's *t*-test is used to determine the significance of paired observations. Differences are considered as significant at $P < 005$.

Modifications of the Method

Using the whole-cell configuration of the patch-clamp technique, Grissmer et al. (1994) analyzed the biophysical and pharmacological properties of five cloned voltage-gated K^+ channels stably expressed in mammalian cell lines.

Sanchez-Chapula (1999) studied the block of the transient outward K^+ channel (I_{to}) by disopyramide in isolated rat ventricular myocytes using whole-cell patch-clamp techniques.

Using the patch-clamp technique, Cao et al. (2001) investigated the effects of a centrally acting muscle relaxant and structurally related compounds on recombinant small-conductance Ca^{2+}-activated K^+ channels (rSK2 channels) in HEK mammalian cells.

Tagliatela et al. (2000) discussed the block of the K^+ channels encoded by the human *ether-á-go-go-related* gene (HERG), termed $K_{V(r)}$, which are the

molecular determinants of the rapid component of the cardiac repolarizing current $I_{K(Vr)}$, involved in the cardiotoxic potential and CNS effects of first-generation antihistamines and may be therapeutic targets for antiarrhythmic agents (Vandenberg et al. 2001; Zhou et al. 2005).

Chabbert et al. (2001) investigated the nature and electrophysiological properties of Ca^{2+}-independent depolarization-activated potassium currents in acutely isolated mouse vestibular neurons using the whole-cell configuration of the patch-clamp technique. Three types of currents were identified.

Furthermore, Longobardo et al. (1998) studied the effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents stably expressed in Ltk^- cells using the whole-cell configuration of the patch-clamp technique.

Moreno et al. (2003) studied the effects of a selective angiotensin II type 1 receptor antagonist on cloned potassium channels involved in human cardiac repolarization.

Sanchez-Chapula et al. (2002) investigated the voltage-dependent block of wild-type and mutant HERG K^+ channels by the antimalarial compound chloroquine.

Anson et al. (2004) published molecular and functional characterization of common polymorphism in HERG (KCNH2) potassium channels.

For more information on the evaluation of HERG potassium channels in safety pharmacology, Champeroux et al. (2013).

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Studies on Kv1.5 Channel

Purpose and Rationale

Treatment of atrial fibrillation/flutter with available potassium channel blockers (class III antiarrhythmic agents which mainly block the delayed rectifier current I_{kr}) is associated with ventricular proarrhythmia. Prolongation of ventricular repolarization leads to early afterdepolarization from which torsades de pointes can evolve. Therefore,

blockade of a cardiac current of exclusive relevance in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. The ultrarapid delayed rectifier potassium current (I_{kur}) seems an ideal atrial antiarrhythmic target since it is found to contribute to the action potential in the atrium but not in the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current is the potassium channel Kv1.5, which therefore gained much interest (Li et al. 1996; Longobardo et al. 1998; Perchenet and Clément-Chomienne 2000; Caballero et al. 2000, 2001, 2004; Bachmann et al. 2001; Kobayashi et al. 2001; Matsuda et al. 2001; Choi et al. 2002; Moreno et al. 2003; Choe et al. 2003; Fedida et al. 2003; Godreau et al. 2002, 2003; Peukert et al. 2003, 2004; Plane et al. 2005).

For in vivo studies on atrial fibrillation, see sections "Experimental Atrial Fibrillation," "Atrial Fibrillation by Atrial Pacing in Dogs," "Atrial Fibrillation in Chronically Instrumented Goats," and "Influence on Ultrarapid Delayed Rectifier Potassium Current in Pigs," chapter "► Anti-Arrhythmic Activity".

Gögelein et al. (2004) studied the effects of the antiarrhythmic drug AVE0118 on cardiac ion channels.

Procedure

Molecular Biology and Cell Culture

Human Kv1.5 cDNA was subcloned into the eukaryotic expression vectors pcDNA3.1 and pcDNA3.1/zeo (Invitrogen, Groningen, the Netherlands), cDNA encoding human Kv4.3 long (Kv4.31; Dilks et al. 1999) was subcloned into pcDNA3.1, and the cDNA encoding human KChIP2 short (KChIP2.2; Decher et al. 2001) was subcloned into pcDNA3.1/zeo expression vector. Chinese hamster ovary (CHO) cells were transfected with either hKv1.5 or hKv4.3 and KChIP2.2 expression constructs. Transfection was carried out using lipofectamine (Life Technologies/Gibco BRL, Karlsruhe, Germany) according to the manufacturer's instructions. To boost Kv1.5 channel expression, CHO cells were consecutively transfected with both Kv1.5 expression constructs. Both hKv1.5 and hKv4.3 + hKChIP2.2 were stably expressed in CHO cells, which were maintained in ISCOVE's medium (Biochrom KG, Berlin, Germany), supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 350 µg/ml Zeocin (Invitrogen), and 400 µg/ml G418 (PAA Laboratories). HERG, the potassium channel underlying I_{Kr} currents in human hearts, was cloned and transfected into CHO cells as described previously (Rampe et al. 1997). Cells used for patch clamping were seeded on glass or plastic coverslips 12–36 h before use.

Northern Blot Analysis of Kv1.5 in the Pig Heart and Cloning of Pig Kv1.5

Polyadenylated RNA was isolated from pig cardiac tissues with the Oligotex mRNA purification kit (Qiagen), and 10 µg per tissue was resolved by denaturing formaldehyde electrophoresis and blotted on a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled riboprobe (DIG RNA labeling kit, Roche) encompassing the entire coding sequence of human Kv1.5 and exposed on a Lumi-Imager (Roche). The pig Kv1.5 was cloned by 5′-rapid amplification and 3′-rapid amplification of cDNA ends (RACE) reactions. An adapter-ligated, double-stranded cDNA library was prepared from pig heart mRNA with the Marathon cDNA Amplification Kit (Clontech). The 5′-RACE and 3′-RACE reactions were performed with oligonucleotide primers derived from a partial pig Kv1.5 nucleotide sequence (GenBank accession number AF348084). Overlapping cDNA clones were obtained by repeated reactions and the DNA sequence determined by automated DNA sequencing on both strands (ABI 310, PerkinElmer). A full-length cDNA clone was established by recombinant PCR. It encodes an open reading frame of 1,083 bp and a protein with 86 % overall sequence similarity to the human Kv1.5 protein. The sequence of the pig Kv1.5 cDNA was submitted to GenBank (accession number: AY635585).

For *Xenopus* oocyte expression, cDNAs encoding Kv1.5, Kv4.3, and KChip2.2 were cloned into the oocyte expression vector pSGEM (Villmann et al. 1997), and capped cRNA was

synthesized using the T7 mMessage mMachine kit (Ambion, Austin, Tex., USA).

Voltage-Clamp Experiments in Xenopus Oocytes

Handling and injection of *Xenopus* oocytes were performed according to Bachmann et al. (2001). Adult female *Xenopus laevis* frogs were anesthetized with 3-aminobenzoic acid ethyl ester solution (1 g/l) and intact ovary lobes were removed. The oocytes were defolliculated by treatment with 40 mg collagenase dissolved in 20 ml buffer (in mM: NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5, titrated to pH 7.5 with NaOH) for 120–150 min at 18 °C. Oocytes were injected with 50 nl cRNA using a microinjector (World Precision Instruments, Sarasota, Fla., USA). Oocytes were stored under gentle shaking at 18 °C in a buffer containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, theophylline 0.5, and gentamicin 50 µg/ml, titrated to pH7.5 with NaOH. They were used for experiments 1–3 days after injection.

Two-electrode voltage-clamp recordings were performed at room temperature in a medium containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and HEPES 5, at pH 7.5 with NaOH. Microelectrodes were pulled from filament borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). After filling with 3 M KCl, pipettes had a resistance of 0.3–1.3 MΩ. To activate hKv1.5 and hKv4.3 channels, oocytes were clamped from a holding potential of −80 mV to 40 mV for 500 ms. Data were recorded with a Turbo Tec 10CX amplifier (NPI, Tamm, Germany) using an ITC-16 interface (Instrutech Corporation, Long Island, USA) and the Pulse software (HEKA Elektronik, Lambrecht, Germany).

Patch-Clamp Experiments with CHO Cells

Cells expressing Kv1.5 or Kv4.3 plus KChIP2.2 were assayed using the standard whole-cell patch-clamp technique (Hamill et al. 1981). Cells were mechanically removed from the tissue culture flask and placed in a perfusion chamber with a solution containing (in mM) NaCl 140, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 1.1, and HEPES 10, at pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM) NaCl 10, KCl 120, EGTA 1, HEPES 10, and $MgCl_2$ 1.1 (pH 7.2 with potassium hydroxide, KOH), pipettes had resistances of 2–3 MΩ. Experiments were carried out at 36 ± 1 °C. For the recording of hKv1.5, voltage pulses of 450 ms duration were applied from the holding potential of −30 mV to +20 mV at a frequency of 1 Hz. For recording of the hKv4.3 +KChIP2.2, the holding potential was −50 mV and test pulses of 200 ms duration were applied to −10 mV at a frequency of 1 Hz. Data were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik) and the Pulse software (HEKA Elektronik) and stored on a PC for later analysis. Series resistance was in the range of 4–9 MΩ and was compensated by 80 % by means of the EPC9's compensation circuit. The experiments were performed under continuous superfusion of the cells with solution heated to 36 ± 1 °C.

HERG channel currents were recorded at room temperature using the whole-cell configuration of the patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments). Briefly, electrodes (3–6 MΩ resistance) were fashioned from TW150F glass capillary tubes (World Precision Instruments) and filled with pipette solution (in mM: potassium aspartate 120, KCl 20, Na_2ATP 4, HEPES 5, $MgCl_2$ 1, pH 7.2 adjusted with KOH). HERG currents were initiated by a positive voltage pulse (20 mV) followed by a negative pulse (−40 mV) and were recorded for off-line analyses. Once HERG current from a cell perfused with control external solution (in mM: NaCl 130, KCl 5, sodium acetate 2.8, $MgCl_2$ 1, HEPES 10, glucose 10, $CaCl_2$ 1 at pH 7.4 adjusted with NaOH) was stabilized, the cell was perfused with external solution containing the compound at a specific concentration for percentage inhibition. For each concentration from each cell, peak amplitude of the steady-state HERG tail current at −40 mV was measured. The peak amplitude for each concentration was compared with that for the control solution from the same cell and expressed as percent control.

Isolation of Porcine Atrial Myocytes

Male pigs weighing 15–30 kg of the German Landrace were anesthetized with pentobarbital exactly as described previously (Wirth and Knobloch 2001). After a left thoracotomy the lung was retracted, the pericardium was incised, and the heart was quickly removed and placed in oxygenated nominally Ca^{2+}-free Tyrode solution containing (in mM) NaCl 143, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.25, HEPES 5, and glucose 10, at pH adjusted to 7.2 with NaOH. The hearts were then mounted on a Langendorff apparatus and perfused via the left circumflex coronary artery with Tyrode solution (37 °C) with constant pressure (80 cmH_2O). All coronary vessels descending to the ventricular walls were ligated, ensuring sufficient perfusion of the left atrium. When the atrium was clear of blood and contraction had ceased (≈5 min), perfusion was continued with the same Tyrode solution, which now contained 0.015 mM $CaCl_2$ and 0.03 % collagenase (type CLS II, Biochrom KG, Berlin, Germany), until atrial tissue softened (≈20 min). Thereafter, left atrial tissue was cut into small pieces and mechanically dissociated by trituration. Cells were then washed with storage solution containing (in mM) L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 1, glucose 10, HEPES 10, and EGTA 2 (pH 7.2 with KOH) and filtered through a nylon mesh. The isolated cells were kept at room temperature in the storage solution.

Isolation of Guinea Pig Ventricular Myocytes

Ventricular myocytes were isolated by enzymatic digestion according to Gögelein et al. (1998). Dunkin–Hartley–Pirbright white guinea pigs (weight about 400 g) were sacrificed by cervical dislocation. The hearts were dissected and perfused retrogradely via the aorta at 37 °C with the same solutions as used for isolation of pig atrial myocytes.

Electrophysiological Recordings from Cardiac Myocytes

Whole-cell currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik) as described above for CHO cells. A small aliquot of cell-containing solution was placed in a perfusion chamber, and after a brief period allowing for cell adhesion to the chamber, the cells were perfused with (in mM) NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.0, HEPES 10, and glucose 10, at pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM) KCl 130, $MgCl_2$ 1.2, HEPES 10, EGTA 10, K_2ATP 1, GTP 0.1, and phosphocreatine 5 (pH 7.2 with KOH), pipettes had a resistance of 2–3 MΩ. Series resistance was in the range of 6–12 MΩ and was compensated by 60–70 %. Offset voltages generated when the pipette was inserted in NaCl solution (1–5 mV) were zeroed before formation of the seal.

Effects of AVE0118 on the I_{KACh} were recorded from pig left atrial myocytes by applying voltage pulses of 500 ms duration from the holding potential of −80 mV to −100 mV. Carbachol (10 μM) was added in order to evoke the I_{KACh}. After stabilization of the I_{KACh} (3 min), AVE0118 was added in increasing concentrations in the continuous presence of carbachol. The current was measured at the end of the pulse after 3 min of incubation at each concentration, and inhibition of the carbachol-activated current was calculated. In some experiments, AVE0118 was washed out before application of the next higher concentration.

Also the L-type Ca^{2+} current was investigated in pig left atrial cells. In these experiments, KCl in the pipette was replaced by CsCl, and voltage pulses of 300 ms duration were applied from the potential of −40 mV to 0 mV. Possible effects of AVE0118 on the currents I_{K1}, I_{Ks}, I_{Kr}, and I_{KATP} were investigated in guinea pig ventricular myocytes. I_{K1} currents were recorded by a voltage step from −80 mV to −120 mV lasting for 200 ms. When I_{Ks} and I_{Kr} currents were recorded, 1 μM nisoldipine was added to the bath to block the L-type Ca^{2+} current. I_{Ks} was assessed by voltage pulse to +60 mV for 3 s, starting from −40 mV. I_{Kr} was evaluated as the tail current evoked by a voltage pulse from −10 mV to −40 mV. I_{KATP} was evoked by adding 1 μM rilmakalim (Krause et al. 1995) to the bath and

by applying voltage ramps from −130 mV to +80 mV for 500 ms. The rilmakalim-activated current was recorded at the potential 0 mV. All patch-clamp experiments were performed under continuous superfusion of the cells with solution heated to 36 ± 1 °C.

Evaluation

All averaged data are presented as the mean ±SEM. The Student's *t*-test was used to determine the significance of paired or unpaired observations. Differences were considered significant at $P < 0.05$. The values for half-maximal inhibition (IC_{50}) and the Hill coefficient were calculated by fitting the data points of the concentration–response curves to the logistic function:

$$f(x) = (a - d)/[1 + (x/c)^n] + d$$

where a represents the plateau value at low drug concentration, d the plateau value at high drug concentration, c the IC_{50} value, and n the Hill coefficient. The curve fitting and the Student's *t*-test were performed with the computer program *Sigma-Plot* 5.0.

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Voltage-Clamp Studies on Calcium Channels

Purpose and Rationale

Calcium influx through voltage-gated Ca^{2+} channels mediates a range of cytoplasmatic responses, including muscle contraction, release of neurotransmitters, Ca^{2+}-dependent gene transcription, and the regulation of neuronal excitability, and has been reviewed by several authors (Augustine et al. 1987; Bean 1989; Miller 1987; Zamponi 1997; Snutch et al. 2001). In addition to their normal physiological function, Ca^{2+} channels as calcium antagonists are also implicated in a number of human disorders (see also "▶ Calcium Uptake Inhibition Activity").

Using patch-clamp techniques, the structure and regulation of voltage-gated Ca^{2+} channels has been studied by many authors (Sculptoreanu et al. 1993; Peterson et al. 1997; Catterall 2000).

Berjukow et al. (2000) analyzed the role of the inactivated channel conformation in molecular mechanism of Ca^{2+} channel block by a dihydropyridine derivative in L-type channel constructs and mutants in *Xenopus* oocytes and described the electrophysiological evaluation.

Procedure

Inward barium currents (I_{Ba}) are studied with two-microelectrode voltage clamp of *Xenopus* oocytes 2–7 days after microinjection of approximately equimolar cRNA mixtures of constructs of L-channel mutants. All experiments are carried out at room temperature in a bath solution with the following composition: 40 mM $Ba(OH)_2$, 50 mM NaOH, 5 mM HEPES, and 2 mM CsOH (pH adjusted to 7.4 with methanesulfonic acid). Voltage-recording and current-injecting microelectrodes are filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, and 10 mM HEPES (pH 7.4) with resistances of 0.3–2 MΩ. Resting channel block is estimated as peak I_{Ba} inhibition during 100-ms test pulses from −80 to 20 mV at a frequency of 0.033 Hz until steady state is reached. The dose–response curves of I_{Ba} inhibition were fitted using the Hill equation:

$$\frac{I_{\text{Ba, drug}}}{I_{\text{Ba, control}}}(\%) = \frac{100 - A}{1 + \left(\frac{C}{IC_{50}}\right)^{nH}} + A$$

where IC_{50} is the concentration at which I_{Ba} inhibition is half maximal, C is the applied drug concentration, A is the fraction of I_{Ba} that is not blocked, and nH is the Hill coefficient.

Recovery from inactivation is studied at a holding potential of −80 mV after depolarizing Ca^{2+} channels during a 3-s prepulse to 20 mV by applying 30-ms test pulses (to 20 mV) at various time intervals after the conditioning prepulse. Peak I_{Ba} values are normalized to the peak current measured during the prepulse, and the time course of I_{Ba} recovery from inactivation is fitted to a mono- or biexponential function:

$$I_{\text{Ba, recovery}} = A \times \exp\left(\frac{-t}{\tau_{\text{fast}}}\right) + B \times \exp\left(\frac{-t}{\tau_{\text{slow}}}\right) + C$$

Voltage dependence of inactivation under quasi-steady-state conditions is measured using a multistep protocol to account for rundown (less than 10 %). A control test pulse (50 ms to 20 mV) is followed by a 1.5-s step to −100 mV followed by a 30-s conditioning step, a 4-ms step to −100 mV, and a subsequent test pulse to 20 mV (corresponding to the peak potential of the I–V curves).

Inactivation during the 30 s conditioning pulse is calculated as follows:

$$I_{\text{Ba, inactivation}} = \frac{I_{\text{Ba, test}}(20\text{mV})}{I_{\text{Ba, control}}(20\text{mV})}$$

The pulse sequence is applied every 3 min from a holding potential of −100 mV. Inactivation curves are drawn according to the following Boltzmann equation:

$$I_{\text{Ba, inactivation}} = I_{SS} + (1 - I_{SS})\left(1 + \exp\left(\frac{V - V_{0.5}}{k}\right)\right)$$

where V is the membrane potential, $V_{0.5}$ is the midpoint voltage, k is the slope factor, and I_{SS} is the fraction of non-inactivating current.

Steady-state inactivation of the mutate channels at −80 mV is estimated by shifting the membrane holding potential from −80 to −100 mV. Subsequent monitoring of the corresponding changes in I_{Ba} amplitudes until steady state reveals the fraction of Ca^{2+} channels in the inactivated state at −80 mV. Steady-state inactivation of different L-type channel constructs at −30 mV is estimated by fitting time course of current inactivation to a biexponential function.

The I_{Ba} inactivation time constants are estimated by fitting the I_{Ba} decay to a mono- or biexponential function.

Evaluation

Data are given as the means ±SE. Statistical significance is calculated according to Student's unpaired *t*-test.

Modifications of the Method

Besides *Xenopus* oocytes (Waard and Campell 1995; Hering et al. 1997; Kraus et al. 1998), several other cell types and constructs, such as CHO cells (Sculptoreanu et al. 1993; Stephens et al. 1997); HEK293 (human embryonic kidney) cells (Lacinová et al. 1999); tsA-201 cells, a subclone of HEK293 (Peterson et al. 1997; McHugh et al. 2000); cardiac myocytes from rats (Scamps et al. 1990; Tohse et al. 1992; Gomez et al. 1994) and rabbits (Xu et al. 2000); isolated atrial myocytes from failing and non-failing human hearts (Cheng et al. 1996); skeletal muscle myotubes from mice and rabbits (Johnson et al. 1994); myocytes of guinea pig mesenteric artery (Morita et al. 1999); dendrites from rat pyramidal and olfactory bulb neurons (Markram and Sakmann 1994; Stuart and Spruston 1995; Koester and Sakmann 1998; Margie et al. 2001); and rat amygdala neurons (Foehring and Srcoggs 1994; Young et al. 2001), were used to study the function of calcium channels.

Using the whole-cell variation of the patch-clamp technique, Yang et al. (2000) studied cellular T-type and L-type calcium channel currents in mouse neuroblastoma N1E115 cells. The cells were cultured in Dulbecco's Modified Eagle's Medium containing 10 % fetal bovine serum at 37 °C in a humidified atmosphere of 5 % CO_2 in air. The medium was changed every 3–4 days. After mechanical agitation, 3×10^4 cells were replanted in 35-mm tissue culture dishes containing 4 ml of bath solution. After cell attachment, the dish was mounted on the stage of an inverted phase-contrast microscope for Ca^{2+} channel current recording. These cells expressed predominantly T channel currents. In experiments where L channels were specifically sought, the cells were grown and maintained at confluence for 3–4 weeks under the same culture conditions with the addition of 2 % dimethyl sulfoxide (Narahash et al. 1987). Three to 5 days before use, the cells were replanted with the same medium. These cells expressed predominantly L channel currents. A small number of these cells also expressed T channel currents. Hence, cells were selected so that at a holding potential of −40 mV, the T channel component was very small and the inward current measured was conducted predominantly by L channels.

By using whole-cell and perforated patch-clamp techniques, Wu et al. (2000) showed that mifrabidile, a non-dihydropyridine compound, has an inhibitory effect on both T- and L-type Ca^{2+} currents in pancreatic β-cells.

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Patch-Clamp Studies on Chloride Channels

Purpose and Rationale

Cl^- channels are a large, ubiquitous, and highly diverse group of ion channels involved in many physiological key processes including regulation of electrical excitability, muscle contraction, secretion, and sensory signal transduction. Cl^- channels belong to several distinct families characterized in detail: voltage-gated Cl^- channels, the cAMP-regulated channel CFTR (cystic fibrosis transmembrane conductance regulator), ligand-gated Cl^- channels that open upon binding to the neurotransmitters GABA or glycine, and Cl^- channels that are regulated by the cytosolic Ca^{2+} concentration (Jentsch and Günther 1997; Frings et al. 2000).

Cliff and Frizel (1990) studied the cAMP- and Ca^{2+}-activated secretory Cl^- conductances in the Cl^--secreting colonic tumor epithelial cell line T84 using the whole-cell voltage-clamp technique.

Procedure

T84 cells are used 1–3 days after plating on collagen-coating coverslips. The cells are maintained at 37 °C. At this temperature, the responsiveness of the cells to secretagogues, particularly to cAMP-dependent agonists, is improved. Increases in Cl^- and K^+ conductances are the major electrical events during stimulation of Cl^- secretion. Accordingly, bath–pipette ion gradients are chosen so that transmembrane Cl^- and K^+ currents can be monitored independently at clamp voltages equal to the reversal potentials of these ions. The pipette solution is 115 mM KCl, 25 mM *N*-methyl-D-glucamine (NMDG) glutamate, 0.5 mM EGTA, 0.19 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 0.05 mM Na_3GPT, and 5 mM HEPES, at pH 7.2. The bath solution is 115 mM NaCl, 40 mM NMDG glutamate, 5 mM potassium glutamate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, at pH 7.2. Bath Na^+ and Cl^- concentrations are reduced by substituting NMDG chloride or sodium glutamate for NaCl. When Na^+- and K^+-free solutions are used, Na^+ and K^+ are replaced by $NMDG^+$, and Cl^- is reduced by replacing Cl^- by glutamate.

During whole-cell recording, the membrane potential is clamped alternately to three different voltages, each for 500-ms duration. Computer-controlled voltage-clamp protocols are used to generate current–voltage (I–V) relations when the transmembrane currents are relatively stable by stepping the clamp voltage between −100 mV and +100 mV at 20 mV intervals.

Test drugs (e.g., 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, A23187, forskolin, or ionomycin) are solubilized in stock solutions (ethanol of DMSO) and diluted.

Evaluation

Instantaneous relations are constructed from currents recorded 6 ms after a voltage step.

Modifications of the Method

Maertens et al. (2000) used the whole-cell patch-clamp technique to study the effect of an antimalarial drug on the volume-regulated anion channel (VRAC) in cultured bovine pulmonary artery endothelial cells. They also examined the effects on other Cl^- channels, i.e., the Ca^{2+}-activated Cl^- channel and the cystic fibrosis transmembrane conductance regulator, to assess the specificity for VRAC.

Pusch et al. (2000) characterized chloride channels belonging to the ClC family. Chiral clofibric acid derivates were tested on the human ClC-1 channel, a skeletal muscle chloride channel, after heterologous expression in *Xenopus laevis* oocytes by means of two-microelectrode voltage-clamp recordings.

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Inhibition of Hyperpolarization-Activated Channels

Purpose and Rationale

The hyperpolarization-activated cation currents (termed I_f, I_h, or I_q) play a key role in the initiation of cardiac and neuronal pacemaker depolarizations. Unlike most voltage-gated channels, they are activated by hyperpolarizing voltage steps to potentials negative to −60 mV, near the resting potential of most cells. This property earned them the designation of I_f for "funny" or I_q for "queer." The funny current, or pacemaker (I_f) current, was first described in cardiac pacemaker cells of the mammalian sinoatrial node as a current that slowly activates on hyperpolarization at voltages in the diastolic voltage range and contributes to the generation of cardiac rhythmic activity and to its control by sympathetic and parasympathetic innervations (DiFrancesco et al. 1986; Accili et al. 1997, 2002; Robinson and Siegelbaum 2003; Baruscotti et al. 2005). In sinoatrial cells, f-channels are modulated by cAMP independently of phosphorylation, through a mechanism involving direct interaction of cAMP with the intracellular side of the channels (DiFrancesco and Tortora 1991; Bois et al. 1996). A significant advancement in the study of molecular properties of pacemaker channels was achieved when a new family of channels was cloned, the HCN (hyperpolarization-activated, cyclic nucleotide-gated) channels (Ishii et al. 1999; Kaupp and Seifert 2001; Biel et al. 2002; Macri et al. 2002). The HCN family is related to the cyclic nucleotide-gated channel and *eag* potassium channel family and belongs to the superfamily of voltage-gated cation channels. HCN channels are characterized by six membrane-spanning segments (S1–S6) including voltage-sensing (S4) and pore (between S5 and S6) regions. In the C-terminal region, they contain a consensus sequence for binding of cyclic nucleotides. In the heart, neurotransmitter-induced control of cardiac rhythm is mediated by I_f through its

second-messenger cAMP, whose synthesis is stimulated and inhibited by β-adrenoceptor and muscarinic agonists, respectively.

Inhibition of the I_f channel was recommended for induction of bradycardia and treatment of coronary disease (Thollon et al. 1994, 1997; Simon et al. 1995; Bois et al. 1996; Deplon et al. 1996; Acilli et al. 1997; Rocchetti et al. 1999; Monnet et al. 2001, 2004; Bucchi et al. 2002; Cerbai et al. 2003; Rigg et al. 2003; Vilaine et al. 2003; Albaladejo et al. 2004; Colin et al. 2004; DiFrancesco and Camm 2004; Moreno 2004; Mulder et al. 2004; Vilaine 2004; Chatelier et al. 2005; Leoni et al. 2005; Romanelli et al. 2005; Schipke et al. 2006).

Romanelli et al. (2005) reported the design, synthesis, and preliminary biological evaluation of zatebradine analogues as potential blockers of hyperpolarization-activated current, and Chatelier et al. (2005) described that a calmodulin antagonist directly inhibits f-type current in rabbit sinoatrial cells.

Procedure

Sinoatrial Cell Isolation

Sinoatrial node myocytes of the rabbit were isolated (DiFrancesco et al. 1986). Cells were allowed to settle in Petri dishes and were superfused with normal Tyrode solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, D-glucose 5.5, and HEPES–NaOH 5, at pH 7.4.

Electrophysiology

In macro-patch experiments the temperature was kept at 27–28 °C and the patch pipette solution contained (in mM) NaCl 70, KCl 70, $CaCl_2$ 1.8, $MgCl_2$ 1, $BaCl_2$ 1, $MnCl_2$ 2, and HEPES–KOH 5, at pH 7.4. The control solution perfusing the intracellular side of the membrane patches contained (in mM) potassium aspartate 130, NaCl 10, $CaCl_2$ 2, EGTA 5, and HEPES–KOH 10, at pH 7.2, pCa = 7. In some experiments, the calcium concentration of the bath solution was reduced to 0.1 nM according to the calculation of Fabiato and Fabiato (1979) and the correction of Tsien and Rink (1980).

Macro-patches containing hundreds of f-channels were formed using a large-tipped pipette (0.5–2 MΩ) (DiFrancesco and Tortora 1991). The test compound or calmodulin (Calbiochem) was dissolved in either distilled water and ethanol (50/50) or distilled water, respectively, divided into aliquots, and stored at −20 °C until use. Ethanol was added to control solutions at the same concentration used in test solutions (lower than 0.1 %).

Evaluation

The time course of macro-patch I_f under the influence of the modifying compounds was recorded by applying hyperpolarizing steps of 3 s duration at a frequency of 1/15 Hz. At steady state, the voltage dependence of I_f was described by the equation $I_f(E) = g_f(E) \cdot (E - vE_f) = g_{fmax} \cdot y_\infty(E) \cdot (E - E_f)$, where g_f is the conductance, g_{fmax} the fully activated conductance, $y\infty$ (E) the steady-state activation parameter, and E_f the reversal potential (DiFrancesco and Noble 1985). Steady-state current–voltage (I–V) curves were measured by applying 1-min-long hyperpolarizing voltage ramps with a rate of −115 mV/min from a holding potential of −35 mV. Conductance–voltage (g_f/E) relations were then obtained from the above equation as ratios between steady-state I–V curves (i_f/E) and $E - E_f$, where E_f was set to −12.24 mV (DiFrancesco and Mangoni 1994). Conductance curves were fitted by Boltzmann function, $g_f(E) = g_{fmax} \cdot y_\infty(E) = g_{fmax} \cdot 1/[1 + \exp(E - E_{1/2})/p]$, where $E_{1/2}$ is the half-maximal voltage of activation and p is the inverse-slope factor. This allowed estimation of the shifts of the voltage dependence of conductance (i.e., of the activation parameter y_∞) measured as changes in $E_{1/2}$. Shifts of the I_f activation curve caused by cAMP were also determined by a quicker method not requiring measurement of the conductance–voltage relation (Accili and DiFrancesco 1996). Shifts were obtained by applying hyperpolarizing steps from −35 mV to near the midpoint of the I_f activation curve and adjusting the holding potential (−35 mV in the control solution) until the cAMP-induced change

in I_f was compensated and the control I_f magnitude fully restored. Since the compensation involved a change of the test voltage (from E to $E + s_m$, where s_m is the measured displacement of the holding potential in mV), a correction was introduced to obtain the shift of the activation curve (s, mV), according to the relation: $s = s_m \cdot [+(y_\infty/(dy_\infty/dE)]/(E - E_f)$.

When comparing different sets of data, statistical analysis was performed with either the Student's t-test or analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant. Statistical data were given as mean ±SEM values.

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Measurement of Cytosolic Calcium with Fluorescent Indicators

Purpose and Rationale

Intracellular free Ca concentration can be measured in cultured endothelial cells with a fluorometric method (Tsien et al. 1982; Grynkiewicz et al. 1985; Lückhoff et al. 1988; Busse and Lamontagne 1991; Hock et al. 1991).

Procedure

Cultured endothelial cells from the pig are seeded on quartz coverslips and grown to confluence. The cells are loaded with the fluorescent probe indo-1 by incubation with 2 μmol indo-1/AM and 0.025 % Pluronic F-127, a nonionic detergent. Thereafter, the coverslips are washed and transferred to cuvettes, filled with HEPES buffer.

Evaluation

Fluorescence is recorded in a temperature controlled (37 °C) spectrofluorophotometer (excitating wavelength 350 nm, emission wavelength simultaneously measured at 400 nm and 450 nm).

Modifications of the Method

Lee et al. (1987) measured cytosolic calcium transients from the beating rabbit heart using indo-1 AM as indicator.

Yanagisawa et al. (1989) measured intracellular Ca^{2+} concentrations in coronary arterial smooth muscle of dogs with fura-2.

Makujina et al. (1995) measured intracellular calcium by fura-2 fluorescence simultaneously with tension in everted rings of porcine coronary artery denuded of endothelium.

Hayashi and Miyata (1994) described the properties of the commonly used fluorescent indicators for intracellular calcium: fura-2, indo-1, and fluo-3.

Monteith et al. (1994) studied the Ca^{2+} pump-mediated efflux in vascular smooth muscles in spontaneously hypertensive rats.

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Phosphodiesterases and the Effects of Forskolin

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Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and/or cGMP. They function with adenyl and guanylyl cyclases to regulate the amplitude and duration of responses triggered by the second messengers cAMP and cGMP. The enzyme phosphodiesterase (PDE) exists in various forms. At least 11 families of phosphodiesterases have been identified (Torphy and Page 2000; Francis et al. 2001; Maurice et al. 2003; Lugnier 2006). The properties and functions of GAF domains in cyclic nucleotide phosphodiesterases are reviewed by Zoraghi et al. (2004).

Hofmann et al. (2006) reviewed the nomenclature and structure–function relationships of cyclic nucleotide-regulated channels.

Mongillo et al. (2004) reported fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes, revealing distinct functions of compartmentalized phosphodiesterases. Studying real-time monitoring of PDE2 activity in live cells, Nikolaev et al. (2005) found that hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis.

Snyder et al. (2005) studied the role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis.

Zhang et al. (2004) described a glutamine switch mechanism for nucleotide selectivity by phosphodiesterases.

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Inhibition of Phosphodiesterase

Purpose and Rationale

The inhibition of cAMP-PDE and cGMP-PDE by various test compounds can be measured using a two-step radioisotopic procedure.

Procedure

Materials

[8-^{3}H]cAMP (28 Ci/mmol), [8-^{3}H]-cGMP (15 Ci/mmol), and [U^{14}C]guanosine (528 mCi/mmol) are obtained from du Pont de Nemours (Paris, France). Unlabeled cyclic nucleotides, 5′-nucleotidase (Ophiophagus hannah venom), are from the Sigma Chemical Co. (La Verpillière, France).

Tissue Preparations

Male Sprague–Dawley rats (250–300 g) are decapitated. Hearts are perfused with 0.15 M NaCl through the aorta to remove the blood. The ventricles are minced in 5 vol. of 10 mM Tris–HCl buffer containing 0.32 M sucrose, 1 mM Ethylenediaminetetraacetic acid (EDTA), 5 nM DL-Dithiothreitol (DTT), and 0.1 mM Phenylmethanesulfonyl fluoride (PMSF) at pH 7.5. The suspension is homogenized in a glass–glass Potter–Elvehjem. The homogenate is then centrifuged at 105,000 g for 60 min. The 105,000 g supernatant is stored at −75 °C until injection on the HPLC column.

Isolation of PDEs

The cytosolic fraction from rat ventricles (5–8 mg of protein) is loaded at the rate of 1 ml/min on a Mono Q HPLC column which has been previously equilibrated with buffer A (50 mM Tris–HCl, 2 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5). Under these conditions, greater than 95 % of the PDE activity is bound to the column. PDE activity is eluted at a flow rate of 1 ml/min using the following steps and linear gradients of NaCl in buffer A: 25 ml of 0.16 M NaCl, 20 ml of 0.23 M

NaCl, 30 ml from 0.23 to 0.29 M NaCl, 15 ml of 0.29 M NaCl, and 30 ml from 0.29 to 0.50 M NaCl. The separation is done at 4 °C. Fractions of 1 ml are collected and stored at −75 °C in the presence of 20 % glycerol. The fractions are tested for PDE activity, and the peaks containing the different isoenzymes are identified. Fractions containing preferentially one isoenzyme are pooled.

PDE Assay

PDE activity is assayed by a two-step radioisotopic procedure according to Thompson et al. (1974), Boudreau and Drummond (1975), Prigent et al. (1981). cAMP-PDE and cGMP-PDE activities are measured with a substrate concentration of 0.25 µM. To evaluate the cGMP-stimulated PDE activity, assays are performed with 5 µM cAMP in the absence or presence of 5 µM cGMP. Xanthine derivates are dissolved in Dimethyl sulfoxide (DMSO). The stock solutions are appropriately diluted with 40 mM Tris–HCl buffer so that the final DMSO concentration in the PDE assay does not exceed 1 %. At this concentration, DMSO has no significant effect on the PDE activity of any of the fractions. The inhibitory potency of the xanthine derivates is examined on each separated isoform.

Evaluation

The IC_{50} values (concentration of a drug which inhibit 50 % of the enzymatic activity) are calculated by plotting the percentage of residual enzymatic activity versus the logarithmic concentration of the drug. Confidence limits (95 %) for the IC_{50} values are determined by linear regression analysis.

Modifications of the Method

Phosphodiesterase activity can be determined using ^{32}P-labeled Guanosine 5′-triphosphate (GTP) (Reinsberg 1999). The reaction is performed using an Eppendorf cup with 10 mM GTP, the test solution, and [^{32}P]GTP. The reaction is started with 1 mM sodium nitroprusside solution. After an incubation period of 20 min at 37 °C, the reaction is stopped with 500 µl of 120 mM sodium carbonate and 400 µl of zinc acetate, whereby the zinc carbonate and the substrate GTP are almost (about 90 %) all precipitated and so separated from cGMP. The precipitate is separated by centrifugation and the supernatant submitted to chromatography on an aluminum oxide column. The column is first treated with Tris–HCl buffer. From the supernatant, 900 ml is applied. After two washing periods, the cGMP is eluted in five fractions. These fractions are measured, and the highest activities are pooled.

The PDE activity is calculated from the difference between applied [^{32}P]GTP minus unaltered [^{32}P]GTP.

Rich and Karpen (2005) describe an optical assay for monitoring cAMP signals and PDE in living cells, by using a genetically engineered cyclic nucleotide-gated channels as cAMP sensors.

Younès et al. (2011) adapted a bioluminescence method to measure phosphodiesterase activity in a one-step technique. The method employs a four-enzyme system to generate ATP, with measurement of the concomitant luciferase light emission. The method enables detection of PDE activity in cell and tissues extract containing 0.25–10 µg proteins.

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Description of Phosphodiesterase Isoforms Phosphodiesterase 1

PDE1 family variants are activated upon Ca^{2+}/calmodulin binding (Kakkar et al. 1999; Goraya and Cooper 2005; Sharma et al. 2006). The PDE1 subfamily consists of three different gene products (PDFE1A, PDE1B, and PDE1C) which differ in their regulatory properties, substrate affinities, specific activities for calmodulin, tissue distribution, and molecular weights (Yan et al. 1996; Yu et al. 1997). PDE1 is present in brain, cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells (Maurice et al. 2003). Inhibition has been described for nimodipine (Epstein et al. 1982) and for vinpocetine (Hagiwara et al. 1984).

Phosphodiesterase 2

A single *PDE2* gene encodes three PDE2 variants (Rosman et al. 1997). Martinez et al. (2002) studied the crystal structure of murine PD2A and found that the two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and cGMP binding. PDE2 is found in various areas of the brain (Lugnier 2006), in adrenal medulla, heart, rat ventricle (Yanaka et al. 2003), liver, and brown adipose tissue (Coudray et al. 1999). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was shown to specifically act on PDE2 by inhibiting cCMP-activated PDE2 (Podzuweit et al. 1995).

Chambers et al. (2006) described a new chemical tool for exploring the physiological function of the PDE2 isozyme.

Phosphodiesterase 3

The PDE3 family is composed of two genes, *PDE3A* and *PDE3B*. *PDE3A* mRNA is enriched in blood vessels, heart, megakaryocytes, and oocytes, whereas *PDE3B* is highest in adipocytes, hepatocytes, brain, renal collecting duct epithelium, and developing spermatocytes (Reinhardt et al. 1995). PDE3 inhibitors have been extensively investigated and developed as non-glycoside, non-sympathomimetic, cardiotonic agents for the treatment of heart failure. Milrinone is the most studied and most extensively used PDE3 inhibitor and is used in the acute treatment of heart failure (Criuckshank 1993). Trequinsin (HL 725) inhibits PDE3 in a nanomolar range (Ruppert and Weithmann 1982); it also inhibits PDE1, PDE2, and PDE4 in submicromolar concentrations (Stoclet et al. 1995).

Boswell-Smith et al. (2006) studied the pharmacology of two long-acting trequinsin-like phosphodiesterase 3/4 inhibitors, RPL554, and RPL565.

Hambleton et al. (2005) studied isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium.

Masciarelli et al. (2004) described mice deficient of cyclic nucleotide phosphodiesterase 3A as a model of female infertility.

Adachi et al. (2005) reported the effects of a phosphodiesterase 3 inhibitor, olprinone, on rhythmical change in the tension of human gastroepiploic artery.

Abbott and Thompson (2006) performed analysis of anti-PDE3 activity of 2-morpholinochromone derivates revealing multiple mechanisms of antiplatelet activity.

Phosphodiesterase 4

PDE4 is mainly present in the brain (Houslay et al. 1998), inflammatory cells (Tenor and Schudt 1996), cardiovascular tissues (Stoclet et al. 1995), and smooth muscles, but is lacking in the platelets. Four *PDE4* genes (*PDE4A–D*) yield a large number of distinct PDE4 variants. These enzymes, which result from the use of alternate promoters and extensive splicing of PDE4 mRNAs, are stratified into long or short forms (Conti et al. 2003).

One *PDE4A*, three *PDE4B* (PDE4B1, PDE4B2, and PDE4B3), and three *PDE4D* (PDE4D1, PDE4D2, PDE4D3) variants are expressed in rat and human cardiac tissue (Houslay and Adams 2003). Two *PDE4D* gene-derived variants, PDE4D3 and PDE4D6, are expressed in human and rat aortic, mesenteric, and femoral contractile/quiescent and synthetic/activated vascular smooth muscle cells (Liu et al. 2000).

The role of cAMP-specific PDE4 phosphodiesterases in cellular signaling was reviewed by Houslay and Adams (2003) and Conti et al. (2003).

Huai et al. (2003) studied the three-dimensional structures of PDE4D in a complex with roliprams and the implication for inhibitor selectivity.

Baillie et al. (2003) found that β-arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from G_s to G_i.

The antidepressant compound rolipram (ZK 62711) is a selective inhibitor of PDE4 (Schwabe et al. 1976; Komas et al. 1989). O'Donnell and Zhang (2004) described the antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Many analogs were studied as PDE4 inhibitors for treatment of asthma and chronic obstructive pulmonary disease (COPD) as well as anti-inflammatory drugs; however, most failed due to emetic side effects (Giembycz 2002).

Phosphodiesterase 5

Selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (**PDE5**) were found to be effective in the treatment of erectile dysfunction in men (Jeremy et al. 1997; Ballard et al. 1998; Chuang et al. 1998; Stief et al. 1998; Corbin and Francis 1999; Turko et al. 1999; Wallis et al. 1999; Hosogai et al. 2001; Rotella 2001; Saenz de Tejada et al. 2001; Ukita et al. 2001).

The PDE5 family consists of a single *PDE5* gene that can encode three distinct proteins (PDE5A1–3) (Loughney et al. 1998).

Wang et al. (2001) characterized type 5 phosphodiesterases in the corpus cavernosum of several species.

Qiu et al. (2000) demonstrated that rabbit corpus cavernosum smooth muscle shows a different phosphodiesterase profile than human corpus cavernosum.

Kim et al. (2001) compared the inhibition of cyclic GMP hydrolysis in human corpus cavernosum smooth muscle cells by vardenafil with that by sildenafil.

The discovery of novel, potent, and selective PDE5 inhibitors was reported by Bi et al. (2001).

Corbin et al. (2004) described the structural basis for the higher potency of vardenafil compared with sildenafil in inhibiting cCMP-specific phosphodiesterase 5 (PDE5).

PDE5 inhibitors such as sildenafil can be used in the treatment of pulmonary arterial hypertension (Galié et al. 2005).

Cohen et al. (1996) found that inhibition of cGMP-specific phosphodiesterase selectively vasodilates the pulmonary circulation in rats made chronically hypoxic by exposure to a simulated high altitude. Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization

in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling (Jernigan et al. 2004).

Thompson et al. (2001) studied the effect of sildenafil on corpus cavernosal smooth muscle relaxation and cGMP formation in the diabetic rabbit.

Tantini et al. (2005) found an antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells.

Other Phosphodiesterases

The families of **PDE6 to PDE11** have been identified as a result of bioinformatics-based genomic screening (Soderling and Beavo 2000). The search for inhibitors is just beginning.

Gillespie and Beavo (1989) discussed inhibition and stimulation of photoreceptor phosphodiesterases (later on termed **PDE6**) by dipyridamole and M&B 22,948.

D'Armours et al. (1999) investigated the potency and mechanism of action of E4021, a type 5 phosphodiesterase isozyme-selective inhibitor, on the photoreceptor phosphodiesterase.

Zhang et al. (2005) described the efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (**PDE6**) in retinal photoreceptors.

Smith et al. (2004) reported the discovery of a selective inhibitor of **phosphodiesterase 7**.

The discovery of thiadiazoles as a novel structural class of potent and selective **PDE7 inhibitors** was reported by Vergne et al. (2004a, 2004b).

Lorthiois et al. (2004) and Bernadelli et al. (2004) described spiroquinazolinones as potent and selective **PDE7** inhibitors.

The first potent and selective **PDE9** inhibitor was characterized by Wunder et al. (2005) using a cGMP reporter cell line.

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Stimulation of Heart Membrane Adenylate Cyclase

Purpose and Rationale

Metzger and Lindner (1981) discovered that the positive inotropic and vasodilatory effects of forskolin were correlated with the stimulation of adenylate cyclase and cAMP-dependent protein kinase. Subsequent studies by Seamon et al. (1981) demonstrated that forskolin, unlike hormones, guanine nucleotides, fluoride, or cholera toxin could stimulate cyclase activity in the absence of the guanine nucleotide regulatory protein. Since those reports, hundreds of papers have been published on the effects of forskolin in numerous mammalian organ and cell systems. Several comprehensive review articles have also been published (Seamon and Daly 1981, 1983; Daly 1984).

While forskolin has proven to be an invaluable research tool for investigations of adenylate cyclase systems (Salomon et al. 1974; Seamon et al. 1981, 1983), reports on its effects on cardiovascular (Lindner et al. 1978), pulmonary (Chang et al. 1984), and ocular physiology (Caprioli and Sears 1983; Caprioli 1985) suggest a therapeutic potential (Seamon 1984) as well.

Described here is an in vitro assay which can be used to compare the potency of forskolin, forskolin analogs, or other direct or indirect adenylate

cyclase activators for the stimulation of adenylate cyclase in heart membranes. The purpose of this assay is to determine and compare the potency of direct or indirect activators of adenylate cyclase for an ability to stimulate heart membrane adenylate cyclase in vitro.

Procedure

The hearts of the Wistar rat, Hartley guinea pig, golden Syrian hamster, or cardiomyopathic hamster (CHF-146) are used as a source of adenylate cyclase for this assay.

Reagents

1. 0.5 M Tris buffer, pH 7.4
2. 0.05 M Tris buffer, pH 7.4, containing 0.1 M $CaCl_2$
3. Tris buffer mixture

   0.05 M Tris buffer, containing 1 mM IMBX (isobutylmethylxanthine), 0.2 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP (Na_2 ATP $\times$ $3H_2O$), and 20 mM creatine phosphate (Na_2 creatine–PO_4 $\times$ $5H_2O$) (final concentrations in the incubation media).
4. Creatine phosphokinase (ATP: creatine *N*-phosphotransferase, EC 2.73.2), type I, from rabbit muscle is obtained from Sigma Chemical Co.

   The concentration of enzyme in the incubation media is 40 U/ml.
5. Test compounds

   For most assays, a 30 mM stock solution is made up in a suitable solvent (ethanol, ethyl acetate, or DMSO for most forskolin analogs). Serial dilutions are made such that the final concentration in the assay ranges from 3×10^{-4} to 3×10^{-7} M. The concentration of vehicle in the assay is 1 %.

Tissue Preparation

One entire heart from rat, guinea pig, or hamster is dissected, weighed, and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer and pH 7.4 containing 0.1 mM $CaCl_2$ (reagent 2) using a Brinkman Polytron (setting 7 for 15 s). The homogenate is centrifuged at 10,000 g for 20 min at 4 °C. The resulting pellet is resuspended in 50 volumes of homogenizing buffer and recentrifuged as before. The supernatant of this spin is discarded, and the resulting pellet is finally resuspended in 10 volumes of the homogenizing buffer, for rat and hamster and 60 volumes for guinea pig tissue. This final preparation is filtered through a thin layer of gauze and kept on ice until used in the assay. The protein concentration is approximately 250–350 mg/ml.

Protein concentrations from an aliquot of the tissue suspension are determined on the day of the experiment by the method of Bradford (1976) using the Bio-Rad assay kit.

Assay

300 µl	Tris buffer mixture, pH 7.4 (reagent 3)
50 µl	Creatine phosphokinase
5 µl	Vehicle or appropriate concentration of test drug
95 µl	H_2O
50 µl	Tissue suspension

The tubes are incubated for 5 min at 37 °C, and the reaction is then stopped by placing the tubes into a boiling water bath for 4 min. The tubes are then centrifuged at 1,000 g for 10 min and cAMP levels determined in a 15 µl aliquot of the supernatant using a radioimmunoassay kit (Code TRK432) obtained from Amersham according to the manufacturer's protocol.

Test principle: The method is based on the competition between unlabeled cAMP and a fixed quantity of ^{3}H-labeled cAMP for binding to a protein which has high specificity and affinity for cyclic AMP. The amount of labeled cAMP–protein complex formed is inversely related to the amount of unlabeled cAMP present in the assay sample. The concentration of cAMP in the unknown is determined by comparison with a linear standard curve.

Evaluation

The data are expressed as pmol cAMP/mg protein/min, and dose–response stimulation curves

are subjected to logic analysis to determine the concentration of compound which exhibits 50 % of maximal stimulation (ED_{50}).

The β-blocking activities of compounds can be determined by their activities, by which they counteract the isoproterenol and GTP-induced stimulation of rat heart membrane-bound adenylate cyclase (Greenslade et al. 1979).

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^{3}H-Forskolin Binding Assay

Purpose and Rationale

This assay is used to identify compounds which demonstrate high affinity [nM] for association with forskolin binding sites in vitro as a preliminary screen in conjunction with stimulation of adenylate cyclase to determine the potential for cardiac chronotropic and inotropic and other effects of forskolin. Guinea pig heart tissue and rat brain tissue are used as sources of binding assays.

Procedure for Guinea Pig Heart Tissue

A. Reagents
   1. 0.05 M Tris–HCl buffer, pH 7.4
   2. 0.05 M Tris–HCl, pH 8.0 containing 1 mM EGTA, 1 mM $MgCl_2$, and 0.32 M sucrose
   3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear. Final concentration in the assay is approximately 15 nM.
   4. Forskolin and test compounds Forskolin and forskolin analogs are diluted to 40 μM. A 100 μl addition of this solution to the final incubate of 200 μl tissue and 100 μl ^{3}H-forskolin results in a final concentration of 10^{-5} M.

      Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

   Male Hartley guinea pigs (300–350 g) are sacrificed by decapitation. The heart is immediately removed, weighed, rinsed, diced, and homogenized in 10 volumes of ice-cold 0.05 M buffer (reagent2) using a Polytron homogenizer (setting 10, 30 s). The resulting homogenate is centrifuged at 12,000 g for 15 min at 4 °C. The clear supernatant of this spin is discarded; the remaining pellet (P_2) is resuspended in 5 volumes of the same ice-cold buffer and rehomogenized. This final suspension (approximately 0.8–1.0 mg protein/ml) is kept on ice until use. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

   To generate a dose–response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

   200 μl tissue suspension

   100 μl ^{3}H-forskolin

   100 μl appropriate concentration of forskolin or forskolin analog, or buffer

   Tubes are incubated at 30 °C for 10 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris–HCl buffer, pH 7.4 (reagent 1) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed 3 times with 5 ml aliquots of Tris–HCl buffer (reagent 1), added to 10 ml scintillation cocktail, and analyzed for radioactivity.

Procedure for Rat Brain Tissue

A. Reagents
   1. 0.32 M sucrose buffer.
   2. 0.05 M Tris–HCl buffer, pH 7.5.
   3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear. Final concentration in the assay is approximately 10 nM.
   4. Forskolin and test compounds Forskolin and forskolin analogs are diluted to 40 μM. A 100 μl addition of this solution to the final incubate of 200 μl tissue and 100 μl ^{3}H-forskolin results in a final concentration of 10^{-5} M.

      Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

Male Sprague–Dawley rats (200–250 g) are sacrificed by decapitation. The brain is rapidly removed and dissected on ice. Striata are homogenized in 50 volumes of ice-cold 0.32 M sucrose buffer (reagent 1) using a Polytron homogenizer (setting 7, 15 s). The resulting homogenate is centrifuged at 1,000 g for 10 min at 0–4 °C. The supernatant is retained and recentrifuged at 20,000 g for 10 min at 0–4 °C. The clear supernatant of this spin is discarded, and the remaining pellet (P_2) is resuspended in ice-cold Tris–HCl buffer, pH 7.5 such that the final protein concentration is approximately 3.0–4.0 mg/ml. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose–response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 µl tissue suspension

100 µl ^{3}H-forskolin

100 µl appropriate concentration of forskolin or forskolin analog or buffer

Tubes are incubated at 23 °C for 60 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris–HCl buffer, pH 7.4 (reagent 2), and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed three times with 5 ml aliquots of Tris–HCl buffer (reagent 2), added to 10 ml scintillation cocktail, and analyzed for radioactivity.

Evaluation

Specific binding is defined as the difference between binding of ^{3}H-forskolin in the absence and presence of 10 µM forskolin and represents 85–90 % of total binding at 10 nM ^{3}H-forskolin.

Results of the dose–response inhibition curves are analyzed by determining the concentration of competing compound which inhibits 50 % of the ^{3}H-forskolin binding sites (IC_{50}). This value is determined by computer-derived log-probit analysis.

The activities of various forskolin analogs are based on IC_{50} values and are categorized as follows:

	IC_{50}[M]
0 = Not determined	
1 = No activity	$>10^{-5}$
2 = Slight activity	10^{-5}–10^{-6}
3 = Moderate activity	10^{-6}–10^{-7}
4 = Marked activity	$<10^{-7}$

To compare the activity of various compounds from experiment to experiment, an inhibition constant (K_I) is determined as described by Cheng and Prusoff (1973). The K_I is determined from the equation

$$K_I = IC_{50}/1 + LC/K_D$$

IC_{50} = concentration of competing compound which inhibits 50 % of the ^{3}H-forskolin binding sites

LC = determined ^{3}H-forskolin concentration (approximately 10 nM)

K_D = dissociation of affinity constant for ^{3}H-forskolin determined previously to be approximately 13.4 nM for rat striatum and 196 nM for guinea pig heart

Standard data:

Binding inhibition values for forskolin

	Striatum (nM)	Heart (nM)
IC_{50}	43.1 ± 4.9	34.2 ± 5.0
K_I	26.0 ± 3.1	31.6 ± 4.6

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Renin–Angiotensin System

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General Considerations

The rennin-angiotensin system (RAS) regulates blood pressure, fluid volume, and sodium and potassium balance (Ferrario and Strawn 2006). Dysregulation of RAS system is implicated in cardiovascular disease – hypertension, atherosclerosis, left ventricular hypertrophy, myocardial infarction, stroke, congestive heart failure – and renal disease (reviewed by Ferrario and Strawn 2006). The classical RAS was initially described by Goldblatt et al. (1934), and since then, innumerous contributions (Braun-Menendez et al. 1940; Page and Helmer 1940; Haber et al. 1969; Boyd et al. 1969; Khairallah et al. 1970; Pals et al. 1971; Wong et al. 1988; Lin and Goodfriend 1970) have added to a better understanding of the complexity of the system. Historical perspectives of the RAS are presented in several reviews (Aurell 1998a, b; Page 1987; Robertson 1993; Skeggs 1986; Pickering 1982; Inagami 1998; Hall 2003). In the classical view, the RAS signaling pathway begins with angiotensinogen (an alpha-2-globulin) produced mainly by the liver, which is cleaved by renin, an enzyme expressed in the kidney and released in the bloodstream. This reaction forms angiotensin I, an inactive decapeptide, which is cleaved by the angiotensin-converting enzyme (ACE) into Ang II, a biologically active octapeptide. Ang II exerts its biological activities through its interaction with AT1 receptors and AT2 receptors; however, most of the known cardiovascular effects of Ang II are mediated by AT1 receptors (Matsusaka and Ichikawa 1997; Allen et al. 2000). Advances from the last three decades have revealed a more complex RAS with multiple active peptides (angiotensin IV, angiotensin-(1-7), angiotensin-(3-4), angiotensin A, and alamandine), enzymes (aminopeptidases, carboxypeptidases, endopeptidases, angiotensin-converting enzyme 2, and neprilysin), and receptors (AT1, AT2, Mas receptor, and AT4) (Schiavone et al. 1988; Santos et al. 2003; Donoghue et al. 2000; Ferreira and Santos 2005; Bader 2007; Ocaranza and Jalil 2012; Rabelo et al. 2011; Ferrario and Varagic 2010; Albiston et al. 2001; Braszko 2010).

Pharmacological inhibition of RAS can be accomplished by many routes besides the classical inhibition of ACE, neprilysin, and AT1 receptor blockade. More recently, inhibition of RAS can be accomplished by the direct rennin inhibitors (DRIs), AT4 receptor blockade, activation of the ACE2/Ang-(1-7)/Mas.

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Inhibition of the Angiotensin-Converting Enzyme In Vitro

Purpose and Rationale

ACE was discovered in the mid-1950s and found to release the carboxyl-terminal histidyl-leucine of angiotensin I in producing angiotensin II (Lentz et al. 1956; Skeggs et al. 1956). Erdös and Yang (1967) also identified ACE as bradykinin-degrading enzyme from hog kidney named the enzyme kininase II. Studies in mice treated with an ACE inhibitor or in mice genetically lacking ACE expression show that ACE is responsible for at least 90 % of angiotensin I conversion to angiotensin II in the blood, kidney, heart, lung, and brain (Campbell et al. 2004). These studies also showed that blood bradykinin (1–9) levels were increased 6.4- to 8.4-fold in animals treated with an ACE inhibitor. ACE also acts as a C-terminal dipeptidase for other small peptide hormones, including neurotensin, substance P, enkephalins, *N*-formyl-Met-Leu-Phe, acetyl Ser-Asp-Lys-Pro (AcSDKP), and angiotensin 1-7 (Rousseau et al. 1996; Deddish et al. 1998; Rice et al. 2004; Azizi et al. 1996; Bernstein et al. 2011; Defendini et al. 1982).

ACE is found in most tissues but the highest concentrations are found in the kidney and lung (Skidgel and Erdös 1993). Soluble ACE is derived by the enzymatic cleavage of tissue-bound ACE. ACE is also found in virtually all body fluids, including serum, urine, cerebrospinal fluid, seminal fluid, and amniotic fluid (Das et al. 1977; El-Dorry et al. 1983; Schweisfurth and Schiöberg-Schiegnitz 1984; Yasui et al. 1984; Hooper 1991).

The first ACE inhibitor was a peptide identified in extracts from Bothrops venom by Ferreira et al. (1970). Further pharmacologic development culminated in the synthesis in the 1970s of the first oral agents, captopril and subsequently enalapril, and lisinopril in the early to mid-1980s (Ondetti and Cushmen 1981; Patchett and Cordes 1958; Menard and Patchett 2001; Erdös 2006; Patchett et al. 1980). ACE inhibitors were the first drugs to be used to interrupt the RAS and have an established role as the first-line treatment for a number of cardiovascular and renal diseases (Sica and Gehr 2005). The many beneficial effect of ACE inhibitors is linked to its ability to prevent the formation of angiotensin II and increase bradykinin and, in turn, the production of NO_2 and prostacyclin (Zhang et al. 1997; Wiemer et al. 1991).

Sensitive in vitro methods are used to evaluate ACE activity in the tissue or blood and screen effective compounds. ACE activity is usually determined by monitoring the fluorescence generated by an artificial substrate in the presence or absence of the inhibitor.

Procedure

Reagents

1. 50 mM Tris–HCl buffer, pH8.0 + 100 mM NaCl
2. 10 mM potassium phosphate buffer, pH8.3
3. Substrate: *O*-aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline (molecular weight 482) (Bachem-Gentec. Inc., Torrance, California, USA)
   (a) Stock solution: 10 mg substrate in 10 ml 50 mM Tris–HCl buffer, pH8.0 + 100 mM NaCl
   (b) Working solution: 2 ml stock solution is added to 18 ml 50 mM Tris–HCl buffer, pH8.0 + 100 mM NaCl; final concentration in the assay is 170.2 μM
4. Test compounds

   Compounds are made up to a concentration of 1 mM in 50 mM Tris–HCl buffer, pH 8.0 + 100 mM NaCl, or 10 % methanol in Tris/NaCl if insoluble in aqueous buffer alone. This will give a final concentration in the assay of 0.1 mM. If inhibition is seen, further dilution in Tris/NaCl should be made.

Enzyme Preparation

Lung tissue from 10 rats is diced and homogenized in a blender with 3 pulses of 15 s each. The homogenate is centrifuged at 5,000 g for 10 min. The pellet is discarded, and the supernatant is dialyzed against three 1 l changes of 10 mM potassium phosphate buffer, pH 8.3 overnight in the cold, and then centrifuged at 40,000 g for 20 min. The pellet is discarded, and 390 mg $(NH_4)_2SO_4$ is added for each ml of supernatant. This will give 60 % saturation. The solution is stirred on ice for 15 min. The pellet formed is dissolved in 15 ml potassium phosphate buffer, pH8.3, and dialyzed against the same buffer overnight in the cold with three 1 l changes. Some protein will precipitate during dialysis. The suspension is centrifuged at 40,000 g for 20 min and the supernatant is discarded. The final solubilized enzyme preparation can be aliquoted and stored at −20 °C at least 6 months.

Enzyme Inhibition Studies

1. Enzyme activity is measured with a Perkin Elmer LS-5 Fluorescence Spectrophotometer or equivalent at an excitation wavelength of 357 nm and an emission wavelength of 424 nm.
2. Enzyme assay.
   50 µl vehicle or inhibitor solution and 40 µl enzyme are preincubated for 5 min, and then 410 µl substrate working solution is added.

Samples are mixed by drawing fluid back up into the pipette and by pipetting into the cuvette. For the initial control run of the day, the auto zero is pushed immediately after placing the sample in the cuvette.

Evaluation

The individual fluorescence slope is measured and % inhibition is calculated as follows:

$$\%\,\text{inhibition} = \%\,\text{inhibition} = \left(100 - \frac{\text{slope in presence of inhibitor}}{\text{control slope}}\right) \times 100$$

Inhibitor concentrations on either side of the IC_{50} should be tested to generate a dose–response curve. The IC_{50} is calculated using Litchfield–Wilcoxon logprobit analysis.

Standard data:

- IC_{50} values for inhibition of angiotensin I-converting enzyme
- Compound IC_{50} [M]
- Captopril 6.9×10^{-9}

Modifications of the Method

Other assays use the cleavage of hippuric acid from tripeptides (Hip-Gly-Gly or Hip-His-Leu) whereby hippuric acid is either tritium labeled or determined spectrophotometrically (Cushman and Cheung 1969, 1971; Friedland and Silverstein 1976; Santos et al. 1985; Hecker et al. 1994).

Bünning (1984) studied the binding and inhibition kinetics of ramipril and ramiprilate (Hoe 498 diacid) with highly purified angiotensin-converting enzyme using furanacryloyl-Phe-Gly-Gly as substrate.

The importance of tissue converting enzyme inhibition in addition to inhibition in plasma has been verified in several studies (Unger et al. 1984, 1985; Linz and Schölkens 1987).

Araujo et al. used internally quenched fluorogenic bradykinin-related peptide substrates for continuous measurements of the ACE enzymatic activity.

Doig and Smiley (1993) developed an HPLC method to separate the synthetic substrate hippuryl-L-histidyl-L-leucine from the hydrolysis product hippuric acid. The separation was accomplished by direct injection of biological assay mixtures onto a shielded hydrophobic phase column with isocratic elution. This method was used to determine ACE activity in serum and tissue extracts.

More recently, Wu et al. (2002) described an HPLC assay to determine the activity of ACE in the presence of inhibitory peptides present in soybean protein hydrolysates. The method uses HPLC to separate and quantify hippuryl-histidyl-leucine

(HHL) and hippuric acid (HA). Geng et al. (2010) improved the method by shortened running time (3.5 min), lowering limit of detection (5 pg), and limit of quantification (18 pg).

Micellar electrokinetic chromatography (MEKC) was employed previously to estimate the ACE inhibitory activity by separating the hippuric acid liberated in the presence of captopril (Watanabe et al. 2003).

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Quantitative Autoradiographic Localization of Angiotensin-Converting Enzyme

Purpose and Rationale

Cardiac angiotensin-converting enzyme can be quantified in the tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized in vitro autoradiography (Kohzuki et al. 1996).

Procedure

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see section "Myocardial Infarction After Coronary Ligation in Rrodents," chapter "▶ Coronary Drugs"). After various time intervals (1–8 months), the animals are decapitated and the hearts rapidly removed and snap-frozen in isopentane at −40 °C. Frozen section (20 μm) are cut in a cryostat at −20 °C. The sections are thaw mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4 °C, and then stored at −80 °C.

Quantitative Autoradiography

Radioligand: MK351A is a tyrosyl derivative of lisinopril, a potent competitive inhibitor of ACE. MK351A is iodinated by the chloramine T method and separated free from ^{125}I by SP Sephadex C25 column chromatography.

^{125}I-MK351A binding: The sections are preincubated in 10 mmol/L sodium phosphate buffer, pH7.4, containing 150 mmol/L NaCl and 2 % bovine serum albumin for 15 min at 20 °C. The sections are then incubated with 11.1 KBq/ml ^{125}I-MK351A in the same buffer for 60 min at 20 °C. Nonspecific binding is determined in the presence of 10^{-6} mol/L MK351A or lisinopril. Binding isotherms are determined using a set of serial sections incubated with 10^{-12}–10^{-6} mol/L lisinopril for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and stained with hematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

Evaluation

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, the intraventricular septum, the infarcted area in the left ventricle, and the non-infarcted area in the left ventricle are

estimated by an iterative nonlinear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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Inhibition of Neutral Endopeptidase (Neprilysin)

Purpose and Rationale

Neutral endopeptidase (NEP) is an endothelial, membrane-bound metallopeptidase that cleaves various peptides, such as enkephalins, kinins, chemotactic peptide, atrial natriuretic peptide, and substance P. NEP is widely distributed in endothelial cells, smooth muscle cells, cardiac myocytes, renal epithelial cells, and fibroblasts. NEP is also found in the lung, gut, adrenal glands, brain, and heart. Reviews on neutral endopeptidase 24.11 (enkephalinase) were given by Erdös and Skidgel (1989) and by Roques et al. (1993).

The combined inhibition of NEP and ACE by vasopeptidase inhibitors blocks angiotensin II synthesis and simultaneously prevents the degradation of ANP, BNP, and bradykinin. Promoting vasodilatation, diuresis and improving myocardial function (Burnett 1999; Bralet and Schwartz 2001; Duncan et al. 1999; Dumoulin et al. 1998, 2001).

Omapatrilat was the first vasopeptidase inhibitor; it has been proved more potent than candoxatril in lowering blood pressure (BP) and improving hemodynamics in patients with HF (Kostis et al. 2004; Rouleau et al. 2000). Long-lasting hypotensive effects of omapatrilat were observed in low-, normal-, and high-renin models of hypertension, and the effects were greater than those elicited by selective inhibition of either enzyme alone (Trippodo et al. 1998). The higher effectiveness of omapatrilat as well as other vasopeptidase inhibitors, as compared to ACE inhibitors, has been extensively demonstrated; however, omapatrilat clinical development has been stopped due to the high occurrence and greater severity of angioedema (Messerli and Nussberger 2000; Packer et al. 2002).

Several enzymatic assays have been developed for measuring neutral endopeptidase activity, such as radiolabeled methods (Vogel and Altstein 1977; Llorens et al. 1982), colorimetric assays (Almenoff et al. 1981; Almenoff and Orlowsky 1984), and fluorometric assay (Florentin et al. 1984; Goudreau et al. 1994). Burrell et al. (1997) and Hubner et al. (2001) used the selective NEP inhibitor radioligand 125I-labeled RB104. Cavalho et al. (1995, 1996) described a highly selective assay for neutral endopeptidase based on the cleavage of a fluorogenic substrate related to leuenkephalin. Heath et al. (1995) described the quantification of a dual ACE-I-converting enzyme-neutral endopeptidase inhibitor and the active thiol metabolite in dog plasma by high-performance liquid chromatography with ultraviolet absorption detection.

Procedure

A recombinant soluble form of NEP (rNEP) was expressed using a baculovirus/insect–cell system and purified by immunoaffinity.

The substrate (10 nmol) was incubated with rNEP (100 ng) in a final volume of 100 µl of 50 mM Tris–HCl buffer, pH 7.4, at 37 °C for 30 min. For the inhibition assays, the enzyme was preincubated with 1 µM thiorphan or 1 µM captopril for 20 min before its incubation with the substrate. The reaction was stopped by heating for 5 min at 100 °C. After centrifugation at 10,000 g for 10 min, the supernatant fraction was injected into an HPLC column and eluted with a 20–40 % gradient of acetonitrile containing 0.05 % trifluoroacetic acid over a period of 30 min, at a flow rate of 1 ml/min. The substrate and products, detected by both UV absorbance (220 nm) and fluorescence ($\lambda_{em} = 420$ nm, $\lambda_{ex} = 320$ nm) with the detectors arranged in series, were collected to identify the cleavage site by amino acid analysis.

Evaluation

Kinetic parameters for the NEP-catalyzed hydrolysis were determined from the double-reciprocal Lineweaver–Burk plots.

Modifications of the Method

Sulpizio et al. (2004) described the determination of **NEP activity in tissues** after in vivo treatment of rats with ACE inhibitors. After sacrifice of the animals, approximately 250 mg of kidney tissue was homogenized in six volumes of 0.1 M KH_2PO_4, pH8.3, 0.3 M NaCl, and 1 µM $ZnSO_4$, using a Teflon-glass motor-driven pestle. NEP activity was measured by adding 35 µl of homogenate to wells containing 5 µl buffer or 10 phosphoramidon. Next, 10 µl of 2.5 mM *N*-dansyl-D-ala-gly-*p*-nitro-phe-gly substrate (Florentin et al. 1984) was added to each sample to yield a 0.5 mM final concentration and incubated for 4 min at 37 °C. Subsequently, 100 µl of 10 % TCA was added and plates were centrifuged to pellet precipitated proteins. Then 50 µl of supernatant was added to 100 µl of 100 % ethanol and 50 µl of 1 N NaOH in a black fluorometric plate. After 10 min, plates were read at 590 nm emission and 320 nm excitation in a fluorometer.

Zhang et al. (1994) described an ELISA for the neuropeptide endopeptidase 3.4.24.11 in human serum and leukocytes.

Gros et al. (1989) studied the protection of atrial natriuretic factor against degradation and the diuretic and natriuretic responses after in vivo inhibition of enkephalinase (EC 3.4.24.11) by acetorphan. Increased tissue neutral endopeptidase 24.11 activity in spontaneously hypertensive hamsters was reported by Vishwanata et al. (1998). Graf et al. (1998) studied regulation of neutral endopeptidase 24.11 in human vascular smooth muscle cells by glucocorticoids and protein kinase C.

Pham et al. (1992) described the effects of a selective endopeptidase inhibitor on renal function and blood pressure in conscious normotensive Wistar and hypertensive DOCA-salt rats.

NEP is involved in organ systems other than the cardiovascular system, for example, the brain and lung.

Ratti et al. (2001) studied the correlation between neutral endopeptidase (NEP) in serum and the degree of bronchial hyperreactivity.

Shirotani et al. (2001) found that neprilysin degrades both amyloid β peptides 1–40 and 1–42 very rapidly and efficiently. Newell et al. (2003) found that thiorphan-induced neprilysin inhibition raises amyloid β levels in rabbit cortex and cerebrospinal fluid.

Facchinetti et al. (2003) described the ontogeny and regional and cellular distribution of metalloprotease neprilysin 2 (**NEP2**) in the rat in comparison with neprilysin and endothelin-converting enzyme-1.

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Angiotensin-Converting Enzyme 2 Activity

Purpose and Rationale

ACE2 was discovered in 2000 as a homologue enzyme of ACE (Donoghue et al. 2000; Tipnis et al. 2000). Despite their similarities, ACE and ACE2 function differently; ACE releases a C-terminal dipeptide from its substrate (dipeptidylpeptidase), whereas ACE2 cleaves a single amino acid (monocarboxypeptidase). ACE-2 is not blocked substantially by current ACE inhibitors and is expressed mainly in the heart, kidney, testis, endothelium of coronary, intrarenal vessels, and renal tubular epithelium (Donoghue et al. 2000; Tipnis et al. 2000; Gallagher et al. 2008). Soluble form of ACE2 is also found in plasma and urine (Epelman et al. 2009 and Wysocki et al. 2013). The relevance of ACE2 in cardiovascular has been reviewed by Kuba et al. (2013) and Varagic et al. (2014).

ACE2 cleaves Ang I to generate the presumably inactive Ang1–9 peptide, which then can be

converted to the vasodilator peptide Ang-(1–7) by ACE or other peptidases (Donoghue et al. 2000; Tipnis et al. 2000; Rice at al. 2004). ACE2 also metabolizes Ang II to generate Ang-(1–7) with high affinity (Vickers et al. 2002; Rice at al. 2004). ACE2 is the main enzyme that catalyzes the formation of Ang-(1–7) in vitro and in vivo (Zisman et al. 2003) but also cleaves Apelin-13, Apelin-36, dynorphin A-(1–13), and des-Arg bradykinin (Kazemi-Bajestani et al. 2012; Vickers et al. 2002).

Ang-(1–7) peptide has been shown to interact with the G protein-coupled receptor Mas to mediate cardiorenal protective effects (Santos et al. 2003). Ang-(1–7) has been characterized as a biologically active component of the RAS with functional role in counterbalancing the Ang II actions (Ferrario et al. 1997; Ferrario and Varagic 2010). Ang-(1–7) induces systemic and regional vasodilation, diuresis, and natriuresis and exerts antiproliferative and antigrowth effects in vascular smooth muscle cells, cardiac myocytes, and fibroblasts, as well as glomerular and proximal tubular cells (Brosnihan et al. 1996; Freeman et al. 1996; Santos et al. 2003; Ferrario and Varagic 2010).

Transgenic ACE2 overexpression in the vessels of SHRSP rats reduces blood pressure and improves endothelial function, and neuronal overexpression of ACE2 also attenuates hypertension (Rentzsch et al. 2008; Feng et al. 2008). In humans, several studies have shown a strong association of ACE2 polymorphisms to hypertension in female Chinese patients with metabolic syndrome, essential hypertension, or diabetes-associated hypertension (Zhong et al. 2006; Yi et al. 2006; Lu et al. 2012; Patel et al. 2012). Several recent reviews have focused on the cardiovascular and renal actions of the ACE2/A1-7/Mas axis of the RAS and its implication in cardiovascular and renal diseases (Varagic et al. 2014; Passos-Silva et al. 2013; Tikellis et al. 2011).

Novel pharmacological strategy that interacts with specific sites of ACE2 causing its conformational change and, consequently, enhancing its activity is being developed as an antihypertensive therapy (Hernández Prada et al. 2008; Murça et al. 2012).

Procedure

Reagents

1. 150 mM NaCl, 75 mM Tris, 10 μM $ZnCl_2$, 0.01 % Triton X-100, pH 7.2
2. Substrate: 7-methoxycoumarin-4-yl acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH (Mca-APK-Dnp) (molecular weight 696.7) (AnaSpec, Fremont, CA)
3. Working concentration: 200 μM Mca-APK-Dnp.
4. Inhibitor: ACE2 inhibitor MLN-4760 (EMD Millipore, Billerica, Massachusetts USA)
5. Enzyme Source:
   (a) Human recombinant (hr) ACE2 (R&D systems, Minneapolis, MN, USA). rhACE2 is serial diluted with 100 mM glycine+50 μM ZnCl+150 mM+1 % BSA starting with 5 μg/mL. The serially diluted enzyme is further diluted 1:5 with assay buffer 150 mM NaCl+75 mM Tris + 10 μM $ZnCl_2$ + 0.01 % Triton X-100, pH 7.2.
   (b) Cell lysates. Cells are lysed with M-PER lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail. Total protein is measured by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Cell lysates are prepared at 4 mg/mL and diluted 1:5 with 150 mM NaCl, 75 mM Tris, 10 μM $ZnCl_2$, 0.01 % Triton X-100, pH 7.2.

Enzymatic Assay

Cleavage of the substrate 7-Mca-YVADAPK (Dnp) by ACE2 removes the 2,4-dinitrophenyl moiety that quenches the fluorescence of the 7-methoxycoumarin moiety, thus resulting in increased fluorescence.

1. Enzyme assay: 50 μL of the diluted enzyme was transferred to a black 96-well microtiter plates with a 100 μL total volume, and then 50 μL of substrate at 200 μM was added to each well (final substrate concentration was 100 μM). Samples are mixed and the plates are read immediately. For cell lysates 50 μL of the diluted cell lysate is incubated with

50 µL of stock 200 µM substrate at 37 °C for 20 min.
2. Enzyme activity is measured with a FluRex (Molecular Devices, Sunnyvale, CA) or equivalent at an excitation wavelength of 320 nm and an emission wavelength of 405 nm within 60 min.
3. For the inhibition assays, the enzyme was preincubated with inhibitors for 10 min before its incubation with the substrate.
4. Results after subtraction of the inhibition value are expressed as RFU (relative fluorescent units) per µg of protein and per hour (RFU/µg/h).

Modifications of the Method

Other assays use the hydrolysis of natural substrate angiotensin I whereby ACE2 hydrolyzes Ang I to produce Ang-(1–9) and leucine. The released leucine can then be monitored by the activity of leucine dehydrogenase with concomitant conversion of NAD+ to NADH. The production of NADH is coupled to the diaphorase-catalyzed reduction of resazurin to resorufin, which can be monitored on a fluorescence reader (Huang et al. 2003).

ACE2 activity in tissue extract and intact cells was previously evaluated by Wysocki et al. (2006) and Keidar et al. (2005).

Liao et al. (2013) developed and optimized an enzymatic assay for the detection of neutralizing antibodies directed against the ACE2 enzyme. The assay provided a direct measurement of ACE2 catalytic activity for the detection of neutralizing antibodies in clinical samples.

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Renin Activity

Purpose and Rationale

Renin is an aspartyl protease synthesized as prorenin, a proenzyme that contains an additional 43-amino acid N-terminal fragment. Prorenin is converted into active renin in the juxtaglomerular cells of the kidney (Sealey et al. 1977). Renin cleaves the N-terminus of circulating angiotensinogen to form angiotensin I which is then transformed in angiotensin II. Angiotensinogen is the only known substrate for renin. The rate-limiting step in the RAS is Ang I generation. Ang II inhibits renin release through stimulation of the AT1 receptor. High levels of renin are observed with ACE inhibitors or AT1 receptor blockade and associated with worse outcome (latini et al. 2004; Rouleau et al. 1994).

Renin inhibitors bind to the active site of renin rendering the entire RAS pathway quiescent. Renin inhibitors do not cause stimulation of bradykinin or prostaglandins and reduce angiotensin II formed by non-ACE pathways. Aliskiren is the only oral renin inhibitor approved for use in humans. Aliskiren decreases plasma-renin activity and Ang II, and Ang II in hypertensive patients, besides the urinary aldosterone, was reduced (Azizi et al. 2004; Gallagher et al. 2008; Cagnoni et al. 2010).

The effect of various agents on renin concentration can be evaluated by determining the plasma-renin activity or the plasma-renin concentration. Plasma-renin activity (PRA) measures the enzymatic activity of renin and quantifies the amount of angiotensin I generated from renin activity. PRA is also a predictor of response to various antihypertensives (Olson et al. 2012). Plasma active renin, also known as active renin concentration (ARC), allows quantification of the amount of renin present, regardless of its activity.

A method to quantify plasma-renin activity is described below.

Procedure

The synthetic substrate represents the first fourteen amino acids of the N-terminus of human angiotensinogen: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn. The assay mixture is composed of phosphate buffer (pH 7.5), bovine serum albumin, 3 mM EDTA, 0.01 mM phenylmethylsulfonyl fluoride (PMSF), 0.002 % Genapol PF 10, test compound (dissolved in DMSO), substrate (3 μM), and purified human kidney renin (Calbiochem GmbH, Frankfurt/M.,

Germany; cat. no. 553861). The mixture is incubated for 2 h at 37 °C. Then the reaction is stopped by transfer of 450 µl into preheated (95 °C) Eppendorf tubes. The amount of angiotensin I liberated is measured by RIA (Renin-MAIA kit, Serono Diagnostika GmbH, Freiburg, Germany).

Human angiotensinogen (0.2 µM) may be used as a substrate instead of the tetradecapeptide. The pH value of the incubation mixture may be lowered to 6.0 by using a maleic acid buffer; this results in higher renin activity. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) may be substituted for phosphate in the pH 7.5 buffer.

Evaluation

Renin activity, i.e., angiotensin I production (ng/ml × 2 h), is corrected for an angiotensin I – like immunoreactivity which can be measured in the assay samples even in the absence of added renin. IC_{50} values are determined from a plot of renin activity (as percent of control) versus molar concentration of the test compound.

Modifications of the Method

Wang et al. (1993) described a continuous fluorescence assay of renin activity employing a new fluorogenic peptide substrate.

Inhibition of plasma-renin activity in blood samples from various species can be determined in order to evaluate the species specificity of a renin inhibitor (Linz et al. 1994).

Blood samples are obtained from dogs, sheep and rhesus monkeys by venipuncture. Wistar rats and guinea pigs are anesthetized with Nembutal (60 mg/kg intraperitoneally) and the blood is collected by puncture of the abdominal aorta. Human blood is collected from volunteers (Donafix blood collecting set, Braun Melsungen AG, Melsungen, FR Germany) in cooled bottles. All blood samples are anticoagulated with Na-EDTA (final concentration 10–15 mM). The renin is dissolved in DMSO as 10^{-2} M stock solution and diluted before each experiment in DMSO. The endogenous formation of ANG I in plasma during incubation at 37 °C is determined as the measure of renin activity. Generation and quantitation of ANG I are performed using a commercial radio immunoassay kit (Renin-MAIA, Serono Diagnostika GmbH, Freiburg, FR Germany). Plasma samples are thawed on ice and centrifuged after addition of 100 µl PMSF solution (kit) per 10 ml. The assay mixture contains 450 µl plasma plus 1 %(v/v) PMSF solution, 45 µl buffer (phosphate buffer, pH = 7.4, + 10^{-5} M ramiprilat), and 5 µl renin inhibitor solution (diluted in DMSO as required) or pure DMSO for controls. The assay is incubated for an appropriate time (2–3 h) at 37 °C. ANG I is measured in 100 µl samples (triplicate determinations). Basal ANG I immunoreactivity of the plasma is determined from an unincubated control assay (0 °C). This preincubation value is subtracted from all measurements. The renin activity in the presence of the renin inhibitor is calculated as percent activity in relation to control samples containing only DMSO. The IC_{50} value is determined from a semilogarithmic plot of percent renin activity versus concentration of the renin inhibitor.

Wood et al. (1990) determined the activity of a synthetic renin inhibitor against rat, mouse, dog, guinea pig, rabbit, cat, marmoset, and human renin using plasma pools from these species. Plasma from each species was collected using EDTA as an anticoagulant. Samples of plasma were incubated at 37 °C in the presence or absence of varying concentrations of test compound. The ANG I formed was measured by radioimmunoassay.

Shibasaki et al. (1991) used squirrel monkeys to study the in vivo activity of a specific renin inhibitor after intravenous and oral application.

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Salimbeni et al. (1996) tested the in vitro inhibition of human plasma-renin activity by two synthetic angiotensinogen transition state analogues.

Wood et al. (2003) determined the inhibitory potency of an orally effective renin inhibitor

in vitro against human renin. Human recombinant renin (0.33 ng/ml) was incubated with a synthetic tetradecapeptide substrate (TDP, 13.33 μM) corresponding to the 14 terminal amino acids of human angiotensinogen, in 0.33 M Tes buffer, pH7.2, containing 1 % human serum albumin and 0.1 % neomycin sulfate for 1 h at 37 °C. The enzymatic reaction was stopped by adding 1 ml ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1 % HSA. The angiotensin generated during the incubation was measured by radioimmunoassay. The oral activity was confirmed in hypertensive patients (Gradman et al. 2005).

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Angiotensin Receptor Binding

Purpose and Rationale

ANG II mediates its effects via at least two plasma membrane receptors: AT1 and AT2 receptors. AT_1 and AT_2 have been identified by structurally dissimilar antagonists, by different distribution in organs of various species, and with specific radioligands (Chiu et al. 1989, 1990, 1992, 1993; Chang and Lotti 1991; Gibson et al. 1991; Chansel et al. 1992; Steckelings et al. 1992; Aiyar et al. 1993; Barnes et al. 1993; Bossé et al. 1993; Bottari et al. 1993; Dzau et al. 1993; Feuillan et al. 1993; van Meel et al. 1993; Alexander et al. 2001). These two types of receptors have been cloned (Sasaki et al. 1991; Murphy et al. 1991; Mukoyama et al. 1993; Kambayashi et al. 1993). Ang II and also Ang III bind with high affinity at the AT1 and AT2 receptors, but Ang IV and Ang 1–7 display lower affinity to both receptors (de Gasparo et al. 2000). The functional correlates of angiotensin II receptors have been discussed by Timmermans et al. (1992, 1993) and Bernstein and Berk (1993). Most effects of angiotensin are mediated via the AT_1 receptors. Evidence for AT_1 receptor subtypes (AT_{1A} and AT_{1B}) has been reported (Iwai and Inagami 1992; Kakar et al. 1992; Balmforth et al. 1994; Matsubara et al. 1994; Bauer and Reams 1995; de Gasparo et al. 1998).

The second major angiotensin receptor isoform is the AT2 receptor, although the exact signaling pathways and the functional roles of AT2 receptors are unclear; these receptors may antagonize, under physiological conditions, AT1-mediated actions by inhibiting cell growth and by inducing apoptosis and vasodilation (Yamada et al. 1998; Wiemer et al. 1993a, b; Keiser et al. 1992).

Two other angiotensin receptors named AT3 and AT4 have been described (de Gasparo et al. 1998; Swanson et al. 1992). AT4 binding site shows highest selectivity for angiotensin IV, a metabolic product of Ang II (Swanson et al. 1992; Hall et al. 1995). Chai et al. (2004) described the properties of the angiotensin IV/AT4 receptor. Recently the receptor for Ang IV was purified and identified by mass spectrometry as insulin-regulated aminopeptidase (IRAP) (Albiston et al. 2001).

Recently Santos et al. (2003) identified the G protein–protein-coupled receptor Mas as a receptor for Ang-(1–7). Most of A-17 actions are mediated by Mas and blocked by the selective antagonist A-779 (Ferreira et al. 2012).

(Pro)renin receptor was discovered in human mesangial cells in vitro and cloned in 2002

(Nguyen et al. 2002). Proteolytic removal of prosegment of prorenin to form active renin and initiate the RAS cascade is the first necessary step. Binding of prorenin to the (pro)renin receptor and subsequent removal of the prorenin prosegment from the active enzymatic cleft have been suggested as a non-proteolytic alternative pathway (Nguyen et al. 2002). Prorenin receptor (PRR) is a multifunctioning protein possessing many different roles besides enhancing the tissue renin-angiotensin system. (Pro)renin receptor functions was reviewed by Sihn et al. (2013), Binger and Muller (2013), and Oshima et al. (2014).

The assay described below is used to determine the affinity of test compounds to the angiotensin II receptor by measuring their inhibitory activity on the binding of ^{3}H-angiotensin II to a plasma membrane preparation from rat or bovine adrenal cortex.

Procedure

Fresh bovine adrenal glands are obtained from the local slaughter house. For rat adrenal glands, male Sprague–Dawley rats weighing 250–300 g are sacrificed. The adrenals are separated from fat tissue and the medullae removed. The cortices are minced and homogenized in 5 mM Tris buffer containing 1 mM $MgCl_2$ and 250 mM sucrose, pH 7.4, using a chilled Potter homogenizer. The homogenate is centrifuged at 3,000 g and 4 °C for 10 min. The supernatant is recentrifuged at 39,000 g and 4 °C for 10 min. The pellets are resuspended in 75 mM Tris buffer containing 25 mM $MgCl_2$, pH 7.4, and recentrifuged twice at 39,000 *g* and 4 °C for 10 min. After the last centrifugation, the pellets are suspended in 75 mM Tris buffer containing 25 mM $MgCl_2$ and 250 mM sucrose, pH 7.4. Samples of 0.5 ml are frozen in liquid nitrogen and stored at −70 °C.

In the competition experiment, 50 µl ^{3}H-angiotensin II (one constant concentration of 0.5–1 × 10^{-9} M), 50 µl test compound (six concentrations, 10^{-5}–10^{-10} M), and 100 µl membrane suspension from rat or bovine adrenal cortex (approx. 250 mg wet weight/ml) per sample are incubated in a bath shaker at 25 °C for 60 min. The incubation buffer contains 50 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, and 0.2 % bovine serum albumin, pH 7.4.

Saturation experiments are performed with 12 concentrations of ^{3}H-angiotensin II (15–0.007 × 10^{-9} M). Total binding is determined in the presence of incubation buffer; nonspecific binding is determined in the presence of non-labeled angiotensin II (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after the addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

Evaluation of Results

The following parameters are calculated:

- Total binding of ^{3}H-angiotensin II
- Nonspecific binding: binding of ^{3}H-angiotensin II in the presence of mepyramine or doxepin
- Specific binding = total binding – nonspecific binding
- % inhibition of ^{3}H-angiotensin II binding: 100 – specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-angiotensin II versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

Modifications of the Method

Olins et al. (1993) performed competition studies in rat uterine smooth muscle membranes and rat adrenal cortex membranes using [^{125}I] labeled angiotensin II.

Membranes from cultured rat aortic smooth muscle cells and from human myometrium were used for binding studies with [^{125}I]-labeled angiotensin II by Criscione et al. (1993).

Wienen et al. (1993) used membrane preparations from rat lung and adrenal medulla for binding studies with [^{125}I]-labeled angiotensin II.

Bradbury et al. (1993) used a guinea pig adrenal membrane preparation to study non-peptide angiotensin II receptor antagonists.

Cazaubon et al. (1993) prepared purified plasma membranes from rat livers for [^{125}I] AII binding assays.

Noda et al. (1993) described the inhibition of rabbit aorta angiotensin II (AII) receptor by a non-peptide AII antagonist.

Kushida et al. (1995) tested AT II receptor binding in particulate fractions of rat mesenteric artery and rat adrenal cortex and medulla with ^{125}I-AT II.

Chang et al. (1995) used rabbit aorta, rat adrenal and human AT_1 receptors in CHO cells, and AT_2 receptors from rat adrenal and brain to characterize a non-peptide angiotensin antagonist.

Aiyar et al. (1995) tested inhibition of [^{125}I]-angiotensin II or [^{125}I]angiotensin II (Sar^1, Ile^8) binding in various membrane and cell preparations, such as rat mesenteric artery, rat adrenal cortex, rat aortic smooth muscle cell, human liver, recombinant human AT_1 receptor, bovine cerebellum, and bovine ovary.

Caussade et al. (1995) tested [^{125}I]Sar^1, Ile^8-angiotensin II binding to rat adrenal membranes and rat aortic smooth muscle cells.

Using [^{125}I]Sar^1, Ile^8-angiotensin II as radioligand, de Gasparo and Whitebread (1995) compared the affinity constants of valsartan and losartan in the liver and adrenal of rat and marmoset, in human adrenal, and in rat aortic smooth muscle cells.

Webb et al. (1993) transfected the vascular angiotensin II receptor cDNA (AT_{1A}) into Chinese hamster ovary cells to generate the stable cell line CHOAT_{1A} and recommended these cells as a useful model to study AT_{1A} receptor domains, which are critical to signaling pathways.

Kiyama et al. (1995) used COS cells transfected with a cDNA encoding a human AT_1 angiotensin II receptor to evaluate non-peptide angiotensin II receptor antagonists.

Mizuno et al. (1995) used bovine adrenal cortical membranes, and Nozawa et al. (1997) used membrane fractions from rat aorta, bovine cerebellum and human myocardium and [^{125}I]angiotensin II as radioligand.

Renzetti et al. (1995a, b) used membranes from rat adrenal cortex and bovine cerebellum for binding assays with [^{3}H]angiotensin II as radioligand.

Interspecies differences in angiotensin AT_1 receptors were investigated by Kawano et al. (1998).

The angiotensin II receptor subtype having a high affinity for losartan has been designated angiotensin AT_1 receptor and the receptor having a high affinity for PD123177 (1-(3-methyl-4-aminophenyl) methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazo[3,5-c]pyridine-6-carboxylic acid) as angiotensin AT_2 receptor (Pucell et al. 1991; Nozawa et al. 1994; Chang et al. 1995).

In order to determine affinity for the angiotensin AT_1 subtype in a radioligand binding assay with ^{125}I-sarcosine^1, isoleucine^8 angiotensin II, Chang and Lotti (1991), Chang et al. (1995), and Wong et al. (1995) incubated membranes of tissues with both AT_1 and AT_2 receptors in the presence of 1 μM PD121981 (which occupied all the AT_2 binding sites) and for the angiotensin AT_2 subtype in the presence of 1 μM losartan (which occupied all the AT_1 binding sites).

Hilditch et al. (1995) used membranes from rat livers and [^{3}H]-AT II for the determination of binding affinity at AT_1 receptors or membranes from bovine cerebellum and [^{125}I]-Tyr^4-AT II for AT_2 receptors.

Lu et al. (1995) studied the influence of freezing on the binding of ^{125}I-sarcosine^1, isoleucine^8 angiotensin II to angiotensin II receptor subtypes in the rat. The results suggested that studies of AII receptor subtypes that involve freezing of the tissue underestimate the density and affinity of the AT_1 receptor subtype.

Nielsen et al. (1997) found that in myometrium from nonpregnant sows, the Ang II receptors were almost exclusively AT_2 receptors. Binding of [^{125}I]Ang II to myometrium membranes was conducted in the presence of losartan to block binding to AT1 receptors.

Whitebread et al. (1991) described the radioligand CGP 42112A as a high-affinity and

highly selective ligand for the characterization of angiotensin AT_2 receptors. Heemskerk and Saavedra (1995) performed quantitative autoradiography of Ang II AT_2 receptors with [^{125}I]CGP 42112.

Heerding et al. (1997) performed mutational analysis of the Ang II type 2 receptor in order to study the contribution of conserved extracellular amino acids.

Hoe et al. (2003) reported the molecular cloning, characterization, and distribution of the gerbil Ang II AT_2 receptor.

Utsunomiya et al. (2005) described Ang II AT_2 receptor localization in cardiovascular tissues by its antibody developed in AT_2 gene-deleted mice.

The presence of nuclear angiotensin receptor sites has been described previously (Li and Zhuo 2008; Licea et al. 2002; Pendergrass et al. 2006; Zhuo et al. 2006). Li and Zhuo (2008) used FITC-labeled ANG II and [^{125}I]Val5-ANG II binding assays to demonstrate that the majority of ANG II receptor binding in isolated rat renal cortical nuclei belongs to AT1 receptor.

Demaegdt et al. (2011) described the [^{3}H]Ang IV and its metabolically stable analogue [3H]AL-11, binding to CHO-K1 cells transfected with human IRAP.

Santos et al. (2003) demonstrated that genetic deletion Mas receptor in mice abolishes the binding of ^{125}I-Ang (1–7) in kidney slices.

Angiotensin receptor subtypes in the kidney cortical nuclei, uteroplacental unit, and vascular smooth muscle cells were characterized by the competition of ^{125}I-sarthran binding with selective antagonists: losartan, the AT1 antagonist; DALA, the Ang-(1–7) antagonist; and PD, the AT2 receptor antagonist (Gwathmey et al. 2010; Clark et al. 2001; Yamaleyeva et al. 2013).

Prorenin binding experiment of pre-adsorbed (pro)renin receptors on plastic wells was described previously (Nabi et al. 2012, 2009).

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Angiotensin II-Induced Contraction in Isolated Rabbit Aorta

Purpose and Rationale

The isolated rabbit aorta has been used to evaluate angiotensin II agonists (Liu 1993) and angiotensin II antagonists (Chang et al. 1992, 1994; Noda et al. 1993; Aiyar et al. 1995; Cirillo et al. 1995; Kushida et al. 1995; Mochizuki et al. 1995; Renzetti et al. 1995; Wong et al. 1995; Hong et al. 1998; Kawano et al. 1998).

Procedure

New Zealand white male rabbits weighing 2–3 kg are sacrificed and exsanguinated. The thoracic aorta is removed and cleaned from adherent fat and connective tissue. The vascular endothelium is removed by gently rubbing the intimal surface of the vessel. Spiral aortic strips (2–3 mm wide and 30 mm long) are prepared and mounted in 5 ml organ baths containing Krebs–Henseleit solution (120 mM NaCl, 4.7 mM KCl, 4.7 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, glucose 10 mM, pH 7.4). The organ baths are kept at 37 °C and gassed continuously with 95%O_2/5%CO_2. Strips are attached to isometric transducers connected to a polygraph, and a resting tension of 1 g is applied to each strip. Changes in contraction are analyzed with a digital computer. Aortic strips are allowed to equilibrate for 1 h and washed every 15 min. Two consecutive contractile-response curves to cumulative addition of ATII (0.1–300 mM) are constructed. After each curve the strips are washed four times and allowed to relax to the baseline tension. Afterward, each strip is incubated for 30 min with the vehicle or with a single concentration of the antagonist (1 – 10 – 100 – 1,000 mM) before a third concentration response curve to angiotensin II is obtained.

Evaluation

The result of each concentration is expressed as a percentage of maximum response to AII. The pA_2 and pD'_2 values are calculated (van Rossum 1963).

Modifications of the Method

Isolated guinea pig aortas were used by Mizuno et al. (1995).

Cirillo et al. (1995) evaluated the antagonism against AII-induced vasoconstriction in rat isolated perfused kidney.

Chang et al. (1992, 1994) determined AII-induced aldosterone release in rat adrenal cells and AII-induced [^{3}H]inositol phosphate accumulation in cultured rat aorta smooth muscle cells.

Shibouta et al. (1993) described the pharmacological profile of a highly potent and long-acting Ang II receptor antagonist and its prodrug.

Ojima et al. (1997) studied the mechanisms of the insurmountable antagonism of candesartan, an angiotensin AT_1 receptor antagonist, on Ang II-induced rabbit aortic contraction in contraction and binding studies.

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Angiotensin II Antagonism In Vivo

Purpose and Rationale

The effect of angiotensin receptor antagonists on blood pressure has been measured in anesthetized (Olins et al. 1993; Beauchamp et al. 1995; Kawano et al. 1998), in pithed (Cazes et al. 1995; Christophe et al. 1995; Cirillo et al. 1995; Deprez et al. 1995; Häuser et al. 1998), and in conscious (Junggren et al. 1996; Nozawa et al. 1997; Shibasaki et al. 1997; Hashimoto et al. 1998) normotensive and hypertensive rats.

Procedure

Male Sprague–Dawley rats are anesthetized with 100 mg/kg i.p. Inactin and placed on servo-controlled heating pads to maintain body temperature between 37 °C and 38 °C. PE50 catheters are implanted in the femoral artery and vein to measure arterial blood pressure and administer compounds, respectively. A catheter is placed in the trachea to ensure airway patency. Arterial pressure is measured continuously by connecting the arterial catheter to transducer coupled to a Gould pressure transducer. The output is recorded on a polygraph. Mean arterial pressure is derived electronically. After a 30–45 min stabilization period, autonomic transmission is blocked by treatment with mecamylamine (3 mg/kg i.v.) and atropine (0.4 mg/kg i.v.). After arterial pressure has stabilized, angiotensin is infused i.v. in isotonic saline with a syringe pump. When the pressure response to angiotensin has stabilized, angiotensin II antagonists are given in increasing doses. The doses are given intravenously in a cumulative fashion, i.e., the next highest dose is given at the time of maximum response to the prior dose.

Evaluation

Data are presented as percent inhibition of the angiotensin pressor response to each dose of the antagonists and plotted against the log of the cumulative doses of antagonist. Linear regression is used to calculate the dose at which the response to angiotensin is inhibited 50 % (ID_{50}) for each rat. Means ± SEM are calculated.

Modifications of the Method

Olins et al. (1993) and Cirillo et al. (1995) determined also the antihypertensive effects in conscious spontaneously hypertensive rats and in conscious sodium-deficient dogs.

Stasch et al. (1997) studied the long-term blockade of the angiotensin II receptor in renin transgenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats.

Nishioaak et al. (1998) and Richter et al. (1998) used the (mRen-2)27 transgenic (Tg^+) rat, a hypertensive model dependent on increased expression of the renin-angiotensin system, to explore the role of angiotensin AT_2 receptors in the control of cardiovascular and renal excretory function.

Simoes e Silva et al. (1998) evaluated the effects of chronic administration of an angiotensin antagonist on diuresis and natriuresis in normotensive and spontaneously hypertensive rats.

Kai et al. (1998) examined the effects of an angiotensin II type 1 antagonist on cardiac hypertrophy and nephropathy using Tsukuba hypertensive mice (THM) carrying both human renin and angiotensinogen genes.

Kivlighn et al. (1995a, b) and Gabel et al. (1995) studied angiotensin II antagonists in conscious rats, dogs, rhesus monkeys, and chimpanzees.

Keiser et al. (1995) studied arterial blood pressure in conscious renal hypertensive rats, conscious sodium-depleted dogs, conscious sodium-depleted monkeys, and conscious renal hypertensive monkeys.

Kim et al. (1997) examined the effects of an angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. Cardiac volume overload was prepared by abdominal aortocaval shunt. Cardiac tissue mRNA was measured by Northern blot analysis with specific probes.

Yamamoto et al. (1997) and Ogilvie and Zborowska-Sluis (1998) studied angiotensin II receptor antagonists in acute heart failure induced by coronary artery ligation in anesthetized dogs and in chronic heart failure induced by rapid left ventricular pacing in conscious dogs.

Massart et al. (1998) evaluated the cumulative hypotensive effects of angiotensin II- and endothelin-1-receptor antagonists in a model renovascular hypertension in dogs.

Hayashi et al. (1997) examined the hemodynamic effects of an angiotensin II type 1 receptor antagonist in rats with myocardial infarction induced by coronary ligation.

Kivlighn et al. (1995c) studied the effects of a non-peptide that mimics the biological actions of angiotensin II in anesthetized rats.

Huckle et al. (1996) evaluated angiotensin II receptor antagonists for their ability to inhibit vascular intimal thickening in a porcine coronary artery model of vascular injury.

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Part II

Pharmacological Assays in Thrombosis and Haemostasis

In Vitro Models of Thrombosis

Shaker A. Mousa

Contents

General Introduction

In vitro models for the assessment of antithrombotic activity of an anticoagulant or antiplatelet can be carried out using human blood or blood from various animal species where the in vivo antithrombotic efficacy would be evaluated.

Arterial thrombus, which is formed under relatively high shear, is primarily composed of platelet aggregates strengthened by a small percentage of fibrin. In contrast, venous thrombus, which is formed under low shear, is primarily composed of fibrin, with a low percentage of platelet aggregate. In vitro model systems can assess the blood global factors behind the hyper-coagulation and/or hyper-platelet activity.

This chapter highlights commonly used in vitro model systems in the evaluation of anticoagulant or antiplatelet agents in vitro or ex vivo.

In Vitro Models of Thrombosis

Purpose and Rationale

There is abundant evidence suggesting that platelets play a pivotal role in the pathogenesis of arterial thrombotic disorders, including unstable angina (UA), myocardial infarction (MI), and stroke. The underlying pathophysiological mechanism of these processes has been recognized as the disruption or erosion of a vulnerable atherosclerotic plaque leading to local platelet adhesion

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and subsequent formation of partially or completely occlusive platelet thrombi.

The specific platelet surface receptors that support these initial adhesive interactions are determined by the local fluid dynamic conditions of the vasculature and the extracellular matrix constituents exposed at the sites of vascular injury. Konstantopoulos et al. (1998) and Alveriadou et al. (1993) demonstrated that under high shear conditions, the adhesion of platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is primarily mediated by the platelet glycoprotein (GP)Ib/IX/V complex. This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa. In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels, as shown by Turitto (1982), may be important. Shear-induced platelet aggregation is dependent upon the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation, which also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.

A variety of methods have been utilized to assess the ex vivo and/or in vitro efficacy of platelet antagonists, including photometric aggregometry, whole blood electrical aggregometry, and particle counter methods, as described in the above segments. In photometric aggregometry, a sample is placed in a stirred cuvette in the optical light path between a light source and a light detector. Aggregate formation is monitored by a decrease in turbidity, and the extent of aggregation is measured as percent of maximal light transmission. The major disadvantage of this technique is that it cannot be applied in whole blood since the presence of erythrocytes interferes with the optical responses. Furthermore, it is insensitive to the formation of small aggregates. Particle counters are used to quantitate the size and the number of particles suspended in an electrolyte solution by monitoring the electrical current between two electrodes immersed in the solution. Aggregation in this system is quantitated by counting the platelets before and after stimulation, and it is usually expressed as a percentage of the initial count, as shown by Jen and McIntire (1984). However, the disadvantage of this technique is that it cannot distinguish platelets and platelet aggregates from other blood cells of the same size. Thus, one is limited to counting only a fraction of single platelets, as well as aggregates that are much larger than erythrocytes and leukocytes. The technique of electrical aggregometry allows the detection of platelet aggregates as they attach to electrodes immersed in a stirred cuvette of whole blood or platelet suspensions. Such an attachment results in a decrease in conductance between the two electrodes that can be quantitated in units of electrical resistance. However, a disadvantage of this method is that it is not sensitive in the detection of small aggregates, as demonstrated by Sweeney et al. (1989).

This segment discusses two complementary in vitro flow models of thrombosis that can be used to accurately quantify platelet aggregation in anticoagulated whole blood specimens and to evaluate the inhibitory efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure direct shear-induced platelet aggregation in the bulk phase, as demonstrated by Konstantopoulos et al. (1995), and (2) a parallel-plate perfusion chamber coupled with a computerized videomicroscopy system to quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood flowing over an immobilized substrate (e.g., collagen I), as shown by Konstantopoulos et al. (1995) and Mousa et al. (2002). Furthermore, Mousa et al. (2002) demonstrated a third in vitro flow assay in which surface-anchored platelets are preincubated with a GPIIb/IIIa antagonist, and unbound drug is washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling one to distinguish agents with markedly distinct affinities and receptor-bound lifetimes.

Procedure

Isolation of Human Platelets

The steps described in subsequent subheadings outline the procedure for isolation and purification of platelets from whole blood obtained by venipuncture from human volunteers. Obtain blood sample by venipuncture from an antecubital vein into polypropylene syringes containing either sodium citrate (0.38 % final concentration) or heparin (10 U/ml final concentration). Centrifuge anticoagulated whole blood at 160 × *g* for 15 min to prepare platelet-rich plasma (PRP).

Isolation of Washed Platelets

PRP specimens are subjected to a further centrifugation (1,100 × *g* for 15 min) in the presence of 2 μM PGE_1 (Evangelista et al. 1996).

The platelet pellet is resuspended in HEPES-Tyrode's buffer containing 5 mM EGTA and 2 μM PGE_1.

Platelets are then washed via centrifugation (1,100 × *g* for 10 min), resuspended at 2×10^8 /ml in HEPES-Tyrode's buffer, and kept at room temperature for no longer than 4 h before use in aggregation/adhesion assays.

Materials

- Anticoagulant solution (sodium citrate, porcine heparin, PPACK, etc.)
- Fluorescently labeled platelet-specific antibody
- Dulbecco's phosphate-buffered saline (D-PBS) (with and without Ca^{2+}/Mg^{2+})
- Formaldehyde
- Type I collagen, from bovine Achilles' tendon
- 0.5 mol/L glacial acetic acid in water
- Glass coverslips (24 × 50 mm; Corning; Corning, NY)
- Silicone sheeting (gasket) (0.005-in or 0.010-in thickness; Specialty Manufacturing Inc; Saginaw, MI)
- Quinacrine dihydrocholride
- Prostaglandin E_1 (PGE_1) and EGTA
- Thrombin
- Bovine serum albumin
- HEPES-Tyrode's buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM K_2PO_4, 0.8 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, 10 mM HEPES, 5.6 mM dextrose)
- 3-Aminopropyltriethoxysilane (APES)
- Acetone
- 70 % Nitric acid in water
- THP-1 monocytic cells
- Platelet antagonists such as abciximab

Evaluation

The methods described below outline different dynamic adhesion/aggregation assays used to assess the in vitro and/or ex vivo efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure shear-induced platelet-platelet aggregation in the bulk phase and (2) a perfusion chamber coupled with a computerized videomicroscopy system to visualize in real time and quantify (a) the adhesion and subsequent aggregation of platelets flowing over an immobilized substrate (e.g., extracellular matrix protein) and (b) free-flowing monocytic cell adhesion to immobilized platelets.

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Cone-and-Plate Viscometry Under Shear-Flow Cytometry

Purpose and Rationale

The cone-and-plate viscometer is an in vitro flow model used to investigate the effects of bulk fluid shear stress on suspended cells. Anticoagulated whole blood specimens (or isolated cell suspensions) are placed between the two platens (both of stainless steel) of the viscometer. Rotation of the upper conical platen causes a well-defined and uniform shearing stress to be applied to the entire fluid medium, as described by Konstantopoulos et al. (1998). The shear rate (γ) in this system can be readily calculated from the cone angle and the speed of the cone using the formula

$$\gamma = \left(\frac{2\pi\omega}{60\theta_{cp}}\right)$$

where γ is the shear rate in sec^{-1}, ω is the cone rotational rate in revolutions per min (rev/min), and θ_{cp} is the cone angle in radians. The latter is typically in the range of 0.3–1.0°. The shear stress, τ, is proportional to shear rate, γ, as shown by $\tau = \mu \cdot \gamma$, where μ is the viscosity of the cell suspension (the viscosity of anticoagulated whole blood is ~0.04 cp at 37 °C). This type of rotational viscometer is capable of generating shear stresses from ~2 dyn/cm^2 (venous level) to greater than 200 dyn/cm^2 (stenotic arteries).

Procedure

Single platelets and platelet aggregates generated upon shear exposure of blood specimens are differentiated from other blood cells on the basis of their characteristic forward scatter and fluorescence (by the use of fluorophore-conjugated platelet-specific antibodies) profiles by flow cytometry, as described by Konstantopoulos et al. (1995). This technique requires no washing or centrifugation steps that may induce artifactual platelet activation, and it allows the study of platelet function in the presence of other blood elements. The procedure used to quantify platelet aggregation induced by shear stress is as follows:

- Incubate anticoagulated whole blood with platelet antagonist or vehicle (control) at 37 °C for 10 min.
- Place a blood specimen (typically ~500 μl) on the stationary platen of a cone-and-plate viscometer maintained at 37 °C.
- Take a small aliquot (~3 μl) from the pre-sheared blood sample, and fix it with 1 % formaldehyde in D-PBS (~30 μl).
- Expose the blood specimen, in the presence or absence of a platelet antagonist, to well-defined shear levels (typically 4,000 s^{-1} to induce significant platelet aggregation in the absence of a platelet antagonist) for prescribed periods of time (typically 30–60 s).
- Take a small aliquot (~3 μl) from the sheared blood specimen, and immediately fix it with 1 % formaldehyde in D-PBS (~30 μl).
- Incubate the fixed blood samples with a saturating concentration of a fluorescently labeled

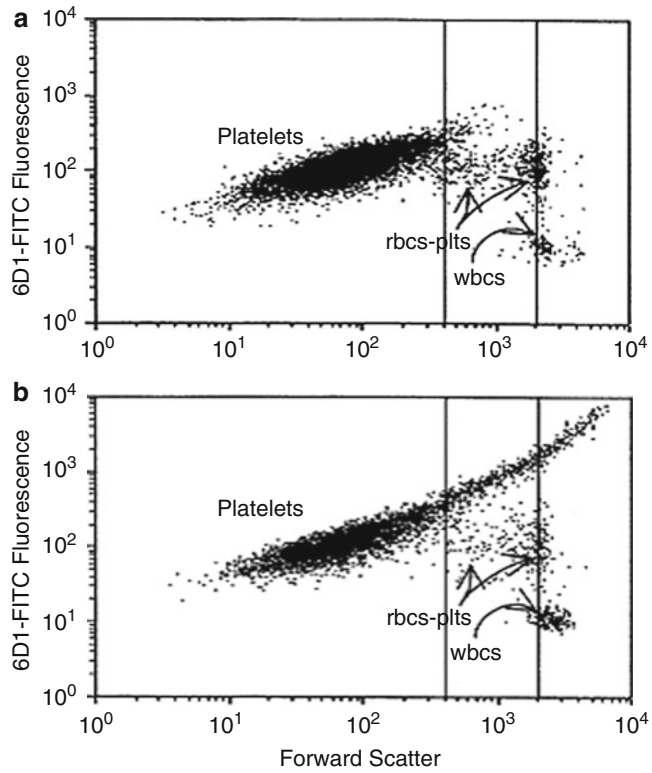

Fig. 1 Quantification of shear-induced platelet aggregation by flow cytometry. *Panel A* corresponds to non-sheared blood specimen. *Panel B* corresponds to a blood specimen that has been subjected to a pathologically high level of shear stress for 30 s. As can be seen in the figure, there are three distinct cell populations. The upper population consists of platelets and platelet aggregates. The "rbcs-plts" population corresponds to platelets associated with erythrocytes and leukocytes. The "wbcs" population consists of some leukocytes that have elevated levels of FITC autofluorescence. The *left vertical line* separates single platelets (≤4.5 μm in diameter) from platelet aggregates, whereas the *right vertical line* separates "small" from "large" platelet aggregates. The latter were defined to be larger than 10 μm in equivalent sphere diameter

platelet-specific antibody, such as anti-GPIb (6D1)-FITC, for 30 min in the dark.

- Dilute specimens with 2 ml of 1 % formaldehyde, and analyze them by flow cytometry.

Flow cytometric analysis is used to distinguish platelets from other blood cells on the basis of their characteristic forward scatter and fluorescence profiles, as shown in Fig. 1. Data acquisition is then carried out on each sample for a set period (usually 100 s), thereby allowing equal volumes for both the pre-sheared and sheared specimens to be achieved. As a result, the percent platelet aggregation can be determined by the disappearance of single platelets into the platelet aggregate region using the formula % *Platelet Aggregation* = (1–*Ns*/*Nc* × 100), where *Ns* represents the single platelet population of the sheared specimen and *Nc* represents the single platelet population of the pre-sheared specimen. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be readily determined.

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Platelet Adhesion and Aggregation Under Dynamic Shear

Purpose and Rationale

The steps described and outlined an in vitro flow model of platelet thrombus formation, which can be used to evaluate the ex vivo and/or in vitro efficacy of platelet antagonists. Thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and von Willebrand factor (vWF), with subsequent platelet activation and aggregation. Konstantopoulos et al. (1995) described the use of a parallel-plate flow chamber that provides a controlled and well-defined flow environment based on the chamber geometry and the flow rate through the chamber. The wall shear stress, τ_w, assuming a Newtonian and incompressible fluid, can be calculated using the formula

$$\tau_W = \frac{6\mu Q}{wh^2}$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, and w is the channel width. A flow chamber typically consists of a transparent polycarbonate block, a gasket whose thickness determines the channel depth, and a glass coverslip coated with an extracellular matrix protein such as type I fibrillar collagen. The apparatus is held together by vacuum. Shear stress is generated by flowing fluid (e.g., anticoagulated whole blood or isolated cell suspensions) through the chamber over the immobilized substrate under controlled kinematic conditions using a syringe pump. Mousa et al. (2002) combined the parallel-plate flow chamber with a computerized epifluorescence videomicroscopy system that enables one to visualize in real time and separately quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood (or isolated platelet suspensions) flowing over an immobilized substrate.

Procedure

Preparation of Collagen-Coated Surfaces

- Dissolve 500 mg collagen type I from bovine Achilles' tendon into 200 ml of 0.5 mol/L acetic acid in water, pH 2.8.
- Homogenize for 3 h.
- Centrifuge the homogenate at 200 × *g* for 10 min, collect supernatant, and measure collagen concentration by a modified Lowry analysis.
- Coat glass coverslips with 200 μl of fibrillar collagen I suspension on all but first 10 mm of the slide length (coated area = 12.7 × 23), and place in a humid environment at 37 °C for 45 min.
- Rinse excess collagen with 10 ml of D-PBS maintained at 37 °C before assembly into the flow chamber (Folie et al. 1988).

Platelet Perfusion Studies

- Add the fluorescent dye quinacrine dihydrochloride to anticoagulated whole blood samples at a final concentration of 10 μM immediately after blood collection.
- Prior to the perfusion experiment, incubate blood with either a platelet antagonist or vehicle (control) at 37 °C for 10 min.
- Perfuse anticoagulated whole blood through the flow chamber for 1 min at wall shear rates ranging from 100 s^{-1} (typical of venous circulation) to 1,500 s^{-1} (mimicking partially constricted arteries) for prescribed periods of time (e.g., 1 min). Platelet-substrate interactions are monitored in real time using an inverted microscope equipped with an epifluorescence illumination attachment and silicon-intensified target video camera and recorded on videotape. The microscope stage and flow chamber are maintained at 37 °C by an incubator heating module and incubator enclosure during the experiment.

Evaluation

Videotaped images are digitized and computer analyzed at 5, 15, and 60 s for each perfusion

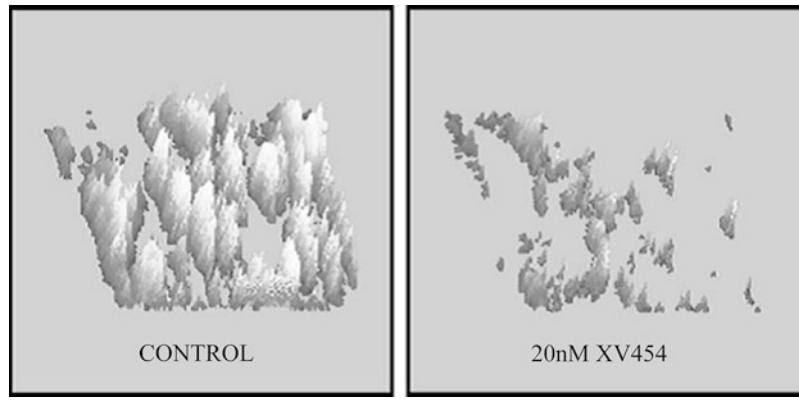

Fig. 2 Three-dimensional computer-generated representation of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused in the absence (control) or presence of a GPIIb/IIIa antagonist (XV454) at 37 °C for 1 min at 1,500 s^{-1}

experiment. The number of adherent individual platelets in the microscopic field of view during the initial 15 s of flow is determined by image processing and used as the measurement of platelet adhesion that initiates platelet thrombus formation. The number of platelets in each individual thrombus is calculated as the total thrombus intensity (area × fluorescence intensity) divided by the average intensity of single platelets determined in the 5-s images. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be determined (Fig. 2). Along these lines, any potential inhibitory effects of a platelet antagonist on platelet adhesion can be readily assessed.

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Cell Adhesion to Immobilized Platelets: Parallel-Plate Flow Chamber

Purpose and Rationale

In this assay, immobilized platelets are pretreated with a GPIIb/IIIa antagonist, and any unbound drug is washed away before the perfusion of monocytic THP-1 cells. Mousa et al. (1998) demonstrated that agents with slow platelet off-rates such as XV454 ($t_{1/2}$ of dissociation = 110 min; K_d = 1 nM) and abciximab ($t_{1/2}$ of dissociation = 40 min; K_d = 9.0 nM) that are distributed predominantly as receptor-bound entities with little unbound in the plasma can effectively block these heterotypic interactions as shown by Abulencia et al. (2001) and by Mousa et al. (2002). In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation = 0.2 min; K_d >110 nM), whose antiplatelet efficacy depends on the plasma concentration of the active drug, do not exhibit any inhibitory effects, as described by Mousa et al. (2002).

Procedure

Preparation of 3-Aminopropyltriethoxysilane (APES)-Treated Glass Slides

- Soak glass coverslips overnight in 70 % nitric acid.
- Wash coverslips with tap water.
- Dry coverslips by washing once with acetone, followed by immersion in a 4 % solution of APES in acetone for 2 min.

- Repeat the step above, followed by a final rinse of the glass coverslips with acetone.
- Wash coverslips 3 times with water, and allow them to dry overnight.

Immobilization of Platelets on 3-APES-Treated Glass Slides

- Layer washed platelets or platelet-rich plasma (2×10^8 cells/ml) on the surface of a coverslip at ~30 μl/cm^2.
- Allow platelets to bind to APES-treated coverslip in a humid environment at 37 °C for 30 min.

Monocytic THP-1 Cell-Platelet Adhesion Assay

- Assemble the platelet-coated coverslip on a parallel-plate flow chamber, which is then mounted on the stage of an inverted microscope equipped with a CCD camera connected to a VCR and TV monitor.
- Perfuse the antiplatelet antagonist at the desirable concentration or vehicle (control) over surface-bound platelets, and incubate for 10 min. The extent of platelet activation can be further modulated by the presence of chemical agonists such as thrombin (0.02–2 U/ml) during the 10 min incubation. The microscope stage and flow chamber are maintained at 37 °C by an incubator heating module and incubator enclosure during the experiment.
- In some experiments, unbound platelet antagonist is removed by a brief washing step (4 min) prior to the perfusion of the cells of interest over the platelet layer. In others, the desirable concentration of the platelet antagonist is continuously maintained in the perfusion buffer during the entire course of the experiment.
- Perfuse cells (e.g., THP-1 monocytic cells, leukocytes, tumor cells, protein-coated beads, etc.) over surface-bound platelets, either in the presence or absence of a platelet antagonist (see above), at the desirable flow rate for prescribed periods of time. THP-1 cell binding to immobilized platelets is monitored in real time and recorded on videotape.
- Determine the extent of THP-1 cell tethering, rolling, and stationary adhesion to immobilized platelets, as well as the average velocity of rolling THP-1 cells. By comparing the corresponding extents of THP-1 cell tethering, rolling, and stationary adhesion to immobilized platelets in the presence and absence of a platelet antagonist (Fig. 3), its antiplatelet efficacy can be determined as shown by Tsao et al. (1995).

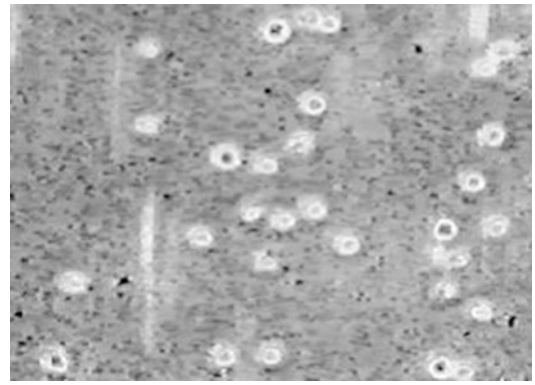

Fig. 3 Phase-contrast photomicrograph of THP-1 cells (phase bright objects) attached to a layer of thrombin-treated platelets (phase dark objects) after THP-1 cell perfusion for 3 min at a shear stress level of 1.5 dyn/cm^2

Evaluation

Low-speed centrifugation results in the separation of platelets (top layer) from larger and denser cells such as leukocytes and erythrocytes (bottom layer). To minimize leukocyte contamination in PRP specimens, slowly aspirate the uppermost two thirds of the platelet layer. Furthermore, certain rare platelet disorders, such as Bernard-Soulier syndrome (BSS), are characterized by larger than normal platelets, which must therefore be isolated by allowing whole blood to gravity separate for 2 h post-venipuncture.

The mechanical force most relevant to platelet-mediated thrombosis is shear stress. The normal time-averaged levels of venous and arterial shear stresses range between 1–5 dyn/cm^2 and 6–40 dyn/cm^2, respectively. However, fluid shear stress may reach levels well over 200 dyn/cm^2 in small arteries and arterioles partially obstructed by atherosclerosis or vascular spasm. The cone-and-plate viscometer and parallel-plate flow chamber are two of the most common devices used to simulate fluid mechanical shearing stress conditions in blood vessels.

Due to the large concentration of platelets and erythrocytes in whole blood, small aliquots (~3 μl) of pre-sheared and post-sheared specimens must be obtained and processed prior to the flow cytometric analysis. This will minimize an artifact produced as a platelet and an erythrocyte pass through the light beam of a flow cytometer at the same time.

The "rbcs-plts" population represents 3–5 % of the displayed cells. A small fraction (~5 %) of this population seems to be leukocyte-platelet aggregates, as evidenced by the use of an anti-CD45 monoclonal antibody. The remaining events correspond to erythrocytes associated with platelets. However, it appears that the majority of the latter population is an artifact generated by the simultaneous passage of a platelet and an erythrocyte through the beam of a flow cytometer. This concept is corroborated by the fact that further dilution of pre-sheared and sheared blood specimens and/or reduction of the sample flow rate during the flow cytometric analysis results in a dramatic relative decrease of the "rbcs-plts" population.

The collagen density remaining on glass coverslips after D-PBS rinsing can be measured by the difference in weight of 20 clean uncoated slides versus 20 collagen-treated slides.

Experiments are optimally monitored ~ 100–200 μm downstream from the collagen/glass interface using a 60 × FLUOR objective and 1 × projection lens, which gives a 3.2×10^4 μm^2 field of view. A field of view closer to the interface may lead to non-reproducible results due to variations in the collagen layering in that region. In contrast, positions farther downstream are avoided in order to minimize the effects of upstream platelet adhesion and subsequent aggregation on both the fluid dynamic environment as well as bulk platelet concentration.

The digitization of a background image (at the onset of perfusion prior to platelet adhesion to the collagen I surface) and its subtraction from a subsequent image acquired 5 s after an initial platelet adhesion event allows the determination of the fluorescence intensity emitted by a single platelet. The intensity level of each single platelet is measured as a mean gray level between 0 (black) and 255 (white) through the use of an image processing software (e.g., OPTIMAS; Agris-Schoen Vision Systems, Alexandria, VA) and is multiplied by its corresponding area (total number of pixels covered by each single platelet). The aforementioned products are then averaged for all single platelet events detected at the 5-s time point, thus enabling us to calculate the average intensity of single platelets.

A single field of view (10×0.55 mm^2) is monitored during the 3 min period of the experiment; at the end, 5 additional fields of view (0.55 mm^2) are monitored for 15 s each. The following parameters can be quantified: (a) the number of total interacting cells per mm^2 during the entire 3 min perfusion experiment; (b) the number of stationary interacting cells per mm^2 after 3 min of shear flow; (c) the percentage of total interacting cells that are stationary after 3 min of shear flow; and (d) the average rolling velocity (μm/s) of interacting cells. The number of interacting cells per mm^2 is determined manually by reviewing the videotapes. Stationary interacting cells per mm^2 are considered as those that move $<$1-cell radius within 10 s at the end of the 3 min attachment assay. To quantify their number, images can be digitized from a videotape recorder using an imaging software package (e.g., OPTIMAS). Rolling velocities can be computed as the distance traveled by the centroid of the rolling THP-1 cell divided by the time interval using image processing.

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In Vivo or Ex Vivo Models of Thromobis

Shaker A. Mousa

Contents

General Introduction

In vitro model systems are limited in assessing the impact of global or systemic factors that propagate coagulation and/or platelet activation. Clinically relevant model systems of thrombosis are crucial in assessing the impact of both local (vascular endothelium, atherosclerosis, and other local factors) and systemic factors responsible for coagulation and platelet activation.

Several in vivo model systems have been used successfully in the preclinical evaluation of novel antiplatelets or anticoagulants. The correlation between blood levels (pharmacokinetics) and antithrombotic efficacy (pharmacodynamics) in vivo and ex vivo provided a predictive dosing simulation in humans in achieving antithrombotic efficacy given the understating of any pharmacogenomic variables.

This chapter highlights commonly used in vivo model systems for the evaluation of antithrombotic strategies.

In Vivo or Ex Vivo Models of Thrombosis

Purpose and Rationale

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of

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blood flow, changes in the properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two, or all three factors of Virchow's triad. Therefore, they differ with respect to the prothrombotic challenge – either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, laser light), insertion of foreign surface, or injection of a prothrombotic factor, and they differ with respect to the vessel type and the animal species.

Roughly, two types of models can be differentiated (Didisheim 1972; Kaiser et al. 1999; Mousa et al. 1998; Perzborn et al. 2005): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor, resulting in fibrin-rich "red" venous type thrombi, and (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis, resulting in platelet-rich "white" mural thrombi. But the differentiation is not strict because platelets and the coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in humans, namely, atherosclerosis or thrombophilias, are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. In spite of these limitations, animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a review by Leadley et al. (2000). Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires preclinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that the second- and third-generation antithrombotic drugs are devoid of in vitro anticoagulant effects, yet in vivo, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit in vitro anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models using species such as rats, rabbits, dogs, pigs, and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat, and guinea pig have also been used. Species variations are an important consideration in selecting a model and interpreting the results because these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thromboses have been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models are well established and can be used for the development of antithrombotic drugs. It is also possible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of in vitro potency, the endogenous effect of antithrombotic drugs can also be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used, including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases, and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models, in particular, have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor

antibodies relies largely on these models. These models are, however, of pivotal value in the development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human usage.

Procedure

Animal Models of Thrombosis

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiologic) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

I. **Stasis-thrombosis model:** Since its introduction by Wessler et al. (1959), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents. This model has also been adapted for use in rats (Meuleman et al. 1991). In the stasis-thrombosis model, a hypercoagulable state is mimicked by the administration of one of a number of thrombogenic challenges, including human serum (Carrie et al. 1994), thromboplastin (Walenga et al. 1986), activated prothrombin complex concentrates (Vlasuk et al. 1991), factor Xa (Millet et al. 1994), and recombinant relipidated tissue factor (Callas et al. 1995). This administration serves to produce a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a thrombus.

II. **Models based on vessel wall damage:** The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a non-thrombogenic surface over which the blood flows. Disruption of the endothelium not only limits the beneficial effects enumerated above but also exposes subendothelial tissue factor and collagen that serve to activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means (clamping, catheter), chemical means (FITC, rose bengal, ferrous chloride), thermal injury, or electrolytic injury.

Evaluation

Each setting in the design of an animal model can answer a specific question in relation to certain thrombotic disorders in humans. However, the ultimate model of human thrombosis is in humans.

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Stenosis- and Mechanical Injury-Induced Coronary Thrombosis: Folts Model

Purpose and Rationale

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction, or sudden death. Treatment with angioplasty, thrombolysis, or bypass grafts can expose new thrombogenic surfaces and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFRs) in stenosed canine coronary arteries. Uchida et al. described a similar model in 1975. The model includes various aspects of unstable angina pectoris (i.e., critical stenosis, vascular damage, downstream vasospasm induced by vasoconstrictors released or generated by platelets). The cyclic variations in coronary blood flow are a result of acute platelet thrombi that may occlude the vessel but that either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts et al. 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (Folts and Rowe 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

Coronary Thrombosis Induced by Stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. CFRs, which are alterations in coronary blood flow, are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder

upon the damaged part of the vessel. In dogs, the stenosis is critical, i.e., the reactive hyperemic response to a 10-s occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i.e., there is a partial reactive hyperemia left (Just and Schönafinger 1991; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis are not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required, leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet-activating factor (PAF), according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

Coronary Thrombosis Induced by Electrical Stimulation

In this preparation, coronary thrombosis is induced by delivery of low-amperage electrical current to the intimal surface of the artery, according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

Procedure

Coronary Thrombosis Induced by Stenosis

Protocol 1: Critical Stenosis. Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approx. 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericardium is opened, and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flow probe, the vessel is squeezed with a 2-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2–1.8 mm (depending on the size of the LCX), is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (two to five times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly repeated CFRs of similar intensity within a pretreatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion or by intraduodenal application. CFRs are registered for 2–4 × 60 min and compared to pretreatment values.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical Stenosis. Male castrated pigs (German landrace, weighing 20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.), and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericardium is opened, and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flow probe, the vessel is squeezed with a 1-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the

constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces cyclic flow reductions. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at reduction of blood flow by lifting the vessel with forceps for a short time.

Only pigs with regularly repeated CFRs of similar intensity within a pretreatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion or by intraduodenal application. CFRs are registered for 2 × 60 min and compared to pretreatment values.

Protocol 3: Stenosis plus Epinephrine Infusion. If protocol 1 does not lead to CFRs, additionally epinephrine (0.2 μg/kg/min) is infused into a peripheral vein twice over 60 min (60 min before and 60 min following drug administration). CFRs are registered and compared in the 60 min postdrug phase to the 60 min predrug phase.

Protocol 4: Stenosis plus PAF Infusion. The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFRs, PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into a cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty minutes later, the test substance is concomitantly administered and a second PAF infusion is started for 30 min.

CFRs are registered and compared in the drug-treated, second PAF phase to the predrug, first PAF phase.

Coronary Thrombosis Induced by Electrical Stimulation

Protocol 5: The LCX is punctuated distal to the flow probe with a chrome–vanadium-steel electrode (3-mm length, 1-mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon-coated wire to a 9-V battery, a potentiometer, and an amperemeter. A disk electrode (cathode) is secured to a subcutaneous thoracic muscle layer to complete the electrical circuit. The intima is stimulated with 150 μA for 6 h. During this time, an occluding thrombosis is gradually formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below) (Fig. 1).

For all protocols, the following preparations and measurements are performed:

- To measure peripheral arterial blood pressure (BP [mmHg]), the right femoral artery is cannulated and connected to a Statham pressure transducer.
- Left ventricular pressure (LVP [mmHg]) is determined by inserting a microtip catheter via the carotid artery retrogradely.
- Left ventricular end-diastolic pressure (LVEDP [mmHg]) is evaluated through sensitive amplification of the LVP.
- Contractility (LV dp/dt max [mmHg/s]) is determined from the initial slope of the LVP curve.
- Heart rate (min^{-1}) is determined from the pulsatile blood pressure curve.
- The ECG is recorded in lead II.
- Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
- Blood hematocrit values (37–40 %) and a number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.
- Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
- Template buccal mucosal bleeding time is carried out using the SimPlate device.

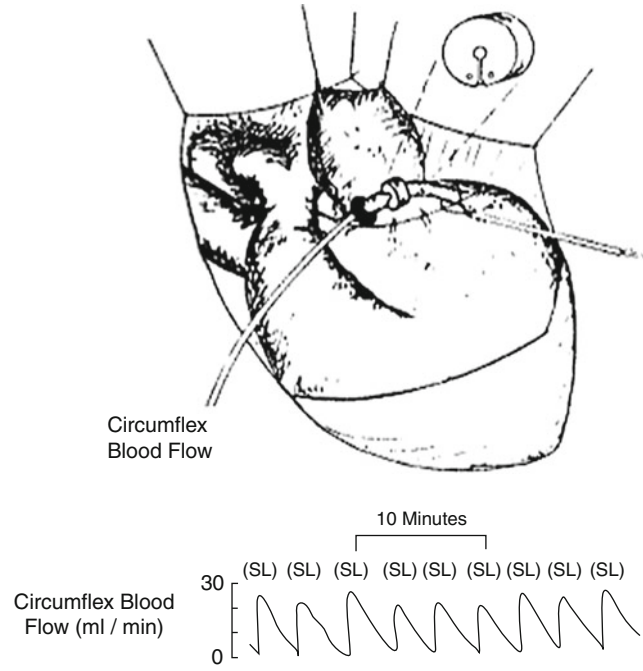

Fig. 1 Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes measure blood flow. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2–3 mm the thrombus must be mechanically shaken loose to restore blood. For detailed application of the Folts model, see Folts and Rowe (1974, 1988) and Folts et al. (1976, 1982)

Evaluation

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic [mmHg]) is determined.

Protocols 1–4: The following parameters are measured to quantify stenosis-induced coronary thrombosis:

- Frequency of CFRs = cycle number per time.
- Magnitude of CFRs = cycle area (mm^2).

Percent change in cycle number and cycle area after drug treatment is calculated compared to pretreatment controls.

Statistical significance is assessed by the paired Student's *t*-test.

Protocol 5: The following parameters are measured to quantify electrically induced coronary thrombosis:

- Occlusion time (min) = time to zero blood flow.
- Thrombus size (mg) = wet weight of the thrombus immediately after removal.

Percent change in mean values for occlusion time and thrombus size in drug-treated groups is compared to the control group.

Statistical significance is assessed by the non-paired Student's *t*-test.

Critical Assessment of the Method

Both the stenosis (Folts) and the electrical (Romson/Lucchesi) models of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e.g., thromboxane, prostacyclin, cyclooxygenase, serotonin, NO donors, and other vasodilators. The effect of an NO donor could be reversed by the NO-scavenger oxyhemoglobin indication that indeed NO was responsible for the antithrombotic action (Just and Schönafinger 1991). Recent mechanisms of antithrombotic drug action that have been studied in either of the two coronary thrombosis models are the oral GP IIb/IIIa antagonist DMP 728 (Mousa et al. 1996); the

low-molecular-weight heparin (LMWH) enoxaparin (Leadley et al. 1998), which inhibited CFRs in contrast to unfractionated heparin; the thrombin inhibitors, PEG-hirudin (Ruebsamen and Kirchengast 1998) and melagatran (Mehta et al. 1998); an anti-P-selectin antibody (Ikeda et al. 1999); and an activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive: e.g., aspirin has no effect, and with some drugs higher dose levels are required; however, in principle, most drug mechanisms act in both models if at all.

Modifications of the Method

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low-amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50 % increase in flow velocity, and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion.

Warltier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis, as well as the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size.

Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts thrombosis has also been applied to carotid arteries in monkeys. Coller et al. (1989) induced CFRs in carotid arteries of anesthetized cynomolgus monkeys and showed abolition by the GP IIb/IIIa antibody abciximab.

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Stenosis- and Mechanical Injury-Induced Arterial and Venous Thrombosis: Harbauer-Model

Purpose and Rationale

Harbauer et al. (1984) and Harbauer and Allendorf (1988) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thromboses are produced in rabbits by the stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an in vivo model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

Procedure

Male chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, intravenous, or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approx. 30 min, p.o. approx. 60 min, i.v. variable).

Sixty-five minutes before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein. A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube. One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion. Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow (ml/min) is measured continuously. After reaching steady state (approx. 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approx. 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. Then a small plastic constricting cylinder 1.2-mm wide and 2-mm long is placed around the site of the endothelial damage.

In addition, the template bleeding time is measured at various time intervals before and after drug treatment (depending on the route of administration) in the shaved inner ear using the SimPlate device. Care is taken to select parts of the skin without larger vessels.

Evaluation

Percent thrombus formation (thrombosis incidence) is judged by measurement of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls. Thrombosis incidence is always 100 % in vehicle controls.

Statistical significance is assessed by means of the Fisher exact test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition, and mean values in dosed groups are compared to controls by means of the unpaired Student's *t*-test. Mean values of occlusion times [min] in dosage and control groups are calculated and compared by means of the *t*-test.

The maximal change in systolic and diastolic blood pressure during the period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mmHg and of diastolic blood pressure by 20 mmHg is quoted as a strong reduction in blood pressure.

Critical Assessment of the Method

Two main factors of arterial thrombosis are essential in this model: high-grade stenosis and vessel wall damage. In the absence of either, no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels thrombus formation is equally dependent on platelet function, as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep-vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model because carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: (i) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, and NO donors (SNP, molsidomine) but not aspirin and thromboxane-synthase inhibitors; (ii) anticoagulants like hirudin, high-dose heparin, and warfarin; and (iii) streptokinase/tissue plasminogen activator (t-PA) (Bevilacqua et al. 1991; Just 1986). In contrast, drugs that only lower blood pressure – such as hydralazine, clonidine, and prazosin – have no effect on thrombus formation in this model.

Modifications of the Method

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH2Cl,

iloprost, and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected and the clot removed for determination of dry weight.

Lyle et al. (1995) searched for an animal model mimicking the thrombotic reocclusion and restenosis occurring in several cases after successful coronary angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ^{111}indium-labeled platelet deposition and thrombosis were assessed 4 h after balloon injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Thrombosis Induced by Cooling

Lindenblatt et al. (2005), Meng (1975), Meng and Seuter (1977), and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized; the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove that was cooled to −15 °C. The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was −12 °C for a period of 5 min, and the compressing weight was 500 g. The wound was closed, and the animal was allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 h, the animals received heparin and were re-anesthetized. The lesioned carotid artery was removed and thrombus wet weight was immediately measured.

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Electrical-Induced Thrombosis

Purpose and Rationale

The use of electrical current to induce thrombosis in hamsters and dogs has been described in the early 1950s by Lutz et al. (1951), Sawyer and Pate (1953), and Sawyer et al. (1953). In general, two different approaches exist. One method produces electrical damage by means of two externally applied hooklike electrodes (Hladovec 1973; Philp et al. 1978). The other method uses a needle electrode that is advanced through the walls of the blood vessels and positioned in their lumen; the second electrode is placed into a subcutaneous site completing the circuit (Salazah 1961; Romson et al. 1980; Benedict et al. 1986).

Procedure

Anesthetized rats weighing 200–300 g are intubated, and a femoral artery is cannulated for the administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm.

A pair of rigid stainless-steel-wire hooklike electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flow meter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

Evaluation

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
- Patency of the blood vessel over 30 min

Critical Assessment of the Method

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed, and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. He concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

Modifications of the Method

The technique described by Salazah (1961) uses a stainless-steel electrode that is inserted into a coronary artery in the dog and that delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control, which complicates the method. The technique was modified by Romson et al. (1980). They placed the electrode directly into the coronary artery of opened-chest anesthetized dogs.

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flowmeter was placed on each common carotid artery proximal to the point of insertion of both an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25 % without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V

nickel–cadmium battery. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by the application of a 150-μA continuous pulse anodal direct current to the intimal surface of the vessel for a maximum duration of 3 h or for 30 min beyond the time of complete vessel occlusion, as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50 %, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 h after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996). A 10-min anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle-treated ferrets within 41 $\pm$ 3 min, with an average weight of 8 $\pm$ 1 mg. Thrombus weight was reduced by aspirin or a thromboxane receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

Sturgeon et al. (2006) adapted the Folts and the electric methods of arterial thrombosis in small animals. Mousa et al. (1999) used the same animal pre- and posttreatment by using left and right arterial sides.

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$FeCl_3$-Induced Thrombosis

Purpose and Rationale

A variety of chemical agents has been used to induce thrombosis in animals. Topical $FeCl_3$ was described by Reimann-Hunziger (1944) and recently by Wang et al. (2006) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evaluation of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

Procedure

Rats weighing between 250 and 300 g are anesthetized with Inactin (100 mg/kg), and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for administration of test agents. The right carotid artery is isolated and an ultrasonic Doppler flow probe (probe 1RB, Transonic, Ithaca, NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm "M" (American Can Co., Greenwich, CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2 × 5 mm), saturated with 25 % $FeCl_3$ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

Evaluation

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between $FeCl_3$ application and the time at which blood flow decreases under 0.3 ml/min
- Thrombus weight after blotting the thrombus on filter paper

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Thrombin-Induced Clot Formation in Canine Coronary Artery

Purpose and Rationale

A canine model of thrombin-induced clot formation was developed by Gold et al. (1984) in which localized coronary thrombosis was produced in

the LAD. This is a variation of the technique described by Collen et al. (1983) who used radioactively labeled fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular vein clots. The vessel was intentionally de-endothelialized by external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later, and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 h, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

Procedure

In the initial study described by Gold et al. (1984), recombinant t-PA was characterized for its ability to lyse 2-h-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 μg/kg/min, i.v, and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a_2-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (1988) demonstrated that streptokinase elicited dose-dependent thrombolysis in this model.

Subsequently, Gold et al. (1986, 1988) modified the model to study not only reperfusion but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45 % of patients (Goldberg et al. 1985). Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

Thrombin-Induced Rabbit Femoral Artery Thrombosis: Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch (Shebuski et al. 1988).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (FABF). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, $CaCl_2$ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 min, the snares are released and FABF is monitored for 30 min to confirm total obstruction of flow by the thrombus.

Evaluation

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent reocclusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (1983) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion (Yasuda et al. 1988). These actions in vivo were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

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Laser-Induced Thrombosis

Purpose and Rationale

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam-lysed erythrocytes because erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary aggregation stimulus following the release reaction induced by the platelets themselves.

Procedure

Apparatus

- 4-W argon laser (Spectra Physics, Darmstadt, FRG); wavelength, 514.5 nm; energy below the objective, 15 mW; duration of exposure, 1/30 or 1/15 s
- Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
- Video camera (Sony, Trinicon tube)
- Recorder (Sony, U-matic 3/4″)
- Video analyzer and correlator to determine blood flow velocity

In Vivo Experiment

Male Sprague–Dawley or spontaneously hypertensive stroke-prone Wistar or Lewis rats with adjuvant-induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anesthetized by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60 or 90 min post dosing. Investigations are performed in arterioles or venules of 13 ± 1 μm in diameter of the fat-free ileocecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37 °C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 s. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of 13 μm or an area of at least 25 μm^2 are evaluated. All measuring procedures are photographed by a video system.

Standard compounds:

- Acetylsalicylic acid (10 mg/kg, per os)
- Pentoxifylline (10 mg/kg, per os)

For detailed description and evaluation of various agents and mechanisms, see the following references: Arfors et al. (1968), Herrmann (1983), Seiffge and Kremer (1984, 1986), Seiffge and Weithmann (1987), and Weichert et al. (1983).

Evaluation

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated.

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Photochemical-Induced Thrombosis

Purpose and Rationale

In 1977, Rosenblum and El-Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the photochemical reaction. He postulated that by exciting the intravascularly administrated fluorescein, singlet oxygen damages endothelial cells, which subsequently leads to platelet adhesion and aggregation.

Procedure

Studies are performed in mesenteric arteries of 15–30-μm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate–dextran 70 (FITC–dextran, Sigma, 10 %, 0.3 ml), the FITC–dextran in arterioles is exposed to ultraviolet light (wavelength of excitation, 490 nm; wavelength of emission, 510 nm).

Evaluation

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

Critical Assessment of the Method

In contrast to other thrombosis induction methods, photochemically induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets; however, the primary target of the photochemical insult is the endothelial cells by means of oxygen radical damage.

Modifications of the Method

Matsuno et al. (1991) report a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wavelength: 540 nm). Blood flow is monitored by a pulsed Doppler flowmeter. Occlusion is achieved after approximately 5–6 min. Pretreatment with heparin dose dependently prolongs the time required to interrupt the blood flow. The model also enables one to study thrombolytic mechanisms, which had been evaluated with t-PA. A comparative data for hirudin in various models was carried out by Just et al. (1991).

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Foreign-Surface-Induced Thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

Wire Coil-Induced Thrombosis

Purpose and Rationale

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in aorta of dogs and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991; Mellot et al. 1993; Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless-steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min, followed by a steady state between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 h following implantation of the wire coil. Thrombosis incidence in untreated control animals in this model is 100 %. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an in vivo model of venous thrombosis in rats.

Procedure

Male Sprague–Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous, or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by intraperitoneal injection of

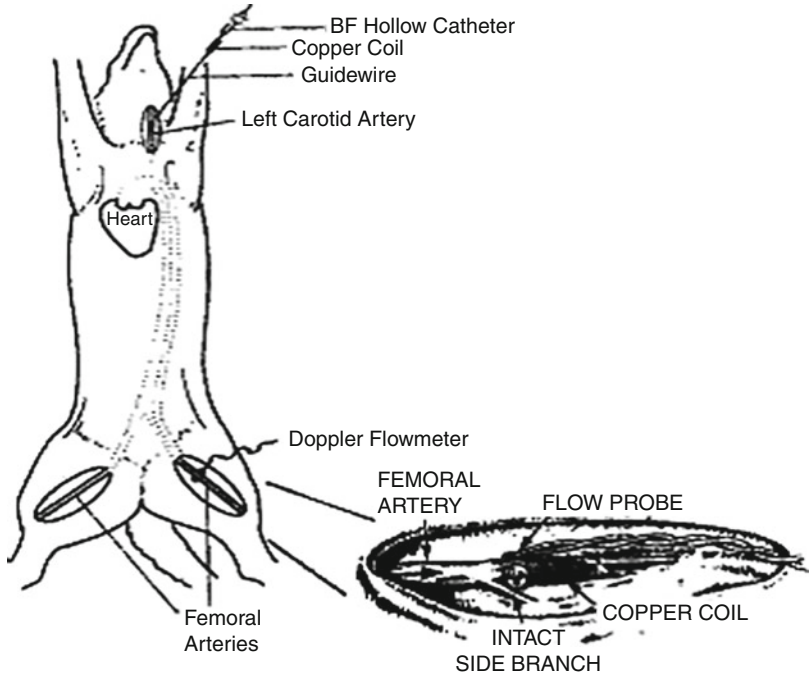

Fig. 2 Schematic diagram of the canine femoral artery copper coil model of thrombolysis. A thrombogenic copper coil is advanced to either femoral artery via the left carotid artery. By virtue of the favorable anatomical angles of attachment, a hollow polyurethane catheter advanced down the left carotid artery nearly always enters the descending aorta, and with further advancement, into either femoral artery without fluoroscopic guidance. A flexible, Teflon-coated guide wire is then inserted through the hollow catheter and the latter is removed. A copper coil is then slipped over the guide wire and advanced to the femoral artery (see inset). Femoral artery flow velocity is measured directly and continuously with a Doppler flow probe placed just proximal to the thrombogenic coil and distal to a prominent side branch, which is left patent to dissipate any dead space between the coil and the next proximal side branch. Femoral artery blood flow declines progressively to total occlusion over the next 10–12 mm after coil insertion

1.3 g/kg urethane. Through a midline incision, the caudal caval vein is exposed and a stainless-steel wire coil (a dental pate carrier, Zipperer size 40 (st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire toward the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37 °C). The wound is reopened after 2 h; the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9 % saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2 % Na_2CO_3 in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry. See figure below (Fig. 2).

Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution, a polyethylene catheter is inserted in the jugular vein. One and a half hours after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 h. The wire coil is then removed and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

Evaluation

Thrombosis incidence (number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content (mg) of the thrombotic material in dosage groups and vehicle

controls is determined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's *t*-test.

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Eversion Graft-Induced Thrombosis

Purpose and Rationale

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4–6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted, and then reimplanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly, leading to complete occlusion of the vessel. This model mimics a deep arterial injury because the adventitial surface is a non-endothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

Procedure

In anesthetized New Zealand white rabbits, the right carotid artery is exposed. After double ligation, a 3-mm segment of the artery is excised, everted, and immersed in pre-warmed (37 °C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double-occluder (2-cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9–0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flowmeter (Model T106, Transonic, Ithaca, NY, USA). The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right jugular vein. Ten minutes after substance administration, the vessel clamps are released and the blood flow is monitored by the flowmeter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration) and at 10, 60, and 120 min after substance administration.

Evaluation

- Time until occlusion (time after restoring of vessel blood flow until occlusion of the vessel, indicated by a flow less than 3.0 ml/min)
- Patency (time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds)

Statistical Analysis
Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p < 0.05$) are calculated by the nonparametric Kruskal–Wallis test.

Critical Assessment of the Method
The eversion graft is very thrombogenic, although technically difficult and time consuming. The deep occlusive thrombi can be prevented only by intra-arterially administered thrombolytics or aggressive antithrombotic treatments such as recombinant hirudin at high dosages or PEG-hirudin. The adventitial surface is a non-endothelial tissue containing tissue factor and collagen. Thus, both the coagulation system and blood platelets are activated.

Modifications of the Method
Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial obstructed left circumflex coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal non-endothelial surface, produces total thrombotic occlusion within 5 min.

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Arteriovenous Shunt Thrombosis

Purpose and Rationale
A method for the direct observation of extracorporeal thrombus formation was introduced by Rowntree and Shionoya (1927) and extensively utilized by others (Rukshin et al. 2003; Tang et al. 2003). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. The A–V-shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs, cats (Best et al. 1938), and nonhuman primates (Yokoyama et al. 1995).

Procedure
Rats are anesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2-cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps that are occluding the A–V-shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCrNi thermocouple, which is fixed distal to the glass capillary. If blood is flowing, the temperature rises from room temperature to body temperature. In contrast, decreases of temperature indicate the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

Critical Assessment of the Method
It has been shown by Best et al. (1938) that the thrombi formed in the A–V-shunt are to a greater part white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts; in those cases, the thrombi tend to be more arterial in character (Chi et al. 1999).

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Thread-Induced Venous Thrombosis

Purpose and Rationale

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducing cotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it, growing to a maximum mass after 2–3 h. The prolonged nonocclusive character of thrombogenesis in this model focuses on the progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

Procedure

Rabbits weighing between 2.5 and 3.5 kg are anesthetized with isoflurane inhalation anesthesia, and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240; inner diameter, 1.67 mm) of 14 cm length is filled with isotonic saline, and a copper wire with five fixed cotton threads (length, 6 cm) is inserted into the tube (after the determination of the net weight of the cotton threads). A laparotomy is performed and the vena cava and iliac vein are dissected free from the surrounded tissue. The test agent is administered by a rabbit intragastric tube 60 min (depending on the ex vivo study) prior to the initiation of thrombus formation. Blood samples are measured at 60, 90, 120, 150, and 210 min after oral administration of the test compound.

Thrombus formation is induced by the inserting the thrombosis catheter into the caval vein via the iliac vein (7 cm). Then the copper wire is pushed forward 3 cm to liberate the cotton

threads into the vessel lumen. One hundred and fifty minutes after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed and longitudinally opened and the content blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

Evaluation

- Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread
- Mean arterial blood pressure
- APTT, Hep test, anti-FIIa, and anti-FXa activity

Critical Assessment of the Method

The composition of the cotton-threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes, thus being in accordance with human deep-vein thrombosis structure. Nonocclusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors, and thrombin inhibitors (Hollenbach et al. 1994, 1995).

Modifications of the Method

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

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Thrombus Formation on Superfused Tendon

Purpose and Rationale

In all models that include vessel wall damage, blood gets in contact with adhesive proteins of the subendothelial matrix, i.e., von Willebrand factor, collagens, fibronectin, laminin, and others. Gryglewski et al. (1978) described an in vivo method where blood of an unanesthetized animal is in contact ex vivo with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs basd on the formation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

Procedure

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37 °C), blood was separated into two streams, each flowing at a speed of 3 ml/min superfusing in parallel two twin strips of the central part of longitudinally cut rabbit Achilles tendon (30 × 3 mm). The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously

recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

Evaluation

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of anti-aggregatory activity.

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Stasis-Induced Thrombosis (Wessler Model)

Purpose and Rationale

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952, 1953, 1955a, b, 1957) and Wessler et al. (1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances that can be used as procoagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

Procedure

Anesthetized rabbits are fixed in supine position on a temperature-controlled (37 °C) heating table. Following cannulation of both carotid arteries (the left in cranial direction) and the right femoral vein, segments of 2-cm length of the two external jugular veins are exposed and isolated between two loose sutures. Then, 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 s followed by injection of 0.5-ml physiological saline within 15 s; 45 s later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 s before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

Evaluation

The size of the clots is assessed using a score system (0, blood only; 1, very small clot piece [s], filling out at most 1/4 of the vessel; 2, larger clot piece[s], filling out at most 1/2 of the vessel; 3, very large clot[s], filling out at most 3/4 of the vessel; 4, one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added, forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum–maximum). Thrombus weight is given as mean ± SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric *U*-Test of Mann and Whitney (thrombus score) or Student's *t*-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p < 0.05$.

Critical Assessment of the Method

Breddin (1989) described the Wessler model because of its static character as the retransformation of an in vitro experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation in vivo. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

Modifications of the Method

There are a number of different procoagulant agents that had been used to induce thrombosis in this model, such as human serum, Russell viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates, and factor X_a (Aronson and Thomas 1985; Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem, some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or post-thrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal antihuman PAI-1 antibody, and Biemond et al. (1996) compared the effect of thrombin and factor X_a inhibitors with a low-molecular-weight heparin.

Venous Reperfusion Model: New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue, and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ^{131}I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). Then, 150 µl of this blood is aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol $CaCl_2$, and 200 µl of the clotting blood is immediately injected into the isolated segment. The vessel clamps are removed 30 min after clot injection, and blood flow is restored. ^{125}I-Radiolabeled fibrinogen (approximately 5 µCi) is injected through the cannula in the carotid artery (in case of the fibrinolysis studies immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ^{131}I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ^{125}I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed as variance analysis and the Newman–Keuls test. Statistical significance is expressed at the level of $p < 0.05$.

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Disseminated Intravascular Coagulation (DIC) Model

Purpose and Rationale

DIC is another model that is also used widely in rats and mice. It is a model of systemic thrombosis or disseminated intravascular coagulation (DIC), which is induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (Herbert et al. 1996; Yamazaki et al. 1994; Sato et al. 1998). After systemic administration of the thrombogenic stimulus, this model can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is the thrombus mass, but when stasis is not used, the readouts are fibrin degradation products, fibrinogen, platelet count, PT, and APTT, among others. As shown by the many and varied parameters, when used without stenosis, the post-experimental analysis can be time consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations such as the ability to withdraw multiple blood samples over the course of the experiment and the difference in activity of at least some FXa inhibitors in human compared to rat plasma in vitro require that compounds be characterized further in more advanced in vivo models of thrombosis.

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Microvascular Thrombosis in Trauma Models

Purpose and Rationale

Successful replantation of amputated extremities is dependent in large degree on maintaining the microcirculation. A number of models have been developed in which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (Fu et al. 1997; Korompilias et al. 1997; Stockmans et al. 1997). In the model of Stockmans et al. (1997), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1,500 g/mm^2, is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for 30 s as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over 35 min by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias et al. (1997) and Fu et al. (1997) examine the formation of arterial

thrombosis in rats and rabbits, respectively. In these models, either the rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points post-anastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

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Cardiopulmonary Bypass Models

Purpose and Rationale

Cardiopulmonary bypass (CPB) models have been described in baboons (Van Wyk et al. 1998), swine (Dewanjee et al. 1996), and dogs (Henny et al. 1985). In each model, the variables that can affect the hemostatic system – such as anesthesia, shear stresses caused by the CPB pumps, and the exposure of plasma components and blood cells to foreign surfaces (catheters, oxygenators, etc.) – are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet functions are altered. The effectiveness of direct thrombin inhibitors (Van Wyk et al. 1998), LMWHs (Murray 1985), and heparinoids (Henny et al. 1985) has been compared to standard heparin. Endpoints have included the measurement of plasmatic anticoagulant levels, the histological determination of microthrombi deposition in various organs, the formation of blood clots in the components of the extracorporeal circuit, and the deposition of radiolabeled platelets in various organs and on the components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatibility of components used to maintain extracorporeal circulation. For detailed protocols and evaluations, see Callas et al. (1995), Carrie et al. (1994), Fu et al. (1997), Korompilias et al. (1997), Meuleman et al. (1991), Millet et al. (1994), Stockmans et al. (1997), Vlasuk et al. (1991), Walenga et al. (1987), and Wessler et al. (1959).

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Extracorporeal Thrombosis Models

Purpose and Rationale

These models employ passing blood over a section of damaged vessel (or other selected substrates) and recording the thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (Badimon and Badimon 1989). This model is interesting because the results can be directly compared to the in vivo deep arterial injury model (Wysokinski et al. 1996) results and to results from a similar extracorporeal model used in humans (Dangas et al. 1998; Ørvim et al. 1995). Dangas et al. (1998) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet glycoprotein IIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvim et al. (1995) also used this model in humans to evaluate the antithrombotic efficacy of rTAP, but instead of evaluating the compound after administration of rTAP to the patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue-factor-coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant. Although this model does not completely represent

pathological intravascular thrombus formation, the use of this "human model" of thrombosis may be very useful in developing new drugs because it directly evaluates the ex vivo antithrombotic effect of a drug in flowing human blood.

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Experimental Thrombocytopenia or Leukocytopenia

Purpose and Rationale

Intravenous administration of collagen, arachidonic acid, ADP, platelet-activating factor (PAF), or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravasal aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of the agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leukocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leukocytopenia as a consequence of in vivo platelet or leukocyte stimulation.

Procedure

Male guinea pigs (Pirbright white) weighing 300–600 g or male NMRI mice (25–36 g) or chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun (i.m.) and placed on an electrically warmed table at 37 °C. The carotid artery is cannulated for blood withdrawal, and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen + adrenaline (injection of the mixture of both within 10 s) or PAF or thrombin. In mice, collagen + adrenaline are injected into a tail vein.

Approximately 50–100 µl blood is collected into potassium-EDTA-coated tubes at times −1, 1, and 2 min (guinea pigs and mice) or 5, 10, and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 h after withdrawal in 10-µl samples of whole blood using a microcell counter suitable for the blood of various animal species.

Evaluation

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100 %.

Percent inhibition of thrombocytopenia (or leukocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

Critical Assessment of the Method

The method of collagen plus epinephrine-induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis/thrombosis. A recent example is the *Gas* 6 −/− mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G-protein G(z) (Yang et al. 2000). The advantage of the method for this purpose is the simple experimental procedure and the small volume of blood necessary. In general, application of the method in small animals (mice, hamsters) needs only small amounts of drug substance. The model is a useful first step of in vivo antithrombotic efficacy of antiplatelet drugs.

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Collagenase-Induced Thrombocytopenia

Purpose and Rationale

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before.

Procedure

Male Sprague–Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin, and 20 min later, approximately 100 µl blood is collected (initial value). Ten minutes later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20, and 30 min following the injection of collagenase, samples of approximately 100 µl blood are collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10-µl samples of whole blood within 1 h after blood withdrawal, using a microcell counter. See Völkl and Dierichs (1986) for details.

Evaluation

The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set at 100 %.

Percent inhibition of thrombocytopenia is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

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Reversible Intravital Aggregation of Platelets

Purpose and Rationale

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation in vivo. Adenosine diphosphate (ADP), platelet-activating factor (PAF), arachidonic acid, thrombin, and collagen are known to induce platelet aggregation. In the following procedure, labeled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation-promoting agents produces an increase in counts in A and a decrease in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The in vivo method can be used to evaluate platelet anti-aggregatory properties of test compounds.

Procedure

Preparation of Labeled Platelets

Blood is obtained from rats by cardio-puncture. After centrifugation at 240 × *g* for 10 min, the platelet-rich plasma (PRP) is transferred into a tube and suspended in calcium-free Tyrode solution containing 250 ng/ml PGE_1. The suspension is centrifuged at 640 × *g* for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium-free Tyrode solution containing 250 ng/ml PGE_1. ^{51}Cr is added to 1 ml of the platelet suspension. Following a 20-min incubation period at 37 °C, the suspension is again centrifuged at 640 × *g* for 10 min. The supernatant is removed, and the labeled platelets are finally resuspended in 1 ml calcium-free Tyrode solution containing 250 ng/ml PGE_1.

In Vivo Experiment

Male Sprague–Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoral is exposed and cannulated. The labeled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual-channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One hour after administration of labeled platelets (when counts in A and B have stabilized), the aggregation-promoting agent (ADP, PAF, arachidonic acid, thrombin, or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 h after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin) or 1 h (collagen) after compound administration, another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

Evaluation

The microcomputer continuously reveals information about aggregation and desegregation of labeled platelets.

The following parameters are recorded:

A = counts over the thorax
B = counts over the abdomen
Difference: $A–B$
Ratio: A/B

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's *t*-test.

Modifications of the Method

Oyekan and Botting (1986) described a method for monitoring platelet aggregation in vivo in rats, using platelets labeled with indium^{3+} oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected

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In Vitro and Ex Vivo Tests of Coagulation and Platelet Function

Shaker A. Mousa

Contents

General Introduction

Thrombosis could be disposed by either local or global factors. Global factors are associated with systemic changes in blood composition, which can be monitored using laboratory tests. In contrast, local factors including vessel wall damage, atherosclerotic plaque, or blood flow stagnation are beyond any functional in vitro laboratory assays but can be monitored in vivo via imaging modalities or specific biomarkers.

Significant advances toward the discovery of specific anticoagulants and antiplatelets were developed over the past two decades, which owed to the early discovery of heparin, warfarin, and aspirin. Advances in understanding heparin anticoagulant mechanisms led to a better understanding of the coagulation cascade because heparin affects the coagulation cascade at various levels (Fig. 1).

It took over 55 years since the introduction of warfarin to introduce novel oral anticoagulants specifically against oral anti-Xa and oral anti-IIa (Figs. 2 and 3).

Similarly, various adenosine diphosphate (ADP) receptor antagonists were introduced to be used in combinations with aspirin.

The development of various in vitro and in vivo clinically relevant thrombosis assays is behind the tremendous success in the discovery of potent and specific anticoagulants and antiplatelets. This chapter and other chapters will review the various assays used in assessing antithrombotic agents and their impact on hemostasis.

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In Vitro and Ex Vivo Tests

Blood Coagulation Tests

Purpose and Rationale

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated conversion of fibrinogen to fibrin, which is one major component of a thrombus. The coagulation cascade can be initiated either by the "exogenous pathway," the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa complex, or by the "endogenous pathway," so-called contact activation leading via factors XII, XI, and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X, which induces the formation of the prothrombinase complex consisting of factor X_a, factor Va, and Ca^{2+} on a phospholipid surface. The latter leads to the activation of thrombin, which, in turn, cleaves fibrinogen to fibrin. The three coagulation tests (prothrombin time [PT], activated partial thromboplastin time [APTT], and thrombin time [TT]) allow one to differentiate between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters PT, APTT, and TT ex vivo. In vitro and ex vivo anticoagulant efficacy can be measured in blood obtained from various species including human or different animal species before and after administration of test agents via different routes.

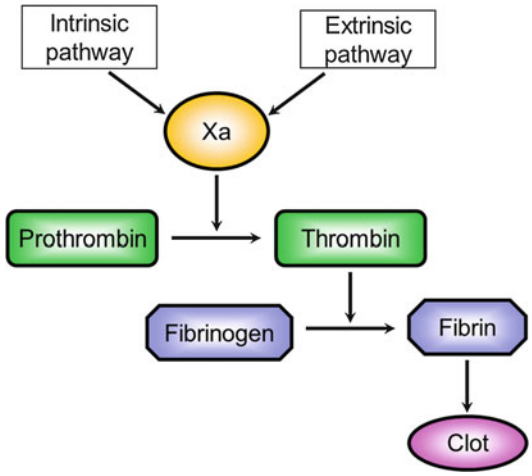

Fig. 1 Simplified coagulation cascade

Procedure

Male Sprague–Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, intravenous, or other route of administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a midline incision or by cardiac puncture, and 1.8 ml blood is collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behringwerke, Marburg). The sample is immediately agitated and centrifuged in a plastic tube at 1,500 × *g* for 10 min. Plasma is transferred to

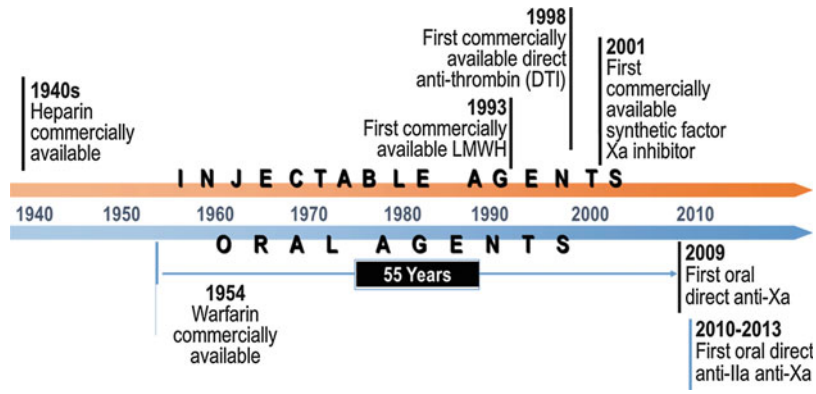

Fig. 2 Historical advances in anticoagulation

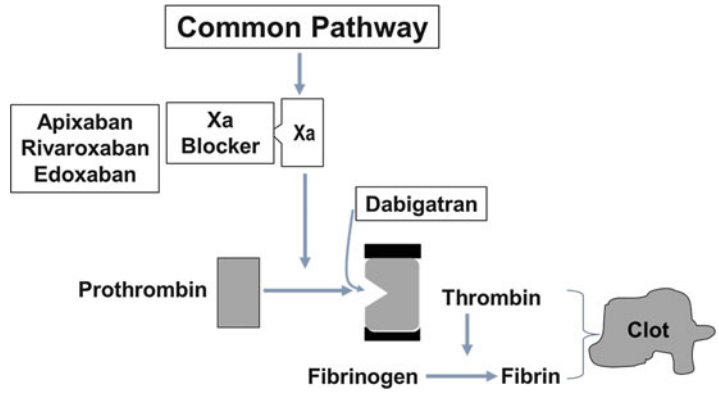

Fig. 3 Novel oral anticoagulants

another plastic tube, and the coagulation tests for the determination of TT, PT, and APTT are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below), and the time to clot formation is determined in the coagulometer (= coagulation time).

For detailed laboratory diagnosis of bleeding disorders and assessment of blood coagulation, see Palmer (1984) and Francis et al. (1994).

Prothrombin Time (PT). An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37 °C. Then 0.2 ml of human thromboplastin (Thromborel, Behringwerke, Marburg FRG) is added and the coagulometer (Schnittger + Gross coagulometer, Amelung, Lemgo-Brake, FRG) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (APTT). To 0.1 ml of citrated plasma, 0.1 ml of human placenta lipid extract (Pathrombin, Behringwerke) is added and the mixture is incubated for 2 min at 37 °C. The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

Thrombin Time (TT). To 0.1 ml of citrated plasma, 0.1 ml of diethyl barbiturate–citrate buffer, pH 7.6 (Behringwerke), is added and the mixture is incubated for 1 min at 37 °C. Then 0.1 ml of bovine test-thrombin (30 IU/ml, Behringwerke) is added and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

Evaluation

Mean values of TT, PT, and APTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's *t*-test.

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Thrombelastography

Purpose and Rationale

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction.

The blood samples are transferred to cuvettes and maintained at 37 °C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermo paper. The modern thrombelastograph transfers the analogous recording to a digital signal that is evaluated by a computer program.

Procedure

TEG can be performed in either whole blood or in citrated platelet-rich or platelet-poor plasma after recalcification. Blood samples are obtained from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.7–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the compound by intravenous (i.v.), subcutaneous (s.c.), or oral administration. Ten or 20 min post dosing (i.v., s.c. administration) or 60, 90, or 180 min post dosing (oral administration) blood is collected. The blood samples are mixed with 3.8 % trisodium citrate solution (1 part citrate solution to 9 parts blood) as anticoagulant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the recalcified whole blood is transferred to the pre-warmed cup of the thrombelastograph. After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

Evaluation

The following measurements are the standard variables of TEG:

1. Reaction time (r): the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
2. Clot formation time (k): the difference from the 1 mm r to 20 mm amplitude. k represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot, caused by fibrin buildup and cross-linking.
3. Alpha angle ($\alpha°$): angle formed by the slope of the TEG tracing from the r to k value. It denotes speed at which solid clot forms.
4. Maximum amplitude (MA): greatest amplitude on the TEG trace. MA represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
5. Clot strength (G in dynes per square centimeter): defined by $G = (5{,}000\ MA)/(96 - sMA)$. In a tissue factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
6. Lysis 30, Lysis 60 ($Ly30$, $Ly60$): Reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis (Table 1).

Modifications of the Method

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by TEG in vitro using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and TEG to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described an endotoxin-induced rabbit model of hypercoagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters, including TEG.

Khurana et al. (1997) introduced tissue factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II). Additionally, Mousa et al. (2005) utilized the TEG in phase II clinical trial in monitoring the efficacy of oral platelet GPIIb/IIIa antagonist on platelet/fibrin clot dynamics.

Table 1 Effect of various stimuli on platelet/fibrin clot dynamics as shown by Mousa et al. (2000)

TEG Parameters	TF (25 ng)	LPS (0.63 ug)	Xa (0.25 nM)	Thrombin (0.3 mU)
	Mean ± SEM			
r (minutes)	29.7 ± 2.3	23.4 ± 1.4	15.6 ± 2.9	3.4 ± 0.6
k (minutes)	5.8 ± 1.0	7.6 ± 0.9	4.8 ± 0.5	5.5 ± 0.8
α (angle)	45.0 ± 2.6	47.8 ± 3.2	61.5 ± 2.1	57.8 ± 2.9
MA (mm)	58.2 ± 1.7	50.0 ± 2.0	65.0 ± 0.8	50.1 ± 2.4

Citrated human whole blood plus 2 mM calcium. Data represent mean for $n = 6 \pm$ SEM

Critical Assessment of the Method

Zuckerman et al. (1981) compared TEG with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count, and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Mousa et al. (2005) and others expanded the use of TEG in differentiating among different antiplatelets, anticoagulants, and optimal combinations of both. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies; it contains additional information on the hemostatic process. This is due to the following: (1) the fact that most laboratory measurements end with the formation of the first fibrin strands, while TEG measures the coagulation process on whole blood from initiation of clotting to the final stages of clot lysis and retraction and (2) the ability of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

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Chandler Loop

Purpose and Rationale

The Chandler loop technique allows the production of in vitro thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to that of human thrombi formed in vivo (Robbie et al. 1997), with platelet-rich upstream

sections ("white heads") that are relatively resistant to tissue plasminogen activator (t-PA)-mediated thrombolysis in contrast to the red blood cell-rich downstream parts ("red tails") (Stringer et al. 1994).

Procedure

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm) tubing. The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23°, and rotated at 17 rpm. At the moment the developing thrombus inside the tube becomes large enough to occlude the lumen, the blood column becomes static and moves around in the direction of rotation of the tube.

Evaluation

Time to occlusion of the tube by the thrombus establishes a definite end point in this system.

Modifications of the Method

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2C8) on the t-PA-induced lysis of Chandler thrombi in vitro. They used citrated blood and supplemented it with 5.8 μM [^{125}I]-labeled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (pre-value). The head and the tail were then subjected to thrombolysis by adding 300 μl phosphate-buffered saline containing plasminogen (2 μM) and t-PA (0.9 nM). During the observation time of 240 min, aliquots of 10 μl were taken at 30, 60, 120, 180, and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the pre-value was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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Platelet Aggregation and Disaggregation in Platelet-Rich Plasma or Washed (Born Method)

Purpose and Rationale

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of unactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V complex at high shear rates and binding of collagen to two receptors, integrin $\alpha 2\beta 1$ and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa ligand binding sites.

These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation, platelets are activated by the addition of one of the following agonists to platelet-rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A2) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, and PAF. The formation of platelet aggregates with stirring leads to changes in optical density that are monitored photometrically, usually for 4 min. The test was developed originally by Born (1962a, b) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation in vitro or ex vivo. For in vitro studies, human PRP is preferred.

Procedure

The test is carried out either ex vivo or in vitro. There are other commercial sources for the various agonists listed (Table 2).

For ex vivo assays, mice, rats, or guinea pigs of either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal, or intravenous administration. At the end of the absorption time, blood is collected by caval venipuncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardio-puncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For in vitro assays, human blood is collected from the antecubital vein of adult volunteers who had not received any medication for the last 2 weeks.

Preparation of PRP, PPP, and WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 150 × *g* for 15 min to obtain platelet-rich plasma (PRP). The PRP supernatant is carefully removed, and the rest is further centrifuged at 1,500 × *g* for 10 min to obtain platelet-poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3×10^8/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5 by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430 × *g*. The pellet is resuspended in the original volume with Tyrode's solution (mM: NaCl 120, KCl 2.6, $NaHCO_3$ 12, NaH_2PO_4 0.39, HEPES 10, glucose 5.5, albumin 0.35 %) and set to platelet count of 3×10^8/ml.

For ex vivo assays, duplicate samples of 320 µl PRP from drug-treated and vehicle control subjects (for rabbits, control samples before drug administration) are inserted into the aggregometer at 37 °C

Table 2 Materials and solutions

Anticoagulation substances	
Hirudin (Sigma) or PPAK	200 µg/ml
Trisodium citrate	0.11 M
Acid-citrate-dextrose (ACD) solution	
Citric acid	38 mM
Sodium citrate	75 mM
Glucose	124 mM
Platelet-aggregating substances (final concentrations)	
ADP: for reversible or biphasic aggregation	0.1–5 µM
ADP: for irreversible aggregation (Sigma)	3–10 µM
Sodium arachidonate (Biodata)	0.3–1 mM
Calcium ionophore A 23187 (Calbiochem)	10 µM
Collagen (Hormonchemie)	3 µg/ml
PAF-acether (C 16-PAF, Bachem)	0.1 µM
Thrombin (Sigma)	0.02–0.05 IU/ml
TRAP (SFLLRNP, Bachem)	1–10 µM
U 46619 (ICN)	1–10 µM
Ristocetin	0.1–1 mg/ml
GPRP (fibrin antipolymerant, Bachem)	0.5 mM
4-Channel aggregometer (PAP 4, Bio Data)	

under continuous magnetic stirring at 1,000 rpm. After the addition of 40 µl physiological saline and 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For in vitro assays, 40 µl of the test solution are added to samples of 320 µl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37 °C for 2 min under continuous magnetic stirring at 1,000 rpm. After the addition of 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, glycine–proline–aspartate–proline (GPRP) is added in order to avoid fibrin formation. In order to measure disaggregation, experimental compounds are added to stimulated PRP at 70 % or 100 % of control aggregation, and monitoring is performed for further 10 min. Disaggregation is measured by the decrease of light transmission (see Haskel and Abendschein 1989).

Studies should be completed within 3 h after blood withdrawal.

Evaluation

The transmission maximum serves as a scale for platelet aggregation (0 % = transmission of PRP, 100 % = transmission of PPP).

For in vitro assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls. Statistical significance is evaluated by means of the unpaired Student's *t*-test.
2. IC_{50} values are determined from the nonlinear curve fitting of concentration-effect relationships. IC_{50} is defined as the concentration of test drug for half maximal inhibition of aggregation.
3. Percent disaggregation is determined at 10 min after addition of compound; IC_{50} is calculated from the concentration-effect relationship.

For ex vivo assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits, control values before drug administration). Statistical significance is evaluated by means of the Student's *t*-test (paired for rabbits, unpaired for others).
2. ED_{50} values are determined from the dose–response curves. ED_{50} is defined as the dose of drug leading to 50 % inhibition of aggregation in the animals.

Critical Assessment of the Method

The assay, introduced by Born (1962a, b), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of a functional parameter in intact human platelets. However, processing of platelets during the preparation of PRP, washed or filtered platelets from whole blood, results in platelet activation and separation of large platelets. Additionally, there is no standardization among the different laboratories due to variation in use of different tubes, different final agonist concentrations, and other technical differences.

Modifications of the Method

Several authors have described modifications of the assay procedure. Breddin et al. (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method of measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratantoni and Poindexter (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96-well microtiter plate method with conventional aggregometry showed similar

dose–response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neil (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor. Mousa et al. (1994, 1998) and others utilized this assay for in vitro screening and ex vivo and antiplatelet efficacy in various species and in humans for platelet GPIIb/IIIa antagonists.

Yamanaka et al. (2005) performed platelet aggregations assays using an eight-channel aggregometer (NBS HEMA TRACER 801, Nikobioscience, Tokyo, Japan) to test structure–activity relationships of potent GPIIb/IIIA antagonists.

Francischetti et al. (2000) used the microplate reader for studying a platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus.*

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Platelet Aggregation After Gel Filtration (Gel-Filtered Platelets, GFP)

Purpose and Rationale

Triggering of platelet activation by low concentrations of ADP, epinephrine, or serotonin – so-called weak platelet agonists – in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet

aggregation induced by thrombin, collagen, or prostaglandin-endoperoxide – so-called strong agonists – is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX-V. Mousa et al. (1994, 1998) and others have extensively utilized this assay to determine the impact of plasma protein binding on antiplatelet efficacy by comparing inhibition of platelet aggregation in GFP versus PRP.

Procedure

Preparation of Gel-Filtered Platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marguerie et al. (1979).

Blood is drawn from healthy adult volunteers who had no medication for the last 2 weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD solution and centrifuged for 10 min at 120 × *g*. The platelet-rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD solution, and centrifuged at 285 × *g* for 20 min. The resulting pellet is resuspended in Tyrode's buffer (approx. 500 µl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode's buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8/ml. Gel-filtered platelets (GFP) are kept at room temperature for 1 h until the test is started (Table 3).

Table 3 Materials and solutions

Acid-citrate-dextrose (ACD) solution	
Citric acid	0.8 %
Sodium citrate	2.2 %
Glucose	2.45 %
Hirudin	0.6 U/ml
Tyrode's solution	
NaCl	137 mM
KCl	2.7 mM
$MgCl_2$	5.5 mM
NaH_2PO_4	3.0 mM
HEPES	3.5 mM
Glucose	5.5 mM
Albumin	0.2 %
Hirudin	0.06 U/ml
Apyrase	40 µg/ml
pH	7.2
ADP	10 µM
Thrombin	0.02–0.05 U/ml
$CaCl_2$	0.5 mM
Fibrinogen (American Diagnostica)	1 mg/ml
von Willebrand factor	10 µg/ml
Sepharose CL 2B (Pharmacia)	
Acrylic glass column (Reichelt Chemietechnik, 3 cm inner diameter, 18 cm length)	
Aggregometer (PAP 4, Biodata)	

Experimental Course

For the aggregation studies, GFP in Tyrode's buffer is incubated with $CaCl_2$ (final concentration 0.5 mM) with or without fibrinogen (final conc. 1 mg/ml) in polystyrene tubes. After 1 min, 20 µl of the test compound or the vehicle (controls) are added, and the samples are incubated for another 2 min. After the addition of 20 µl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37 °C (1,000 rpm) in the aggregometer. Samples with added $CaCl_2$ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregatory response of GFP to 10 µM ADP shows intact platelets (with only minor pre-activation with gel filtration).

Evaluation

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor GFPs; in the case of an anti-aggregating effect, the test is performed with 4–6 GFPs.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student's *t*-test.

The percent inhibition of platelet aggregation in the dosage groups is calculated relative to the vehicle controls.

IC_{50} values (50 % inhibition of aggregation) are determined from the concentration-effect curves.

For detailed methodology and evaluation of different agents, see Marguerie et al. (1979, 1980), Markell et al. (1993), and Mousa et al. (1994, 1998).

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Platelet Aggregation in Whole Blood

Purpose and Rationale

The method uses a whole blood platelet counter that counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anticoagulated human whole blood samples by the addition of the aggregating agents arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC_{50} values are calculated in dosage groups. The effect of compounds on other blood cells that secondarily can influence platelet aggregation is included in this test system. The method has been described by Lumley and Humphrey (1981) and by Cardinal and Flower (1980).

Table 4 Materials and solutions

Anticoagulant: sodium citrate to induce platelet aggregation	3.8 %
Sodium arachidonate (Biodata)	3.6×10^{-4} M
Collagen (Hormonchemie)	10 µg/ml
Serono Hematology System 9000 or Sysmex Micrcellcounter F 800	

Procedure

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers who had not received medication for the last 2 weeks; 9 ml venous blood is anticoagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30–60 min until the start of the test (Table 4).

For the aggregation studies, 10 µl test substance or vehicle (control) is added to 480 µl citrated blood. Samples in closed tubes are preincubated for 5 min in a 37 °C water shaker bath at 75 strokes/min. Then, 10 µl aggregating agent is added, and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ("initial platelet count," "10-min-platelet count" after adding platelet agonist ± antagonist) in a hematology cell counter.

The following samples for the determination of the platelet count are prepared in duplicate:

- Control aggregation = spontaneous aggregation (without aggregating agent): 480 µl blood + 20 µl vehicle. Blood samples with >20 % spontaneous aggregation are not used to test for induced aggregation.
- Maximal aggregation: 480 µl blood + 10 µl vehicle + 10 µl aggregating agent. Values

represent the maximal induced aggregation rate of the blood sample.
- Test substance aggregation: 480 µl blood + 10 µl test substance + 10 µl aggregating agent.

Evaluation

From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\% \text{ maximal aggregation} = 100 - \frac{10 - \text{min platelet count} \times 100}{\text{initial platelet count}}$$

From the samples for test substance-induced aggregation, the percentage of aggregation in dosage groups is calculated according to the following formula:

$$\% \text{ aggregation} = \frac{10 - \text{min platelet count} \times 100}{\text{initial platelet count}}$$

IC_{50} values (50 % inhibition of aggregation) are determined from the dose–response curves (log concentration test substance versus % inhibition of aggregation).

References and Further Reading

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Platelet Micro- and Macro-Aggregation Using Laser Scattering

Purpose and Rationale

- A new highly sensitive method to study platelet aggregation based on the measurement of mean radius or particle size makes it possible to record kinetics of formation of micro- and macroaggregates in real time.
- Sensitivity in measurements of spontaneous aggregation is higher than in routine light transmittance.

Spontaneous platelet aggregation (SPA) measurements without platelet agonists using laser scattering might give us insight about the generation of thrombus within the lumen of the arterial wall at its high shear (Hara et al. 2012). SPA has been shown to be enhanced in patients with type 2 diabetes, which was shown to be reversed after short-term glycemic control or the administration of adiponectin (Suzuki et al. 2014).

Limitations

This technique cannot be applied in whole blood yet but can be used with PRP, washed platelet, or GFP.

Modifications of the Method

For functional characterization of an acid platelet aggregation inhibitor and hypotensive phospholipase A_2 from *Bothrops jararacussu* snake venom, Andrião-Escarso et al. (2002) measured platelet aggregation in rabbit blood using a whole blood lumi-aggregometer.

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Fibrinogen Receptor Binding

Purpose and Rationale

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I-fibrinogen (30–50 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug. Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I-fibrinogen binding at the GPIIb/IIIa receptor.

Procedure

Preparation of Gel-Filtered Platelets

From a healthy volunteer, 200 ml blood is collected. An aliquot of 8.4 ml blood is mixed with 1.4 ml ACD buffer in polystyrol tubes and centrifuged at 150 $\times$ g for 15 min. The resulting platelet-rich plasma (PRP) is collected, and an aliquot is taken for platelet counting. Then, 10 ml PRP is mixed with 1 ml ACD buffer (ACD-PRP, pH $\sim$ 6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at 1,500 $\times$ g for 15 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 µl Tyrode's buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to a Sepharose-packed column that has been eluted with approx. 100 ml degassed Tyrode's buffer B (2 ml/min). The column is closed and eluted with degassed Tyrode's buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4 $\times$ 10^8 platelets/ml with Tyrode's buffer B and kept at room temperature until the start of the test (Mousa et al. 1994, 2001).

Experimental Course

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt). The total volume of each incubation sample is 500 µl. The concentration of ^{125}I-fibrinogen is constant for all samples (10 µg/500 µl).

Competition Experiments

The competition reaction is characterized by one buffer value (bi-distilled water) and various concentrations of non-labeled fibrinogen or test compound.

- 100 µl ^{125}I-fibrinogen
- 100 µl non-labeled fibrinogen or test drug (various concentrations, 10^{-10}–10^{-3} M)
- 5 µl ADP

Nonspecific binding: The nonspecific binding of ^{125}I-fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of non-labeled fibrinogen.

The binding reaction is started by adding 250 µl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 µl aliquot of the incubation sample is transferred to a Microtainer tube containing 400 µl glucose solution. The tubes are centrifuged at 1,500 × *g* for 2 min to separate ^{125}I-fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radioligand. The supernatant is carefully decanted and is allowed to run off for approx. 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3 %.

Materials and Solutions

See Table 5.

Evaluation

The quantity of the specific ^{125}I-fibrinogen binding results from the difference between the total and the nonspecific binding.

Platelet glycoprotein IIb-IIIa receptor binding is given as fmol ^{125}I-fibrinogen/10^8 platelets or ^{125}I-fibrinogen molecules bound per platelet.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{125}I-fibrinogen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K = \frac{K_D{}^{125}\mathrm{I} \times IC_{50}}{K_D{}^{125}\mathrm{I} + [^{125}\mathrm{I}]}$$

IC_{50} = concentration of the test drug, which displaces 50 % of the specifically glycoprotein IIb-IIIa receptor-bound ^{125}I-fibrinogen in the competition experiment.

$[^{125}\mathrm{I}]$ = concentration of ^{125}I-fibrinogen in the competition experiment.

$K_D{}^{125}\mathrm{I}$ = dissociation constant of ^{125}I-fibrinogen, determined from the saturation experiment.

The K_i value of the test drug is the concentration, at which 50 % of the fibrinogen receptors are occupied by the test drug.

For detailed methodology and evaluations of various mechanisms and agents, see the following selected references: Bennett, Vilaire (1979), Kornecki et al. (1981), Marguerie et al. (1979, 1980), Mendelsohn et al. (1990), and Mousa et al. (1994).

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Table 5 Materials and solutions

Solutions for platelet preparation		
Stock solution I	Citrate	0.8 %
	Sodium citrate	2.2 %
Stock solution II	NaCl	120 mM
	KCl	2.8 mM
	NaH_2PO_4	10.0 mM
	HEPES	10.0 mM
ACD buffer	Stock solution I	2.45 %
	+ glucose	
	+ hirudin	0.06 U/ml
Tyrode's buffer A	Stock solution II	
	+ $NaHCO_3$	12 mM
Tyrode's buffer B	Stock solution II	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ bovine albumin	0.35 %
Tyrode's buffers A and B are degassed by aspiration for approx. 1 h after setting the pH to 7.2.		
Tyrode's buffer C	Tyrode's buffer B (degassed)	
	+ apyrase	40 µg/ml
	+ hirudin	0.06 U/ml
Chromatography column	Acryl glass column (200 × 170 mm, 30 mm diameter), closed with 3 perlon filters, pore sizes 63, 90 and 230 µm, and gauze 50 µm filled with degassed Sepharose CL2B suspension (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode's buffer A (2 ml/min)	
Incubation buffer		
Stock solution	NaCl	120 mM
	KCl	2.6 mM
	NaH_2PO_4	0.39 mM
	HEPES	10.0 mM
	$CaCl_2$	0.5 mM
Incubation buffer, pH 7.2	Stock solution	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ human albumin	0.35 %
Glucose solution (in incubation buffer)		
Radioligand	^{125}I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 µCi/mg fibrinogen) (Amersham), 1 mg radiolabeled fibrinogen is dissolved in 10 ml incubation buffer	
Non-labeled fibrinogen (mw 340000, grade L, Sigma; in bi-distilled water)		10^{-3}–10^{-10} M
ADP (in incubation buffer)		10 µM

Euglobulin Clot Lysis Time

Purpose and Rationale

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood according to Gallimore et al. (1971). The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator, and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e.g., plasminogen activators). Thus, compounds that stimulate the release of tissue-type plasminogen activator from the vessel wall can be detected.

Procedure

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37 °C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intraperitoneally. Twenty-five minutes later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

Plasma Preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a midline excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8 % sodium citrate solution. The sample is thoroughly mixed, transferred to a plastic tube, and immediately immersed in ice. Plasma is prepared by centrifugation at 2,000 × *g* for 10 min at 2 °C.

Euglobulin Preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1 % acetic acid. The diluted plasma is kept on ice for 10 min, and the precipitated euglobulin fraction is collected by centrifugation at 2,000 × *g* for 10 min at 2 °C. The supernatant is discharged, and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

Euglobulin Lysis Assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behringwerke) (25 U/ml) is added. The tubes are transferred to a water bath at 37 °C. The time interval between the addition of thrombin and the complete lysis of the clots is measured. For details, see Gallimore et al. (1971) and Singh et al. (2005).

Evaluation

The lysis time (min) is determined. Euglobulin lysis test (ELT) is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage treated groups as compared to controls.

Statistical evaluation is performed by means of the Student's *t*-test.

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Flow Behavior of Erythrocytes

Purpose and Rationale

The deformation of erythrocytes is an important rheological phenomenon in blood circulation according to Teitel (1977) and Nash (1990). It allows the passage of normal red cells through capillaries with diameters smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to two basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

- Addition of calcium ions (increase in erythrocyte rigidity)
- Addition of lactic acid (decrease in pH value)
- Addition of 350–400 mmol NaCl (hyperosmolarity)

- Storing the sample for at least 4 h (cellular aging, depletion of ADP)

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

Procedure

Apparatus

Erythrocyte filtrometer MF4 (Fa. Myrenne, 52159 Roetgen, Germany); membrane filter (Nuclepore Corp.); pore diameter, 5–10 μm; pore density, 4×10^5 pores/cm^2.

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, blood is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at 250 × *g* for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25 % human albumin, and the hematocrit value is fixed at 10 %. The red blood cells are altered by one or several of the stress factors mentioned above.

A sample of 2 ml of the stressed suspension is applied to the filtrometer and the initial flow rate is determined. The filtration curve is plotted automatically.

Evaluation

The cumulative volume of the filtered suspension is recorded per time unit (10 min).

The slope of the curve is determined at different time intervals.

The initial flow rate (10 % of the cell suspension having passed the filter) is recorded.

Statistics

Data of each set are first tested for normal distribution using the Kolmogorov/Smirnov test. The normal distribution hypothesis is eliminated if the data having a significance level of 5 % are not normally distributed. In case that both data sets to be compared are normally distributed, the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5 %. The *t*-test for paired and non-paired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the U-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; U-test = Wilcoxon–Mann–Whitney or Mann–Whitney test).

References and Further Reading

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Filterability of Erythrocytes

Purpose and Rationale

The Single Erythrocyte Rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a synthetic membrane are determined according to Kiesewetter et al. (1982), Roggenkamp et al. (1983), and Seiffge and Behr (1986). The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore, the current is interrupted. The test is used to detect compounds that improve

filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified either by one or by a combination of the following stress factors:

- Addition of calcium ions (increase in erythrocyte rigidity)
- Addition of lactic acid (decrease in pH value)
- Addition of 350–400 mmol NaCl (hyperosmolarity)
- Storing the sample for at least 4 h (cellular aging, depletion of ADP)

Procedure

Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany).

Data: driving pressure, $dp = 70$ Pa (dog, rabbit, rat), $dp = 100$ Pa (man); wall shear stress, $\tau = 3$ Pa

Single pore membrane: length, 30 μm; diameter, 3.5 μm (rat), 4.0 μm (rabbit, dog), 4.5 μm (man).

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood samples are incubated at 37 °C for 5 or 30 min.

The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at 250 × *g* for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in filtrated HEPES buffer containing 0.25 % human albumin, and the hematocrit value is fixed to <1 %. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device, and the passage time of a population of 250 erythrocytes (tm) is determined. Cells remaining in the pore for more than 100 ms (tm >100 ms) lead to a rheological occlusion.

Untreated red blood cell suspensions serve as control.

Evaluation

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes is determined.

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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Erythrocyte Aggregation

Purpose and Rationale

The aggregation of red blood cells into rouleaux and from rouleaux into 3-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood, especially

in disturbed microcirculation. In the following procedure, an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/plate configuration) is transilluminated by light of a defined wavelength. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation according to Kiesewetter et al. (1982) and Schmid-Schoenbein et al. (1973).

Procedure

Apparatus

Selective Erythrocyte Rigidometer (Fa. Myrenne, 52159 Roetgen, Germany).

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood sample is incubated at 37 °C for 5 or 30 min.

Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood. A sample of 40 µl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis, and the extent of erythrocyte aggregation is determined photometrically.

Evaluation

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes (see above).

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Determination of Plasma Viscosity

Purpose and Rationale

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice according to Harkness (1971). In this test, a defined volume of plasma is transferred into a capillary viscometer, and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either ex vivo or in vitro.

Procedure

Ex Vivo

Beagle dogs weighing 12–20 kg, rabbits weighing 2.0–3.0 kg, or Wistar rats weighing 150–300 g of

either sex are used as test animals. Likewise, the test procedure can be performed in humans. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ml blood K^+-EDTA or heparin sodium (5 IU/ml blood) and centrifuged at 250 × *g* for 5 min. The supernatant (plasma) is removed, and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr., LTD, England) provided with a glass capillary of 0.5 mm inside diameter. The temperature during measurement is 37 °C. The flow time, *t*, required for the plasma to flow through the capillary is measured. Untreated plasma serves as control.

Evaluation

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho$$

where η = viscosity of plasma, K = calibration constant of viscometer, t = flow time of 0.9 ml plasma, and ρ = density of plasma.

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's *t*-test.

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Hemostasis Models: Bleeding Models

Shaker A. Mousa

Contents

General Introduction

There is a delicate balance between thrombosis and hemostasis – primary hemostasis (platelet plug), secondary hemostasis (fibrin clot), and fibrinolysis. On the coagulation side, a balance between procoagulants and anticoagulants and pro-fibrinolytics and anti-fibrinolytics is crucial to maintain physiological hemostasis. Similarly, on the platelet side, a balance between endogenous platelet activators and inhibitors is also critical in the maintenance of hemostasis. When using either antiplatelet or anticoagulants to prevent or treat either arterial or venous thrombosis, a risk of bleeding exists due to the shift in the balance between thrombosis and hemostasis.

This chapter focuses on the model systems used to assess hemostasis.

Hemostasis Models: Bleeding Models

Subaqueous Tail Bleeding Time in Rodents

Purpose and Rationale

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

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A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for the bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

Procedure

Anesthetized rats are fixed in supine position on a temperature-controlled (37 °C) heating table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37 °C).

Evaluation

The time until the bleeding stops is determined within a maximum observation time of 600 s.

Critical Assessment of the Method

There are numerous variables that can influence a rodent's bleeding time measurements, as discussed by Dejana et al. (1979): position of the tail (horizontal or vertical), environment (air or saline), temperature, anesthesia, or procedure of injury (SimPlate method, transection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay conditions (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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Arterial Bleeding Time in Mesentery

Purpose and Rationale

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated that inhibit thrombus formation, thus prolonging arterial bleeding time. The test is used to detect agents that interfere with primary hemostasis in small arteries.

Procedure

Male Sprague–Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37 °C.

The abdomen is opened by a midline incision and the mesentery is lifted to display the mesenteric arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode's solution maintained at 37 °C. Bleeding times are determined with small mesenteric arteries (125–250 μm external diameter) at the junction of mesentery with intestines. Adipose tissue

surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16 × 5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

Evaluation

1. Mean values of bleeding times are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.
2. The significance of the results is assessed with the unpaired Student's *t*-test.
3. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.

For further details on methods and evaluations of various mechanisms or agents, see the following: Butler et al. (1982), Dejana et al. (1979), and Zawilska et al. (1982).

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Template Bleeding Time Method

Purpose and Rationale

The template bleeding time method is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the development of a spring-loaded cassette with two disposable blades (SimPlate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method that enables the SimPlate technique to be used as a method to analyze the bleeding time in the oral mucosa of dogs.

Procedure

The dog is positioned in sternal or lateral. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co, Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 s. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.

Evaluation

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between 2 and 4 min.

Critical Assessment of the Method

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989; Klement et al. 1998). Brassard and Meyers (1991) describe the buccal mucosa bleeding time as a test that is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in

animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979; Lind 1991).

Modifications of the Method

The SimPlate device can also be used to perform incisions at the shaved inner ear of rabbits, taking care to avoid major vessels. The normal bleeding time in anesthetized rabbits is approximately 100 s.

Klement et al. (1998) described another ear bleeding model in anesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37 °C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade, avoiding major vessels, and the ear was immediately reimmersed in saline. At different times thereafter (5–30 min) aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine-type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick, to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between 2 and 8 min.

Kubitza et al. (2005) and others extended the use of the model in humans.

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Genetic Models of Hemostasis and Thrombosis

Shaker A. Mousa

Contents

General Introduction

The genes encoding the coagulation factor proteins were among the first human genes to be characterized. Since then significant progress has been made in the application of this information, such as in the case of hemophilia A and B as well as the genetic variations in key coagulation factors. Additionally, the drug metabolizing enzyme (CYPs) variations and the resulting changes in the pharmacokinetic and the pharmacodynamic profiles of the active anticoagulant or antiplatelet moieties can be monitored to determine the unit dose required for optimal efficacy and safety. For various coagulation disorders and pharmacotherapy, genetic characterization of the disease-causing mutations (pharmacogenetics) and pharmacogenomics are currently incorporated into the standard of care for the risk stratification of treatment complications.

The observation of individual differences in platelet response to antiplatelet drugs or coagulation pathway response to an anticoagulant has expanded the spectrum and the possible clinical relevance of the variability of platelet or coagulation functions. The era of "personalized medicine" would benefit from the concepts discussed in this chapter in using clinically relevant models that reflect the impact of different genetic predispositions on the antithrombotic efficacy of the different classes of antiplatelet and anticoagulant.

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Genetic Models of Hemostasis and Thrombosis

Purpose and Rationale

Recent advances in genetic molecular biology have provided tools allowing scientists to design genetically altered animals that are deficient in certain proteins involved in thrombosis and hemostasis (so-called knockouts or nulls) (Carmeliet and Collen 1999; Pearson and Ginsburg 1999). These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examining the phenotype (e.g., spontaneous bleeding, platelet defect, prolonged bleeding after surgical incision, etc.) of a specific knockout strain, scientists can identify the role of the knocked-out protein. Then if the phenotype is favorable (e.g., not lethal), pharmacological agents can be designed to mimic the knockout. More recently, novel gene medicine approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process.

The majority of these gene knockouts result in mice that develop normally, are born in the expected Mendelian ratios, and are viable (as defined by the ability to survive to adulthood). Although seemingly normal, these knockout mice display alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, vWF, and the β_3-integrin (Bi et al. 1996; Denis et al. 1998; Hodivala-Dilke et al. 1999; Wang et al. 1997) all results in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knockout models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann thrombasthenia, respectively). In addition, deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally, but they are unable to survive the perinatal period due to severe hemorrhage, in most cases due to the trauma of birth.

Genetic knockouts have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of the β_3-integrin (Hodivala-Dilke et al. 1999) or of $G_{\alpha q}$ (Offermanns et al. 1997) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein-coding region in the β_3-integrin carboxyl-tail, β_3-DiY, at sites that are thought to be phosphorylated upon platelet activation, also results in unstable platelet aggregation (Law et al. 1999). Deletion of various receptors such as thromboxane A_2, P-selectin, P2Y1, and PAR-3 demonstrates diminished responses to some agonists while other platelet responses are intact (Thomas et al. 1998; Subramaniam et al. 1996; Leon et al. 1999; Kahn et al. 1998). Deletion of PAR-3, another thrombin receptor in mice, has little effect on hemostasis. This indicated the presence of yet another thrombin receptor in platelets and led to the identification of PAR-4 (Kahn et al. 1998).

Given that knockouts of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (Bugge et al. 1995; Ploplis et al. 1995), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and the combined t-PA/u-PA knockout (Carmeliet et al. 1994) result in mice that demonstrate impaired fibrinolysis, susceptibility for thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice may provide a good model of myocardial infarction and heart failure caused by thrombosis (Christie et al. 1999). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, demonstrate no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (Carmeliet et al. 1993), suggesting that inhibition of PAI-1 might be a promising approach for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several of these genes are important

in embryonic development. For example, deletion of tissue factor (Bugge et al. 1996; Toomey et al. 1996; Carmeliet et al. 1996), tissue factor pathway inhibitor (Huang et al. 1997), or thrombomodulin (Healy et al. 1995) results in an embryonic lethal phenotype. These and other (Connolly et al. 1996; Cui et al. 1996) hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. Results of these studies have provided rationale and impetus for attacking certain targets pharmacologically. These types of models have also provided excellent model systems for studying novel treatments for human diseases. For example, these models provided exceptional systems for studying gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the *FIX* gene and its promoter, results in mice that mimic the human phenotype of hemophilia B (Lin et al. 1997). When these mice are treated with adenoviral-mediated transfer of human FIX, the bleeding diathesis is fully corrected (Kung et al. 1998). Similarly, selectively bred dogs that have a characteristic point mutation in the sequence encoding the catalytic domain of FIX also have a severe hemophilia B that is phenotypically similar to the human disease (Evans et al. 1989). When adeno-associated virus-mediated canine FIX gene was administered to these dogs intramuscularly, therapeutic levels of FIX were measured for up to 17 months (Herzog et al. 1999). Clinically relevant partial recovery of whole blood clotting time and APTT was also observed over this prolonged period. These data provided support for initiating the first study of adeno-associated virus-mediated FIX gene transfer in humans (Kay et al. 2000). Preliminary results from this clinical study provided evidence for expression of FIX in the three hemophilia patients studied and also provided favorable safety data to substantiate studying this therapy at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these experiments have provided pharmacological, pharmacokinetic, and safety data that were extremely useful in developing this approach and designing safe clinical trials.

Gene therapy approaches to rescuing patients with bleeding diatheses are further advanced than gene therapy for thrombotic indications. However, promising preclinical data indicates that local overexpression of thrombomodulin (Waugh et al. 1999a) or tissue plasminogen activator (Waugh et al. 1999b) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of tissue factor pathway inhibitor prevented thrombus formation in balloon-injured porcine carotid arteries (Zoldhelyi et al. 2000). These and other studies (Vassalli and Dichek 1997) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully optimized for pharmacokinetics, safety, and efficacy in laboratory animal studies prior to administration to humans.

Genetically Modified Animals

Development and application of animal models of thrombosis has played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. The development and application of small and large animal models of thrombosis to the discovery and development of novel antithrombotic agents is described in this chapter. The methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/

hemostasis is presented using gene therapy for hemophilia as an example of how animal models have aided in the development of therapies that are presently being evaluated clinically.

References and Further Reading

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Knockout Mice

Purpose and Rationale

Genetically modified animals, in particular knockout mice, help in the understanding of the role of various factors in blood clotting, thrombolysis, and platelet function. They are useful to verify the mode of action of new drugs.

Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ∼10 % die shortly after birth and another 40 % around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples failing to clot, or support platelet aggregation in vitro (Suh et al. 1995).

References and Further Reading

Suh TT, Holmback K, Jensen NJ et al (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Factor II (Prothrombin)

Phenotype

Partial embryonic lethality: 50 % between embryonic days (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage a few days after birth; factor II is important in maintaining vascular integrity during development as well as postnatal life (Sun et al. 1998; Xu et al. 1998).

References and Further Reading

Sun WY, Witte DP, Degen JL et al (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602
Xu J, Wu Q, Westfield L et al (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Factor V

Phenotype

Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature, remaining 50 % progress normally to term, but die from massive hemorrhage within 2 h of birth, more severe in mouse than in human (Cui et al. 1996; Yang et al. 2000).

References and Further Reading

Cui J, O'Shea KS, Purkayastha A et al (1996) Fatal hemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68

Yang TL, Cui J, Taylor JM et al (2000) Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 83:70–77

Factor VII

Phenotype

Develop normally but suffer fatal perinatal bleeding (Rosen et al. 1997).

References and Further Reading

Rosen ED, Chan JCY, Idusogie E et al (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Factor VIII

Phenotype

Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness, or reduced activity during the first year of life; have residual clotting activity (APTT), as shown by Bi et al. (1995).

References and Further Reading

Bi L, Lawler AM, Antonarakis SE et al (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10:119–121

Factor IX

Phenotype

Factor IX coagulant activities (APTT): +/+ 92 %, +/− 53 %, −/− <5 %; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized) (Kundu et al. 1998; Wang et al. 1997).

References and Further Reading

Kundu RK, Sangiorgi F, Wu LY et al (1998) Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood 92:168–174

Wang L, Zoppe M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Factor X

Phenotype

Partial embryonic lethality (1/3 died on E11.5–12.5); fatal neonatal bleeding between postnatal day (P) 5–20, as shown by Dewerchin et al. (2000).

References and Further Reading

Dewerchin M, Liang Z, Moons L et al (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Factor XI

Phenotype

APTT prolonged in −/− (158–200 s) compared with +/+ (25–34 s) and +/− (40–61 s); no factor XI activity and antigen, did not result in intrauterine death, −/− similar bleeding as +/+ with a tendency to prolongation (Gailani et al. 1997).

References and Further Reading

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

TF (Tissue Factor)

Phenotype

Abnormal circulation from yolk sac to embryo ~ E8.5 leading to embryo wasting and death; TF has a role in blood vessel development (Bugge et al. 1996; Carmeliet et al. 1996; Toomey et al. 1996, 1997).

References and Further Reading

Bugge TH, Xiao Q, Kombrinck KW et al (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

Carmeliet P, Mackman N, Moons L et al (1996) Role of tissue factor in embryonic blood vessel development. Nature 383:73–75

Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 94:6922–6926

TFPI (Tissue Factor Pathway Inhibitor)

Phenotype

None survive the neonatal period; 60 % die between E9.5–11.5 with signs of yolk sac hemorrhage (Huang et al. 1997).

References and Further Reading

Huang ZF, Higuchi D, Lasky N, Broze GJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Thrombin Receptor

Phenotype

Fifty percent die at E9–10; 50 % survive and become grossly normal adult mice with no bleeding diathesis; −/− platelets strongly respond to thrombin; −/− fibroblast lose their ability to respond to thrombin → second TR must exist, as shown by Connolly et al. (1996) and by Darrow et al. (1996).

References and Further Reading

Connolly AJ, Ishihara H, Kahn ML et al (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Darrow AL, Fung-Leung WP, Ye RD et al (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76:860–866

Thrombomodulin

Phenotype

Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/− mice develop normal without thrombotic complications (Christie et al. 1999; Healy et al. 1995, 1998; Weiler-Guettler et al. 1998).

References and Further Reading

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

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Weiler-Guettler H, Christie PD, Beeler DL et al (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101:1983–1991

Protein C

Phenotype

KO mice appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 h after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable, suggesting fibrinogen depletion and secondary consumptive coagulopathy (Jalbert et al. 1998).

References and Further Reading

Jalbert LR, Rosen ED, Moons L et al (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Plasminogen

Phenotype

Severe spontaneous thrombosis, reduced ovulation and fertility, cachexia and shorter survival, severe glomerulonephritis, impaired skin healing, reduced macrophage and keratinocyte migration (Bugge et al. 1995; Ploplis et al. 1995).

References and Further Reading

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807

Ploplis VA, Carmeliet P, Vazirzadeh S et al (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Alpha$_2$-Antiplasmin

Phenotype

Normal fertility, viability, and development; no bleeding disorder; spontaneous lysis of injected clots; → enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS (Lijnen et al. 1999).

References and Further Reading

Lijnen HR, Okada K, Matsuo O et al (1999) Alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

T-PA (Tissue-Type Plasminogen Activator)

Phenotype

Extensive spontaneous fibrin deposition, severe spontaneous thrombosis, impaired neointima formation, reduced ovulation and fertility, cachexia and shorter survival, severe glomerulonephritis, abnormal tissue remodeling (Carmeliet et al. 1998; Christie et al. 1999).

References and Further Reading

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

PAI-1 (Plasminogen Activator Inhibitor-1)

Phenotype

Reduced thrombotic incidence, no bleeding, accelerated neointima formation, reduced lung inflammation, reduced atherosclerosis. Detailed studies on PAI-1 are reported by Carmeliet et al. (1993), Eitzman et al. (1996), Erickson et al. (1990), Kawasaki et al. (2000), and Pinsky et al. (1998).

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TAFI (Thrombin-Activatable Fibrinolysis Inhibitor)

Not described.

Vitronectin

Phenotype

Normal development, fertility, and survival; serum is completely deficient in "serum spreading factor" and PAI-1 binding activities; delayed arterial and venous thrombus formation (Eitzman et al. 2000; Zheng et al. 1995).

References and Further Reading

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Saunders TL, Camper SA et al (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci USA 92:12426–12430

Urokinase, U-PA (Urinary-Type Plasminogen Activator)

Phenotype

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, and shortened life span; spontaneous fibrin deposits more extensively and in more organs (Carmeliet et al. 1998; Heckel et al. 1990).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intra-abdominal bleeding directly related to transgene expression in the liver and elevated plasma u-PA level; 50 % die between 3 and 84 h after birth; severe hypofibrinogenemia, loss of clotting function.

References and Further Reading

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Heckel JL, Sandgren EP, Degen JL et al (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 62:447–456

uPAR (Urinary-Type Plasminogen Activator Receptor)

Phenotype

Phenotype normal, attenuated thrombocytopenia, and mortality associated with severe malaria (Bugge et al. 1995, 1996; Dewerchin et al. 1996; Piguet et al. 2000).

References and Further Reading

Bugge TH, Suh TT, Flick MJ et al (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Biol Chem 270:16886–16894

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Piguet PF, Da-Laperrousaz C, Vesin C et al (2000) Delayed mortality and attenuated thrombocytopenia associated with severe malaria in

urokinase- and urokinase receptor-deficient mice. Infect Immun 68:3822–3829

Gas6 (Growth Arrest-Specific Gene 6 Product)

Phenotype

Mice are viable and fertile and appear normal, do not suffer spontaneous bleeding or thrombosis, and have normal tail-bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U46619. Arterial and venous thrombosis is inhibited, and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (Angelillo-Scherrer et al. 2001).

References and Further Reading

Angelillo-Scherrer A, DeFrutos PG, Aparicio C et al (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

GPIbalpha (Glycoprotein Ib Alpha, Part of the GPIb–V–IX Complex)

Phenotype

Bleeding, thrombocytopenia, and giant platelets (similar to human Bernard–Soulier syndrome). See Ware et al. (2000) for details.

References and Further Reading

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

GPV (Glycoprotein V, Part of the GPIb–V–IX Complex)

Phenotype

Increased thrombin responsiveness; GpV−/− platelets are normal in size; normal amounts in GpIb–IX; functional in vWF-binding; platelets are hyperresponsive to thrombin → increased aggregation response; shorter bleeding time; →GpV = negative modulator of platelet function (Ramakrishnan et al. 1999).

References and Further Reading

Ramakrishnan V, Reeves PS, DeGuzman F et al (1999) Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

GPIIb (Integrin Alpha IIb, Glycoprotein IIb, Part of the GPIIb–IIIa Complex)

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in human; platelets failed to bind fibrinogen, to aggregate, and to retract a fibrinogen clot; α-granules do not contain fibrinogen (Tronik-Le Roux et al. 2000).

References and Further Reading

Tronik-Le Roux D, Roullot V, Poujol C et al (2000) Thrombasthenic mice generated by replacement of the integrin $alpha_{IIb}$ gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

GPIIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GPIIb–IIIa Complex)

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in human, e.g., defective platelet aggregation, clot retraction, spontaneous bleeding, prolonged bleeding times, dysfunctional osteoclasts, development of osteosclerosis with age (Hodivala-Dilke et al. 1999; McHugh et al. 2000).

References and Further Reading

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

McHugh KP, Hodivala-Dilke K, Zheng MH et al (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

GPIIa (Glycoprotein IIa, Integrin Beta 1, Part of the GPIa–IIa Complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally; platelet count is normal. Collagen-induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major in vivo defects (Nieswandt et al. 2001).

References and Further Reading

Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

vWF (von Willebrand Factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF, highly prolonged bleeding time, hemorrhage, spontaneous bleeding, mice useful for investigating the role of vWF, delayed platelet adhesion in ferric chloride-induced arteriolar injury (Denis et al. 1998; Ni et al. 2000).

References and Further Reading

Denis C, Methia N, Frenette PS et al (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529

Ni H, Denis CV, Subbarao S et al (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

Thromboxane A2 Receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (Thomas et al. 1998).

References and Further Reading

Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001

Prostacyclin Receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses (Murata et al. 1997).

References and Further Reading

Murata T, Ushikubi F, Matsuoka T et al (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

PECAM (Platelet/Endothelial Cell Adhesion Molecule)

Phenotype

Normal platelet aggregation, prolonged bleeding time as described by Duncan et al. (1999) and by Mahooti et al. (2000).

References and Further Reading

Duncan GS, Andrew DP, Takimoto H et al (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030

Mahooti S, Graesser D, Patil S et al (2000) PECAM-1 (CD 31) expression modulates bleeding time in vivo. Am J Pathol 157:75–81

Pallid (Pa)

Phenotype

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky–Pudlak syndrome (the beige mouse is a model of the Chediak–Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution,

kidney lysosomal enzyme elevation serum alpha 1-antitrypsin deficiency, and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino-acid protein pallidin, which interacts with syntaxin 13, a protein mediating vesicle docking and fusion (Huang et al. 1999).

References and Further Reading

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 23:329–332

G Alpha (q) (Guanyl Nucleotide Binding Protein G Alpha q)

Phenotype

Defective aggregation in response ADP, TXA2, thrombin, collagen; shape change normal (Offermans et al. 1997; Ohlmann et al. 2000).

References and Further Reading

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186

Ohlmann P, Eckly A, Freund M et al (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. Blood 96:2134–2139

Gz (Member of the Gi Family of G Proteins)

Phenotype

Impaired platelet aggregation to epinephrine, resistance to fatal thromboembolism, exaggerated response to cocaine, reduced effect of morphine and antidepressant drugs (Yang et al. 2000).

References and Further Reading

Yang J, Wu J, Kowalska MA et al (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Phospholipase C Gamma

Phenotype

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fc_{gamma} receptor signaling, therefore, loss of collagen-induced platelet aggregation (Wang et al. 2000).

References and Further Reading

Wang D, Feng J, Wen R et al (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)

Phenotype

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature in vivo. Platelets fail to aggregate to standard agonists in vitro associated with purinergic P2Y1 receptor desensitization; fibrin deposition at multiple organ sites (Enjyoji et al. 1999).

References and Further Reading

Enjyoji K, Sevigny J, Lin Y et al (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5:1010–1017

Protein Kinase, cGMP-Dependent

Phenotype

Viable, fertile; unresponsive to cGMP and NO; defective VASP phosphorylation; increased adhesion and aggregation of platelets in vivo in ischemic/reperfused mesenteric microcirculation; no compensation by cAMP kinase system (Massberg et al. 1999).

References and Further Reading

Massberg S, Sausbier M, Klatt P et al (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. J Exp Med 189:1255–1264

Vasodilator-Stimulated Phosphoprotein (VASP)

Phenotype

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia; increased collagen/thrombin activation; impaired cyclic nucleotide-mediated inhibition of platelet activation (Aszodi et al. 1999; Hauser et al. 1999).

References and Further Reading

Aszodi A, Pfeifer A, Ahmad M et al (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMBO J 18:37–48

Hauser W, Knobeloch KP, Eigenthaler M et al (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125

Arachidonate 12-Lipoxygenase (P-12LO)

Phenotype

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in ex vivo assays and increased mortality in an ADP-induced mouse model of thromboembolism (Chen et al. 1994; Johnson et al. 1998).

References and Further Reading

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Arachidonate 5-Lipoxygenase (P-5LO)

Phenotype

Develop normally and are healthy. No difference in their reaction to endotoxin shock; however, they resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced (Chen et al. 1994; Johnson et al. 1998).

References and Further Reading

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Thrombopoietin

Phenotype

TPO −/− and c-mpl −/−: both exhibit a 90 % reduction in megakaryocyte and platelet levels, but even with these small platelet levels, the mice do not have excessive bleeding; all platelets that are present are morphologically normal + functionally; in vivo TPO is required for control of megakaryocyte and platelet number but not for their maturation (Lawler et al. 1998).

References and Further Reading

Lawler J, Sunday M, Thibert V et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Thrombospondin 1

Phenotype

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-1 is involved in normal lung homeostasis (Lawler et al. 1998).

References and Further Reading

Lawler J, Sunday M, Thibert V et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Table 1 Genetic models of thrombosis and hemostasis

Knockout	Viable	Embryonic development/survival	References
Coagulation			
Protein C	No	Normal perinatal death	Jalbert et al. (1998)
Fibrinogen	Yes	Normal perinatal death	Suh et al. (1995)
Fibrinogen-QAGVD	Yes	Normal	Suh et al. (1995)
FV	No	Partial embryonic loss, perinatal death	Cui et al. (1996)
FVII	Yes	Normal perinatal death	Rosen et al. (1997)
FVIII	Yes	Normal	Bi et al. (1996)
FIX	Yes	Normal	Wang et al. (1997)
FXI	Yes	Normal	Gailani et al. (1997)
Tissue factor	No	Lethal	Toomey et al. (1996), Bugge et al. (1996)
TFPI	No	Lethal	Huang et al. (1997)
vWF	Yes	Normal	Denis et al. (1998)
Prothrombin	No	Partial embryonic loss, perinatal death	Xue et al. (1998), Sun et al. (1998)
Fibrinolytic			
u-Pa and t-PA	Yes	Normal growth retardation	Carmeliet et al. (1994)
uPAR	Yes	Normal	Dewerchin et al. (1996), Bugge et al. (1995)
Plasminogen	Yes	Normal growth retardation	Bugge et al. (1995), Ploplis et al. (1995)
PA-I	Yes	Normal	Carmeliet et al. (1993)
Thrombomodulin	No	Lethal	Healy et al. (1995)
Platelet			
β_3	Yes	Normal partial embryonic loss	Hodivala-Dilke et al. (1999)
β_3-DiYF	Yes	Normal	Law et al. (1999)
P-selectin	Yes	Normal	Subramaniam et al. (1996)
PAR-1	Yes	Normal	Connolly et al. (1996)
PAR-3	Yes	Normal	Kahn et al. (1998)
$G_{\alpha a}$	Yes	Normal perinatal death	Offermans et al. (1997)
TXA_2 receptor	Yes	Normal	Thomas et al. (1998)
P2Y1	Yes	Normal	Leon et al. (1999)

Mouse knockout models of virtually all of the known hemostatic factors have been reported, as shown in Table 1.

References and Further Reading

Bi L, Sarkar R, Naas T et al (1996) Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. Blood 88:3446–3450

Bugge TH, Suh TT, Flick MJ et al (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Bio Chem 270:16886–16894

Bugge TH, Xiao Q, Kombrinck KW et al (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

Carmeliet P, Stassen JM, Schoonjans L et al (1993) Plasminogen activator inhibitor-1 gene-deficient mice. J Clin Invest 92:2756–2760

Carmeliet P, Schoonjans L, Kieckens L et al (1994) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Connolly AJ, Ishihara H, Kahn ML et al (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Cui J, O'Shea KS, Purkayastha A et al (1996) Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68

Denis C, Methia N, Frenette PS et al (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529

Dewerchin M, van Nuffelen A, Wallays G et al (1996) Generation and characterization of urokinase receptor-deficient mice. J Clin Invest 97:870–878

Dewerchin M, Liang Z, Moons L et al (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) β_3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

Huang ZF, Higuchi D, Lasky N, Broze GJJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Jalbert LR, Rosen ED, Moons L et al (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Kahn ML, Zheng YW, Huang W et al (1998) A dual thrombin receptor system for platelet activation. Nature 394:690–694

Law DA, DeGuzman FR, Heiser P et al (1999) Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. Nature 401:808–811

Leon C, Hechler B, Freund M et al (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest 104:1731–1737

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186

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Rosen ED, Chan JCY, Idusogie E et al (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Subramaniam M, Frenette PS, Saffaripour S et al (1996) Defects in hemostasis in P-selectin-deficient mice. Blood 87:1238–1242

Suh TT, Holmback K, Jensen NJ et al (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Sun WY, Witte DP, Degen JL et al (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602

Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. J Clin Invest 102:1994–2001

Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Wang L, Zoppè M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Xu J, Wu Q, Westfield L et al (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

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Law DA, DeGuzman FR, Heiser P et al (1999) Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. Nature 401:808–811
Leon C, Hechler B, Freund M et al (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest 104:1731–1737
Lin HF, Maeda N, Sithies O et al (1997) A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 90:3962–3966
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Wang L, Zoppè M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566
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Waugh JM, Kattash M, Li J et al (1999b) Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. Proc Natl Acad Sci U S A 96:1065–1070
Zoldhelyi P, McNatt J, Shelat HS et al (2000) Thromboresistance of balloon-injured porcine carotid arteries after local gene transfer of human tissue factor pathway inhibitor. Circulation 101:289–295

Knock-Out Mice: Factor I (Fibrinogen)

Suh TT, Holmback K, Jensen NJ et al (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Knock-Out Mice: Factor II (Prothrombin)

Sun WY, Witte DP, Degen JL et al (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602
Xu J, Wu Q, Westfield L et al (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Knock-Out Mice: Factor V

Cui J, O'Shea KS, Purkayastha A et al (1996) Fatal hemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68
Yang TL, Cui J, Taylor JM et al (2000) Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 83:70–77

Knock-Out Mice: Factor VII

Rosen ED, Chan JCY, Idusogie E et al (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Knock-Out Mice: Factor VIII

Bi L, Lawler AM, Antonarakis SE et al (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10:119–121

Knock-Out Mice: Factor IX

Kundu RK, Sangiorgi F, Wu LY et al (1998) Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood 92:168–174

Wang L, Zoppè M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Knock-Out Mice: Factor X

Dewerchin M, Liang Z, Moons L et al (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Knock-Out Mice: Factor XI

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

Knock-Out Mice: TF (Tissue Factor)

Bugge TH, Xiao Q, Kombrinck KW et al (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

Carmeliet P, Mackman N, Moons L et al (1996) Role of tissue factor in embryonic blood vessel development. Nature 383:73–75

Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci U S A 94:6922–6926

Knock-Out Mice: TFPI (Tissue Factor Pathway Inhibitor)

Huang ZF, Higuchi D, Lasky N, Broze GJJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Knock-Out Mice: Thrombin Receptor

Connolly AJ, Ishihara H, Kahn ML et al (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Darrow AL, Fung-Leung WP, Ye RD et al (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76:860–866

Knock-Out Mice: Thrombomodulin

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

Healy AM, Hancock WW, Christie PD et al (1998) Intravascular coagulation activation in a murine model of thrombomodulin deficiency: effects of lesion size, age, and hypoxia on fibrin deposition. Blood 92:4188–4197

Weiler-Guettler H, Christie PD, Beeler DL et al (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101:1983–1991

Knock-Out Mice: Protein C

Jalbert LR, Rosen ED, Moons L et al (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Knock-Out Mice: Plasminogen

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807

Ploplis VA, Carmeliet P, Vazirzadeh S et al (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Knock-Out Mice: Alpha$_2$-Antiplasmin

Lijnen HR, Okada K, Matsuo O et al (1999) Alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

Knock-Out Mice: T-PA (Tissue-type Plasminogen Activator)

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

Knock-Out Mice: PAI-1 (Plasminogen Activator Inhibitor-1)

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Eitzman DT, McCoy RD, Zheng X et al (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest 97:232–237

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Knock-Out Mice: Vitronectin

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Saunders TL, Camper SA et al (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci U S A 92:12426–12430

Knock-Out Mice: Urokinase, U-PA (Urinary-Type Plasminogen Activator)

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Heckel JL, Sandgren EP, Degen JL et al (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 62:447–456

Knock-Out Mice: uPAR (Urinary-Type Plasminogen Activator Receptor)

Bugge TH, Suh TT, Flick MJ et al (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Bio Chem 270:16886–16894

Bugge TH, Flick MJ, Danton MJ et al (1996) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904

Dewerchin M, van Nuffelen A, Wallays G et al (1996) Generation and characterization of urokinase receptor deficient mice. J Clin Invest 97:870–878

Piguet PF, Da-Laperrousaz C, Vesin C et al (2000) Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. Infect Immun 68:3822–3829

Knock-Out Mice: Gas 6 (Growth Arrest-Specific Gene 6 Product)

Angelillo-Scherrer A, DeFrutos PG, Aparicio C et al (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

Knock-Out Mice: GPIbalpha (Glycoprotein Ib Alpha, Part of the GP Ib-V–IX Complex)

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci U S A 97:2803–2808

Knock-Out Mice: GPV (Glycoprotein V, Part of the GP Ib-V–IX Complex)

Ramakrishnan V, Reeves PS, DeGuzman F et al (1999) Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci U S A 96:13336–13341

Knock-Out Mice: GPIIb (Integrin Alpha IIb, Glycoprotein IIb, Part of the GP IIb–IIIa Complex)

Tronik-Le Roux D, Roullot V, Poujol C et al (2000) Thrombasthenic mice generated by replacement of the integrin alpha$_{IIb}$ gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

Knock-Out Mice: GP IIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GP IIb–IIIa Complex)

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

McHugh KP, Hodivala-Dilke K, Zheng MH et al (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

Knock-Out Mice: GP IIa (Glycoprotein IIa, Integrin Beta 1, Part of the GP Ia–IIa Complex)

Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

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Denis C, Methia N, Frenette PS et al (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529

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Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. J Clin Invest 102:1994–2001

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Murata T, Ushikubi F, Matsuoka T et al (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

Knock-Out Mice: PECAM (Platelet: Endothelial Cell Adhesion Molecule)

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Knock-Out Mice: G Alpha (q) (Guanyl Nucleotide Binding Protein G Alpha q)

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Knock-Out Mice: G z (Member of the Gi Family of G Proteins)

Yang J, Wu J, Kowalska MA et al (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Knock-Out Mice: Phospholipase C Gamma

Wang D, Feng J, Wen R et al (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

Knock-Out Mice: CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)

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Knock-Out Mice: Protein Kinase, cGMP-Dependent

Massberg S, Sausbier M, Klatt P et al (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. J Exp Med 189:1255–1264

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Knock-Out Mice: Arachidonate 12-Lipoxygenase (P-12LO)

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Knock-Out Mice: Arachidonate 5-Lipoxygenase (P-5LO)

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Knock-Out Mice: Thrombopoietin

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Knock-Out Mice: Thrombospondin-1

Lawler J, Sunday M, Thibert V et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Critical Issues in Experimental Models

Shaker A. Mousa

Contents

General Introduction

Cardiovascular, cerebrovascular, and venous thromboembolic disorders continue to be the leading causes of death throughout the world. Over the past two decades, great advances have been made in the pharmacological treatment and prevention of arterial and venous thrombotic disorders (e.g., tissue plasminogen activators, platelet GPIIb/IIIa antagonists, and ADP receptor antagonists such as clopidogrel/prasugrel/ticagrelor, low molecular weight heparins, direct anti-Xa, and direct thrombin inhibitors). New research is leading to the next generation of antithrombotic compounds such as direct coagulation FVIIa inhibitors, tissue factor pathway inhibitors, gene therapy, and orally active direct thrombin inhibitors and coagulation factor Xa (FXa) inhibitors. In vitro assays as well as animal models of thrombosis have played and will continue to play crucial roles in the discovery and validation of novel drug targets, the selection of new agents for clinical evaluation, and the provision of dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. This comprehensive chapter presents the pivotal models that led to the development of drugs that have proven to be effective clinically.

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Critical Issues in Experimental Models

The major issues regarding the use of animal models of thrombosis – such as the use of positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species specificity, and pharmacokinetics – are highlighted. Finally, the use of genetic models in thrombosis/hemostasis research and pharmacology is presented.

Use of Positive Control

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, low molecular weight heparins, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel antithrombotic agents should certainly be required to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose–response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as activated partial thromboplastin time (APTT), prothrombin time (PT), template bleeding time, or other more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (1996), who compared the antithrombotic efficacy of argatroban and dalteparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early in vivo evaluation of compounds that demonstrate acceptable in vitro potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which poly-antithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

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Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well established in terms of efficacy, the preclinical tests for evaluating safety, i.e., bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic or thrombolytic therapy, stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhages are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (Stieg and Kase 1998; Sloan and Gore 1992). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays varies from compound to compound, so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled

incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (Bernardi et al. 1993; Bick 1995; Rodgers and Levin 1990). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg, i.v. bolus, plus 0.2 mg/kg/h (Antman and TIMI 9B Investigators 1996; GUSTO Investigators 1996). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/h), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with Xa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provide valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

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Selection of Models Based on Species-Dependent Pharmacology/Physiology

Species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (Cox et al. 1992) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (Bostwick et al. 1996; Cook et al. 1993; Panzer-Knodle et al. 1993). This discovery led to the widespread use of larger animals (particularly in dogs, whose platelet response to GPIIb/IIIa antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (1996) administered a GPIIb/IIIa antagonist orally and intravenously to rats, and then mixed the platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs.

The mixture was then evaluated in an agonist-induced platelet aggregation assay, and the resulting inhibition of canine platelet aggregation (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065a is much more potent against human FXa (Ki = 78 nM) than against rabbit (Ki = 102 nM) and rat (Ki = 1,980 nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, PTx2, was 0.52 μM) and in squirrel monkey plasma (PT × 2 = 0.46 μM), but was much less potent in rabbit, dog, and rat plasma (PT × 2 = 1.5, 6.5, and 22.2 μM, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (Tidwell et al. 1980; Nutt et al. 1991; Taniuchi et al. 1998). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by in vitro coagulation or platelet aggregation tests prior to evaluation in animal models.

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Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans (Kararli 1995). Due to

resource issues (mainly compound availability) and animal care and use considerations, small rodents, such as rats, are usually considered for primary in vivo evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or nonhuman primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of FXa inhibitor YM-60828 have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4 %, 33 %, 7 %, and 20 % in rats, guinea pigs, beagle dogs, and squirrel monkeys, respectively. Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (Sanderson et al. 1998) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

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Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. The influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in Table 1, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis, but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g., toxicity, narrow therapeutic window, or undesirable pharmacokinetics or pharmacodynamics) that are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. For detailed applications see the following references: Bugge et al. (1995), Bugge et al. (1996), Carmeliet et al. (1993), Carmeliet et al. (1994, 1996), Christie et al. (1999), Connolly et al. (1996), Cui et al. (1996), Evans et al. (1989), Healy et al. (1995), Herzog et al. (1999), Hodivala-Dilke et al. (1999), Huang et al. (1997), Kahn et al. (1998), Kay et al. (2000), Kung et al. (1998), Law et al. (1999), Leon et al. (1999), Lin et al. (1997), Offermanns et al. (1997), Ploplis et al. (1995), Subramaniam et al. (1996), Thomas et al. (1998), Toomey et al. (1996), Vassalli and Dichek (1997), Waugh et al. (1999), and Zoldhelyi et al. (2000).

Table 1 Animal models of thrombosis and their clinical correlates

Compound	Preclinical animal model	Preclinical results	Ref	Clinical indication	Clinical result	Ref
Recombinant tissue plasminogen activator (Activase)	Rabbit pulmonary artery thrombosis	Lysis of preformed pulmonary thrombus	Matsuo et al. (1981)	Acute myocardial infarction – thrombolysis	Improved recanalization	Collen et al. (1984)
Abciximab (ReoPro)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Coller et al. (1986), (1989)	High-risk coronary angioplasty	Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization	EPIC Investigators (1994)
Tirofiban (Aggrastat)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Lynch et al. (1995)	Unstable angina	Reduction in death, myocardial infarction, refractory ischemia	PRISM Investigators (1998)
Eptifibatide (Integrilin)	tPA-induced coronary thrombolysis	Significant improvement in lysis of occlusive thrombus	Nicolini et al. (1994)	Acute myocardial infarction – thrombolysis with tPA	Improvement in incidence and speed of reperfusion	Ohman et al. (1997)
Enoxaparin (Lovenox)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Lynch et al. (1995)	Unstable angina	Significant decrease in death, myocardial infarction, and need for revascularization at 30 days	Cohen et al. (1998)
Hirudin (Refludan)	Rabbit jugular vein thrombus growth	Inhibition of thrombus growth compared to standard heparin	Agnelli et al. (1990)	Deep vein thrombosis after total hip replacement	Significantly decreased rate of DVT	Eriksson et al. (1997)
Argatroban	Canine coronary artery electrolytic injury (tPA-induced thrombolysis)	Accelerated reperfusion and prevented reocclusion	Fitzgerald et al. (1989)	Unstable angina	No episodes of MI during drug infusion	Gold et al. (1993)

These are just some examples but not a full listing of all antithrombotics evaluated

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Safety Assays in Thrombosis and Hemostasis

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Advances in Anticogulants

Shaker A. Mousa

Contents

General Introduction

Utilizing the previously discussed in vitro and in vivo models for the evaluation of new anticoagulants resulted in recent advances in the development of novel oral anticoagulants (NOA). Major advances in the development of NOA progressed well, with the goal of developing safe and effective oral anticoagulants that do not require frequent monitoring or dose adjustment along with minimal food/drug interactions. Indirect inhibitors such as low-molecular-weight heparin (LMWH) and the pentasaccharide fondaparinux represent improvements over traditional drugs such as unfractionated heparin for acute treatment of venous thromboembolism (VTE) with more targeted anticoagulant approaches, predictable pharmacokinetic profiles, and lack of need for monitoring. Vitamin K antagonist, with its inherent limitations of multiple food and drug interactions and frequent need for monitoring, remains the only oral anticoagulant approved for long-term secondary thromboprophylaxis in VTE. The oral direct thrombin inhibitor ximelagatran was withdrawn from the world market due to safety concerns. Newer anticoagulant drugs such as injectables (fondaparinux, idraparinux), oral direct thrombin inhibitors (dabigatran), oral direct factor Xa inhibitors (rivaroxaban, apixaban, and others), and tissue factor/factor VIIa complex inhibitors are "tailor-made" to target specific procoagulant complexes and have the potential to greatly expand oral antithrombotic targets for both acute and

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long-term treatment of VTE, acute coronary syndromes, and prevention of stroke in atrial fibrillation patients.

The questions to be raised based on our early clinical experiences with the NOAs might include the following: Would the NOAs replace warfarin and would the NOAs replace heparin or LMWH?

Recent Advances in Novel Oral Anticoagulants (NOAs)

Warfarin and other vitamin K antagonists were the only class of oral anticoagulants available until 2009 for the prevention and treatment of thromboembolism. However, their narrow therapeutic window and regular INR monitoring due to food and drug interactions resulted in poor patient compliance and their underuse for stroke prevention (Birman-Deych et al. 2006; Hylek et al. 2007).

For the above reason and for over several decades, the search for effective, safe, and convenient oral anti-Xa or anti-IIa anticoagulant to replace warfarin continued (Figs. 1 and 2). The criteria for ideal NOAs were set forward where those NOAs have to be as effective and safe as warfarin, to be given at fixed doses once or twice a day, with rapid onset and offset, and without the need for monitoring (Connolly et al. 2009; Granger et al. 2011; Patel et al. 2011). See Tables 1 and 2 for the characteristics of NOAs.

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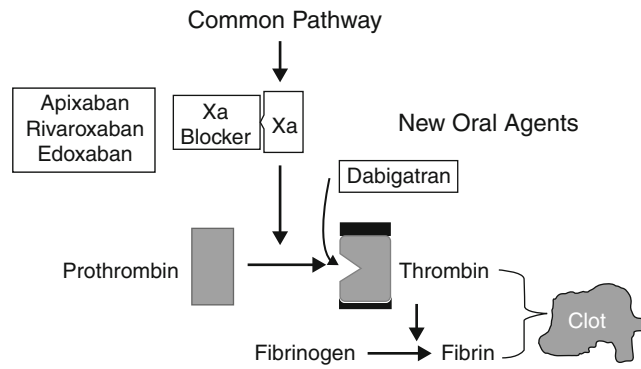

Fig. 1 Novel oral anticoagulants

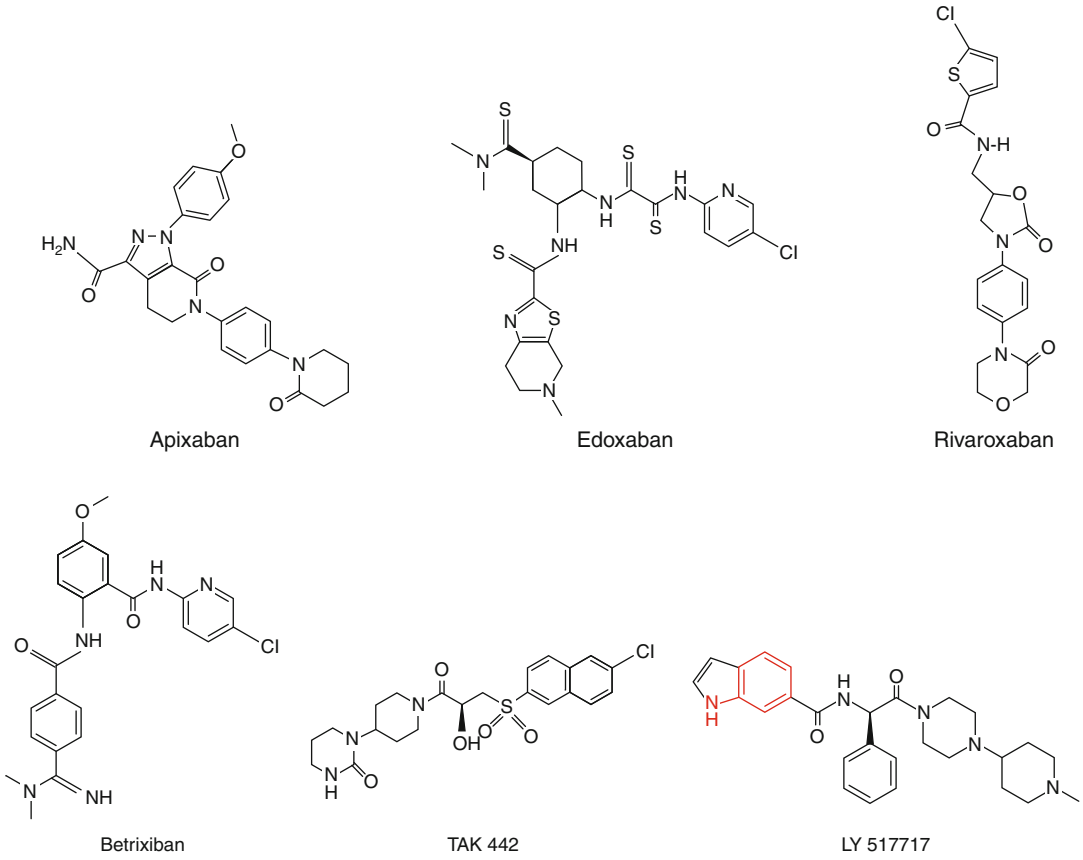

Fig. 2 Chemical structure of various oral anti-Xa agents

Table 1 Features of novel oral anticoagulants

Features	Rivaroxaban	Apixaban	Dabigatran etexilate
Target	Factor Xa	Factor Xa	Factor IIa
Molecular weight	436	460	628
Prodrug	No	No	Yes
Bioavailability (%)	60–80	50–80	6
Time to peak (h)	3	3	2
Half-life (h)	9	9–14	12–17
Renal excretion (%)	65	25	80
Antidote	Under development	Under development	Under development

Patel MR, Mahaffey KW, Garg J, Pan G, Singer DE, Hacke W, Breithardt G, Halperin JL, Hankey GJ, Piccini JP, Becker RC, Nessel CC, Paolini JF, Berkowitz SD, Fox KA, Califf RM, ROCKET AF Investigators (2011) Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. N Engl J Med 365:883–891

Oral Direct Thrombin Inhibitor

Dabigatran

Dabigatran etexilate is a prodrug that converts to the active direct thrombin inhibitor. The study, Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY), compared dabigatran versus

Table 2 Characteristics of novel oral anticoagulants

Immediate onset of action
Fixed dose
No laboratory coagulation monitoring
Minimal drug–drug/drug–food interactions
Short half-life, therefore no "bridging"
Many indications: venous thromboembolism (VTE) prevention/Rx; stroke prevention in atrial fibrillation, acute coronary syndrome

warfarin at targeted controlled international normalized ratio (INR) of 2.0 to 3.0 in patients who had AF and were at increased risk for stroke (Connolly et al. 2009; 2010; Ezekowitz et al. 2009; Larsen et al. 2013). Dabigatran at the two different doses tested (150 and 110 mg) was non-inferior to warfarin with respect to the primary efficacy outcome of stroke or systemic embolism.

Additionally, the rates of intracranial hemorrhage were significantly lower with both doses of dabigatran (150 and 110 mg) as compared to warfarin. However, there was an increase in the rate of gastrointestinal bleeding with the higher dabigatran dose, which might be due to its low oral bioavailability and large residence time in the GI (Connolly et al. 2010; Ezekowitz et al. 2009; Larsen et al. 2013).

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Oral Direct Anti-Xa

Rivaroxaban

Rivaroxaban **O**ral Direct Factor Xa Inhibition **C**ompared with Vitamin **K** Antagonist for Prevention of Stroke and **E**mbolism **T**rial in **A**trial **F**ibrillation (ROCKET AF), a multicenter trial, randomized patients to rivaroxaban (15–20 mg daily) or dose-adjusted warfarin, with a target INR of 2.0–3.0 (Patel et al. 2011). Rivaroxaban was found to be non-inferior to warfarin for the prevention of stroke or systemic embolism. Similar to dabigatran, bleeding from GI sites occurred more frequently in the rivaroxaban group (Piccini et al. 2013).

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Apixaban

Apixaban is another direct oral anti-Xa. **R**eduction **I**n **S**troke and **O**ther **T**hromboembo**L**ic **E**vents in Atrial Fibrillation (ARISTOTLE) was a multicenter trial, randomizing patients to apixaban (15–20 mg daily) or dose-adjusted warfarin, with a target INR of 2.0–3.0, where apixaban significantly reduced the risk of stroke or systemic embolism, major bleeding, and death as compared with warfarin and was associated with a reduction in the rate of GI bleeding (Goto et al. 2014; Hanna et al. 2014).

Reversal of anticoagulant effects of dabigatran, rivaroxaban, or apixaban is under intense clinical investigation (Liotta et al. 2015), but the safety of the reversal agents in tilting the balance toward promoting thrombosis needs to be carefully evaluated.

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Edoxaban

Edoxaban is an oral anticoagulant; The Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation-Thrombolysis in Myocardial Infarction 48 (ENGAGE AF-TIMI 48), in a randomized trial of edoxaban (30 or 60 mg) versus warfarin (INR 2–3), found both edoxaban regimens were non-inferior to warfarin for the prevention of stroke or systemic embolic event (Giugliano et al. 2013; Ogata et al. 2010). The incidence of hemorrhagic stroke, all types of bleed, and the rate of death from cardiovascular causes were significantly lower with both edoxaban regimens than with warfarin (Giugliano et al. 2013; Ogata et al. 2010). Attempts are under way to reverse the anticoagulant effects of edoxaban (Fukuda et al. 2012).

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Summary and Conclusions

Overall, all NOAs showed a favorable safety profile with lesser intracerebral hemorrhage versus warfarin but relatively greater GI bleeding with most NOAs versus warfarin. Another important concern is the absence of antidotes to rapidly reverse the NOAs in the case of life-threatening hemorrhage in contrast to warfarin. However, studies are currently underway to investigate various reversal agents for individual NOAs.

Another overall limitation to the use of the NOAs is short-term anticoagulant coverage due to the rapid offset of 12–24 h, especially if the patient missed a dose in comparison to the long-term anticoagulant coverage with warfarin. Additionally, the cost of NOAs is higher as compared to warfarin even after the costs associated with INR monitoring. However, further independent and objective cost-effectiveness analyses are necessary, which will likely further influence clinical decision making.

For all of these reasons, although NOAs are attractive alternatives, it is likely that warfarin will continue to be used worldwide in many patients with AF.

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Dabigatran

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Advances in Antiplatelets

Shaker A. Mousa

Contents

General Introduction

Considering the previously discussed in vitro and in vivo models, the past decade also witnessed major advances in oral antiplatelet therapies beyond aspirin and clopidogrel, which have been the mainstay for the past decade of antiplatelet management in acute coronary syndromes (ACS) and percutaneous coronary intervention (PCI). However, there is still a need for clinical development of newer and more potent antiplatelet drugs because atherothrombotic events continue to occur in a relevant proportion of populations despite this combination of aspirin and clopidogrel. Two of these drugs, prasugrel and ticagrelor, have been recently approved for clinical use. Other antiplatelet strategies are in clinical development.

Recent Advances in Oral Antiplatelets Utilizing Different Model Systems

Antiplatelet Therapy in the Prevention and Treatment of Cardiovascular Diseases

Aspirin in Primary Prevention of Cardiovascular Diseases

In a comprehensive worldwide meta-analysis of the 6 randomized trials of primary prevention, aspirin produces a statistically significant and clinically important reduction in risk of a first myocardial infarction by about 1/3.

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Dual Antiplatelet Therapy and Increased Risks of Bleeding

In a meta-analysis of 18 randomized trials that included 129,314 patients, those assigned to dual antiplatelet therapy have about a 50 % increase in risks of major bleeding compared with those given single agent therapy. The magnitude of these excess risks are about as high as the approximately 60 % increase observed in the trials comparing single antiplatelet agents to placebo. These excess risks of major bleeding should be considered in relation to the benefits on occlusive cardiovascular disease events in choosing the optimal antiplatelet strategy, especially for long-term treatment of patients with prior events or those at high risk of developing cardiovascular disease.

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Serebruany VL, Malinin AI, Ferguson JJ, Vahabi J, Atar D, Hennekens CH (2008) Bleeding risks of combination vs. single antiplatelet therapy: a meta-analysis of 18 randomized trials comprising 129 314 patients. Fundam Clin Pharmacol 22:315–321

Aspirin + Dipyridamole: Second European Stroke Study (ESPS-2)

A randomized, double-blind, placebo-controlled 2×2 factorial trial with 6602 patients with prior ischemic stroke or TIA was conducted. With acetylsalicylic acid (ASA) (25 mg bid) and/or dipyridamole (200 mg bid sustained release), deaths from stroke were reduced by 13 % by ASA ($p = 0.016$), 15 % by dipyridamole ($p = 0.039$), and 24 % by the combination of ASA and dipyridamole ($p < 0.001$).

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Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A (1996) European stroke prevention study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci 143(1–2):1–13

Clopidogrel + Aspirin

Clopidogrel is an improvement over ticlopidine in terms of safety profiles, and based on large-size trials, clopidogrel increased the benefit of aspirin as evident from the CURE trial, which was a randomized trial of acute MI. It showed that clopidogrel adds to the benefit of aspirin in cardiovascular disease events but increased major bleeding. Figure 1 shows the structure of clopidogrel and ticlopidine as well as the latest approved adenosine diphosphate (ADP) receptor antagonists.

In contrast, COMMIT/CCS-2, a randomized trial of acute coronary syndromes in China, showed that clopidogrel adds to the benefit of aspirin in cardiovascular disease and total mortality but did not increase major bleeding.

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The CHARISMA trial, a randomized, double-blind, placebo-controlled trial of 15,603 patients (79 %) with established cardiovascular disease and 21 % with multiple risk factors, was designed to test whether clopidogrel should be continued beyond 1 year in addition to aspirin. All patients

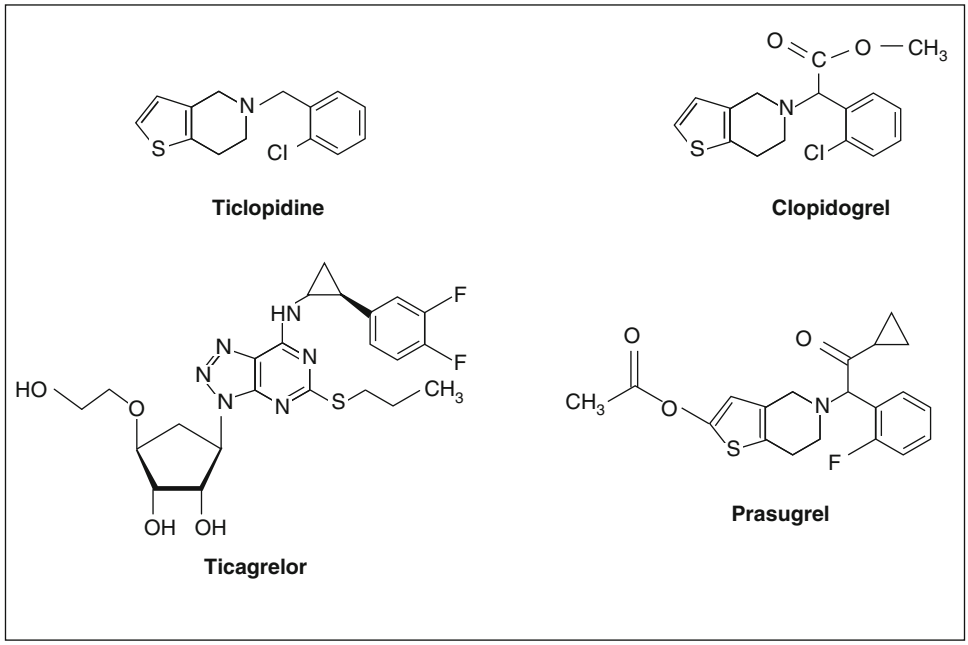

Fig. 1 Structure of approved ADP receptor antagonists

received daily aspirin (75–162 mg) and were randomized to daily clopidogrel (75 mg) or placebo. Clopidogrel patients had an event rate of 6.8 % and placebo patients had an event rate of 7.3 %. CHARISMA demonstrated *no significant benefit long term when clopidogrel was added to aspirin*. Rates of severe bleeding were similar, but clopidogrel patients experienced significantly higher rates of moderate bleeding.

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Issues with clopidogrel include a delayed onset of 4–6 h (after loading dose with 8 × maintenance dose) and offset of 5–7 days. Additionally, variable response is 25–30 % of patients achieve less than 25 % inhibition of platelet activity, and clopidogrel must undergo a 2-step metabolism (CYP3A4 mediated) to the active agent.

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Beyond Clopidogrel

Prasugrel

Prasugrel is a thienopyridine irreversible inhibitor of the P2Y12 receptor (Fig. 1), with a more rapid onset of action than clopidogrel. The Triton-TIMI 38 trial enrolled 13,608 patients with moderate- to high-risk acute coronary syndromes with scheduled PCI. Patients were randomized to prasugrel (60 mg loading dose and a 10 mg daily maintenance dose) or clopidogrel (300 mg loading dose and a 75 mg daily maintenance dose) for 6–15 months. Prasugrel therapy was associated with

significantly reduced rates of ischemic events, but with an increased risk of major bleeding, including fatal bleeding. Overall mortality did not differ significantly between treatment groups.

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Ticagrelor

Ticagrelor is a reversible ADP receptor antagonist (Fig. 1) indicated for use in patients with acute coronary syndromes. Similar to prasugrel, ticagrelor has a rapid onset of action and provides greater inhibition of platelet aggregation than clopidogrel. The PLATO study was a well-designed, double-blind, large (18,624 adult patients with acute coronary syndromes), randomized trial of ticagrelor (180 mg loading dose, 90 mg twice daily thereafter) versus clopidogrel (300–600 mg loading dose, 75 mg daily thereafter), and all patients received ASA 75–325 mg. The results showed that 12 months of treatment with ticagrelor was more effective than clopidogrel in reducing the incidence of the primary composite endpoint of myocardial infarction, stroke, or cardiovascular death. Ticagrelor was generally well tolerated and was not associated with an increased risk of major bleeding relative to clopidogrel. However, the incidences of noncoronary artery bypass grafting-related bleeding and major or minor bleeding, as well as some nonhemorrhagic adverse events, were higher with ticagrelor. There is no head-to-head data among the different ADP receptor antagonists.

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Clopidogrel + Aspirin

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Prasugrel

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Ticagrelor

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Part III

Activity in Urinary Tract

Diuretic and Saluretic Activity

Susan Emeigh Hart

Contents

Diuretic and Saluretic Activity

In Vitro Methods

Carbonic Anhydrase Inhibition In Vitro

Purpose and Rationale

Acetazolamide (Diamox) was one of the first synthetic nonmercurial diuretics. The mode of action was found to be inhibition of carbonic anhydrase. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration (or hydroxylation) of CO_2 to form H_2CO_3 which dissociates nonenzymatically into HCO_3^- and H^+. The enzyme is located within the cytoplasm and at the apical and basolateral membranes of proximal tubules as well as on the apical (lumenal) surface of distal tubules and in the thick ascending limb of the loop of Henle. Its primary function is to enhance H^+ secretion into the urine. At least three isoenzymes, designated as I, II, and II or A, B, and C, are known to exist.

Carbonic anhydrase inhibitors were originally used as diuretics but are no longer used for this purpose except in limited circumstances (metabolic alkalosis accompanied by edema). Chronic inhibition of carbonic anhydrase results in significant hypokalemia and metabolic acidosis due to bicarbonate wasting, and thus inhibition of carbonic anhydrase may be mechanistically important if these effects are noted in in vivo studies or clinically (Okusa and Ellison 2000). Numerous isoenzymes exist, of which CA II is the most

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abundant in proximal tubules and erythrocytes. The activity of carbonic anhydrase in erythrocytes is significantly higher than that in the kidney, and the enzyme is easily harvested by lysing mammalian whole blood. Thus, examination of the degree of inhibition of erythrocyte carbonic anhydrase may serve as a suitable surrogate for examination of effects on renal activity (Maren 1967; Armstrong et al. 1966).

In spite of the fact that newer diuretics are based on other modes of action, the test for inhibition of carbonic anhydrase should be performed for evaluation of a new compound. Moreover, the specific use of carbonic anhydrase inhibitors as antiglaucoma drugs has been described (Friedland and Maren 1984; Caprioli 1985). The mechanism by which carbonic anhydrase inhibitors lower intraocular pressure is through a reduction in aqueous humor formation, by affecting electrolyte and water balance in the nonpigmented ciliary epithelium (Friedland and Maren 1984; Caprioli 1985). Although many methods to measure carbonic anhydrase activity have been developed (Philpot and Philpot 1936), the micro method described by Maren (1960) is relatively simple, sensitive, and reliable. The enzyme sources are red cells, a rich source of the same isoenzymes found in the eye (Maren 1967; Armstrong et al. 1966; Wistrand et al. 1986; Wistrand and Knuuttila 1980).

Procedure

The analytical method is based on the catalysis of the conversion of CO_2 to H_2CO_3 by the enzyme, with resulting decrease in pH being monitored colorimetrically (Philpot and Philpot 1936).

Materials and Solutions

- phenol red indicator solution:
- 12.5 mg phenol red/l 2.6 mM $NaHCO_3$, pH 8.3 + 218 mM Na_2CO_3
- 1 M sodium carbonate/bicarbonate buffer, pH 9.8
- Enzyme: carbonic anhydrase from dog blood; blood is collected into a heparinized tube and diluted 1:100 with deionized water.
- Equipment
  - Reaction vessel – custom made by Labglass Inc., Vineland, NJ, USA
  - Monostat bench-mounted flowmeter
  - 30 % CO_2 – M& G Gases, Branchburg, NJ, USA

Assay

CO_2 flow rate is adjusted to 30 (45) ml/min. The following solutions are added to the reaction vessel:

400 µl phenol red indicator solution

100 µl enzyme

200 µl H_2O or appropriate drug concentration after 3 min for equilibration

100 µl carbonate/bicarbonate buffer is added.

The following parameters are determined in duplicate samples:

T_u = (uncatalyzed time) = time for the color change to occur in the absence of enzyme

T_e = (catalyzed time) = time for the color change to occur in the presence of the enzyme

T_u–T_e = enzyme rate

T_i = enzyme rate in the presence of various concentrations of inhibitor

Evaluation

Percent inhibition of carbonic anhydrase is calculated according to the following formula:

$$\%\,\text{Inhibition} = 1 - \frac{(T_u - T_e) - (T_i - T_e)}{T_u - T_e} \times 100$$

Standard data:

- Compound IC_{50} [M]
- Acetazolamide 9.0×10^{-9}
- Chlorothiazide 9.0×10^{-7}

Critical Assessment of the Method

Determination of carbonic anhydrase inhibition is of value to characterize the activity spectrum of sulfonamide diuretics.

Modifications of the Method

Landolfi et al. (1997) reported a modified procedure for the measurement of carbonic anhydrase

activity. The measure of carbonic anhydrase activity is based on the rate of CO_2 hydration by the enzyme. Such transformation was monitored by a procedure which consists in the measure of time necessary for the pH of an appropriate buffer to decrease from 8 to 7.5 in the presence of a constant CO_2 flow: such time period is dose-dependently reduced by the addition of the enzyme and further modified in the presence of carbonic anhydrase inhibitory compounds. The time required for a specific change in pH can be determined electrochemically as opposed to colorimetrically. This requires the use of a well-calibrated pH meter with a fast-reacting electrode. Activity is expressed in enzyme units, defined as a doubling of the time needed for the pH change in the absence of enzyme. These methods are much more sensitive than the colorimetric determination (Maren 1960; Landolfi et al. 1997; Lukasi 2005).

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In Vivo Methods

Diuretic Activity in Rats (LIPSCHITZ Test)

Purpose and Rationale

A method for testing diuretic activity in rats has been described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animals and compared to rats treated with a high dose of urea. The "Lipschitz-value" is the quotient between excretion by test animals and excretion by the urea control.

Procedure

Male Wistar rats weighing 100–200 g are used. Three animals per group are placed in metabolic cages provided with a wire mesh bottom and a funnel to collect the urine. Stainless-steel sieves are placed in the funnel to retain feces and to allow the urine to pass. The rats are fed with standard diet (Altromin pellets) and water ad libitum. Fifteen hours prior to the experiment, food and water

are withdrawn. Three animals are placed in one metabolic cage. For screening procedures, two groups of three animals are used for one dose of the test compound. The test compound is applied orally at a dose of 50 mg/kg in 5.0 ml water/kg body weight. Two groups of three animals receive orally 1 g/kg urea. Additionally, 5 ml of 0.9 % NaCl solution per 100 g body weight are given by gavage. Urine excretion is recorded after 5 and after 24 h. The sodium content of the urine is determined by flame photometry. Active compounds are tested again with lower doses.

Evaluation

Urine volume excreted per 100 g body weight is calculated for each group. Results are expressed as the "Lipschitz-value," i. e., the ratio T/U, in which T is the response of the test compound and U that of urea treatment. Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found. Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect. Similar to urine volume, quotients can be calculated for sodium excretion. Dose–response curves can be established using various doses. Loop diuretics are characterized by a steep dose–response curve. Saluretic drugs, like hydrochlorothiazide, show Lipschitz values around 1.8, whereas loop diuretics (or high ceiling diuretics) like furosemide, bumetanide, or piretanide reach values of 4.0 and more.

Critical Assessment of the Method

The Lipschitz test has been proven to be a standard method and a very useful tool for screening of potential diuretics.

Modifications of the Method

The method has been modified in various ways by several authors. Cummings et al. (1960) recommended a sequential procedure with criteria for acceptance or rejection of test drugs. Kau et al. (1984) recommended a method for screening diuretic agents in the rat using normal saline (4 % body weight) as hydrating fluid.

Homozygous Brattleboro rats exhibit symptoms of diabetes insipidus (Valtin et al. 1965). The condition is due to the failure of hypothalamic neurons to produce vasopressin, which is due to a single base point deletion in the vasopressin gene (Schmale and Richter 1984). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus is reversed by a vasopressin agonist (Laycock et al. 1994).

These animals can be used to study vasopressin agonism and antagonism and the aquaretic effects of synthetic drugs.

Klatt et al. (1975) described a method of collecting urine excreted by cats. On the basis of urine funnel used in rats, an appropriate larger metabolism cage made out of transparent, rigid polyvinyl chloride was used. The cage was improved by a built-in sieve cone, which assured good separation of urine and feces. A device to measure and record the time and amount of voided urine was attached. Urine was collected in a vessel with a hose connection from the bottom to a pressure sensor. An attached overflow tube could be occluded. The initial pressure of the sensor was fed into a linear recorder. Before the test, the recorder was calibrated with a sufficient amount of distilled water to adjust the number of division intervals for direct measurement of voided urine in milliliters. This allowed calculation of the time point of voiding from the chart speed.

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Saluretic Activity in Rats

Purpose and Rationale

Excretion of electrolytes is as important as the excretion of water for treatment of peripheral edema and ascites in congestive heart failure as well as for treatment of hypertension. Potassium loss has to be avoided. As a consequence, saluretic drugs and potassium-sparing diuretics were developed. The diuresis test in rats was modified in such a way that potassium and chloride as well as osmolality are determined in addition to water and sodium. Ratios between electrolytes can be calculated indicating carbonic anhydrase inhibition or a potassium-sparing effect.

Procedure

Male Wistar rats weighing 100–200 g fed with standard diet (Altromin pellets) and water ad libitum are used. Fifteen hours prior to the test, food but not water is withdrawn. Test compounds are applied in a dose of 50 mg/kg orally in 0.5 ml/100 g body weight starch suspension. Three animals are placed in one metabolic cage provided with a wire mesh bottom and a funnel to collect the urine. Two groups of three animals are used for each dose of a test drug. Urine excretion is registered every hour up to 5 h. The 5-h urine is analyzed by flame photometry for sodium and potassium and argentometrically by potentiometric endpoint titration (Chloride-Titrator Aminco) for chloride. To evaluate compounds with prolonged effects, the 24 h urine is collected and analyzed. Furosemide (25 mg/kg p.o.), hydrochlorothiazide (25 mg/kg p.o.), triamterene (50 mg/kg p.o.), or amiloride (50 mg/kg p.o.) are used as standards.

Evaluation

- The sum of Na^+ and Cl^- excretion is calculated as parameter for saluretic activity.
- The ratio Na^+/K^+ is calculated for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect. Ratios greater than 10.0 indicate a potassium-sparing effect.
- The ratio

$$\frac{Cl^-}{Na^+ + K^+}$$

(ion quotient) is calculated to estimate carbonic anhydrase inhibition.

- Carbonic anhydrase inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios, slight to strong carbonic anhydrase inhibition can be assumed.

Modifications of the Method

Adrenalectomized rats treated with DOCA or aldosterone can be utilized to test **aldosterone antagonists**. Spironolactone has no effect in the absence of a mineralocorticoid but reverses in a dose-related manner the effect of DOCA on the Na^+/K^+ ratio in the urine (Kagawa et al. 1957; Bicking et al. 1965).

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Diuretic and Saluretic Activity in Dogs

Purpose and Rationale

Dogs have been extensively used to study renal physiology and the action of diuretics. Renal physiology of the dog is claimed to be closer to man than that of rats. Oral absorbability of diuretic substances can appropriately be studied in dogs. Using catheters, interval collections of urine can be made with more reliability than in rats. Simultaneously, blood samples can be withdrawn to study pharmacokinetics.

Procedure

Beagle dogs of either sex have to undergo intensive training to be accustomed to accept gavage feeding and hourly catheterization without any resistance. The dogs are placed in metabolic cages. At least four dogs are used as controls receiving water only, as standard controls (1 g/kg urea p.o. or 5 mg/kg furosemide p.o.), or the test drug group. Twenty-four hours prior to the experiment, food but not water is withheld. On the morning of the experiment, the urine bladder is emptied with a plastic catheter. The dogs receive 20 ml/kg body weight water by gavage, followed by hourly doses of 4 ml/kg body weight drinking water. The bladder is catheterized twice in an interval of 1 h and the urine collected for analysis of initial values. Then, the test compound or the standard is applied either orally or intravenously. Hourly catheterization is repeated over the next 6 h. Without further water dosage, the animals are placed in metabolic cages overnight. Twenty-four hours after dosage of the test compound, the dogs are catheterized once more and this urine together with the urine collected overnight in the metabolic cage registered. All urine samples are analyzed by flame photometry for sodium and potassium and by argentometry (Chloride Titrator Aminco) for chloride content. Furthermore, osmolality is measured with an osmometer.

Evaluation

Urine volume, electrolyte concentrations, and osmolality are averaged for each group. The values are plotted against time to allow comparison with pretreatment values as well as with water controls and standards. The nonparametric *U* test is used for statistical analysis.

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Assessment of Renal Function

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In Vitro Methods

Patch Clamp Technique in Kidney Cells (Contribution by H. Gögelein)

Purpose and Rationale

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts), fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). Besides active transport and coupled transport systems, ion channels play an important role in the function of kidney cells. The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode) (Neher and Sakmann 1976; Hamill et al. 1981) allow the investigation of ion channels. In addition, the investigation of other electrogenic transport mechanisms, such as the sodium-coupled alanine transport, can be studied.

Procedure

The patch clamp technique can be applied to cultured kidney cells (Merot et al. 1988), to freshly isolated kidney cells (Hoyer and Gögelein 1991), or to cells of isolated perfused kidney tubules (Gögelein and Greger 1984). The latter method shall be described in more detail.

Segments of late superficial proximal tubules of rabbit kidney are dissected and perfused from one end with a perfusion system (Burg et al. 1966; Greger and Hampel 1981). The non-cannulated end of the tubule is freely accessible to a patch

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pipette. Under optical control (differential interference contrast optics with 400×magnification), the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell-excised mode (Gögelein and Greger 1984; Gögelein and Greger 1986a).

In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 μm). As to facilitate the tearing off, the tubules are incubated for about 5 min in 0.5 g/l collagenase (Sigma, C 2139) at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and gigaseals can be obtained. It was possible to investigate potassium channels (Gögelein and Greger 1987) and nonselective cation channels (Gögelein and Greger 1986b) in these membranes.

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. On the other hand, cotransport systems can only be investigated by the whole-cell method because the transport rate of a single event is much too small to be resolved in a similar manner as single ion channel events. Consequently, cells of rabbit proximal tubules are isolated as described in detail elsewhere (Hoyer and Gögelein 1991; Heidrich and Dew 1977). After cervical dislocation, the kidneys are rapidly excised and placed in ice-cold solution [mmol/l]: 150 K-cyclamate, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.4. The following steps are performed on ice: After decapsulation, superficial cortical slices of about 0.5 mm thickness are dissected and minced with a scalpel. The tissue is homogenized in a Dounce homogenizer by three strokes with a loose-fitting pestle. The homogenate is then poured through graded sieves (250, 75, and 40 μm) to obtain a population of single cells. Since the predominant tubule section of the cortex of the rabbit kidney is the pars convoluta of the proximal tubule, it can be concluded that the majority of the isolated cells in the cell suspension are of proximal tubule origin. By light microscopy, cells are identified by long microvilli distributed over the entire cell surface and can easily be discriminated from remaining erythrocytes, cell detritus, and tubular fragments.

By application of the whole-cell mode of the patch clamp technique to freshly isolated cells of convoluted proximal tubules, the sodium-alanine cotransport system could be investigated in detail (Hoyer and Gögelein 1991).

Evaluation

In isolated perfused renal tubules, concentration response curves of drugs which inhibit ion channels can be obtained with the patch clamp technique. In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent K_m values for sodium and L-alanine can be recorded.

Modifications of the Method

Schlatter (1993) recorded membrane voltages of macula densa cells with the fast or slow whole-cell patch-clamp method. The effects of diuretics and the conductance properties of these cells were examined.

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Perfusion of Isolated Kidney Tubules (Contribution by R. Greger (first edition) and M. Bleich (second edition))

Purpose and Rationale

The various tubule segments such as proximal tubule (PT, S1–S3), descending thin limb of the loop of Henle (DTL), ascending thin limb of the loop of Henle (ATL), thick ascending limb of the loop of Henle (TAL), distal convoluted tubule (DCT), connecting tubule (CNT), cortical collecting duct (CCD), medullary collecting duct (MCD), and papillary collecting duct (PCD) have different functional properties. The in vitro perfusion of isolated tubule segments (Burg et al. 1966) is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies.

Procedure

After its invention by Burg et al. (1966), this technique has been used successfully in the kidney tubule segments of several species: man, rabbit, rat, mouse, hamster, snake, birds, etc. The tubule segments are dissected from thin kidney slices (<1 mm thickness). Usually dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase). The segment is identified by its anatomical location and by its appearance. A 20–50 × lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study. PT, TAL, DCT, CNT, CCD, and MCD can all be dissected quite easily. The dissection of DTL, ATL, and PCD is much more difficult because these segments are damaged easily by the mechanical dissection. Dissection is usually performed at 4 °C in a Ringer type solution.

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400×). The chamber is usually kept at 37 °C, and the bath perfusate is also preheated to this temperature. The bath perfusate will depend on the tubule segment under study. In most instances, it will contain HCO_3^- and will be bubbled with CO_2. The metabolic substrate will be acetate for PT and D-glucose for TAL, CCD, etc. The actual perfusion is performed with two sets of concentric glass pipettes: one set at the perfusion end and other at the collection end of the segment. These pipettes are manufactured with special glass forges. The most refined one has been designed by Hampel and Greger. The glass tube is rotated at approximately 1 rps and is moved in perpendicular direction by a remote control, and the heating filament is moved in xy

direction also by a remote control. The shaping of the glass is observed continuously by a lens (5–50×). The pulling force is provided by weights fixed on the lower end of the glass tube. The pipettes are cut either by a diamond or by the pulling force of a small glass bead, fixed on the edge of a vertical platinum filament and melted sidewise on the pipette. When the heating current of the platinum filament is switched off, the filament retracts and brakes the pipette at the desired site. Greger and Hampel (1981) have modified the original perfusion system of Burg and coworkers. Their device is optimized inasmuch as it guarantees concentric alignment of the various pipettes. The forward and backward movement is controlled by small electric motors. At the perfusion side, they use four concentric pipettes. The outermost one contains sylgard and is driven over the perfused end of the tubule in order to seal this end. The tubule is held by a holding pipette with appropriate dimensions. The tubule is sucked into this pipette up to the constriction. Then the perfusion pipette with a tip diameter smaller than the inner diameter of the perfused segment is advanced into the segment held by the holding pipette. The perfusion pipette is put under hydrostatic pressure of a few to 100 cm to achieve a perfusion rate of 1–20 nl/min. Usually, the collapsed tubule lumen opens when the perfusion pipette is advanced. The pipette is advanced in the lumen until it reaches an area of the segment where it appears intact by inspection (200–400×). Within its lumen the perfusion pipette contains yet another pipette, the fluid exchange pipette. With this pipette the composition of the perfusate can be replaced very rapidly (Greger and Schlatter 1983). The collection end of the tubule segment is sucked into a holding pipette. A sylgard pipette is advanced to seal the collection site. The holding pipette at the collection site will contain mineral oil in flux measurements. Then a collection pipette is advanced through the oil to quantitatively collect the perfusate delivered by the tubule.

The **measured parameters** can be as follows:

Flux measurements (Schafer et al. 1974). The collection rate (V_c, nl/min) can be measured by the constant bore collection pipette by timed collections. Radioactive tracers can be added to the lumen or bath fluid. For instance, radioactively labeled inulin can be added to the perfusate (In_p) and can be used to measure volume absorption (ΔV = perfusion rate (V_i–V_c)). Unidirectional fluxes, bath to lumen and lumen to bath, for any given substance can be quantified, and permeabilities (P_x) can be determined:

$$P_x = (V_i - V_c)L^{-1}\left[\ln\left(x_p \mathrm{In}_c x_c^{-1} \mathrm{In}_p\right) + 1\right]$$

where L is the length of the segmen, x_p and x_c are the concentrations of x in the perfusate and in the collected fluid, and In_c is the inulin concentration in the collected fluid. Net fluxes of x can be determined as the difference of the unidirectional fluxes or by the chemical determination of Δx (perfusate–collected fluid). This requires very sensitive methods. Electron probe analysis of Na^+, K^+, Ca^{2+}, Mg^{2+}, etc. has been used to determine the net transport of these ions in various tubule segments (Wittner et al. 1988). Flux studies are usually performed at low luminal perfusion rates of a few nl/min. Substances under study can be added to the luminal and bath perfusate, and paired data can be obtained under control and experimental conditions (Burg and Green 1973; Stoner et al. 1974; Burg and Orloff 1980; Burg and Stoner 1976; Dillingham et al. 1993).

Transepithelial electrical measurements. The perfusion pipette can be connected to the high impedance input of an electrometer. The voltage is referenced to the grounded bath. The connections are usually made with agar bridges (80 g agar in 1 l Ringer's solution) and appropriate corrections for liquid junction voltages must be applied. With identical solutions in the bath and in the lumen and with high luminal perfusion rates (>10 nl/min), any transepithelial voltage (V_{te}) must be caused by active transport = active transport potential (Frömter 1984). Hence, the effectiveness of putative inhibitors of active transport can also be examined by the measurement of V_{te}. According to Ohm's law, the determination of the flux of ions also requires the measurement of transepithelial resistance. Greger (1981) has introduced a method which utilizes a dual channel perfusion pipette, made of Q-shaped glass. One channel is used for perfusion and the other for

current (I_{te}) injection. The current is defined by a resistor chosen such that the deflection in V_{te} generated by this pulse is in the order of 10–20 mV. Transepithelial resistance (R_{te}) can now be calculated from ΔV_{te} and I_{te}. The ratio of V_{te} and R_{te} is called equivalent short circuit current. It is directly proportional to active transport (Greger 1985). The measurement of V_{te} and R_{te} is much more efficient than flux studies for pharmacological screening, provided that the process under study produces a transepithelial voltage. Several substances can be examined in one single tubule in strictly paired fashion (Schlatter et al. 1983; Wangemann et al. 1986). The time resolution of the measurements is on the order of 1 s, whereas that of flux studies is several minutes at best.

Intracellular electrical measurements. Greger and Schlatter (1983) have developed a method for the use of impalement techniques in the isolated perfused tubule. Very fine tip microelectrodes (Ø < 100 nm) are used to impale the tubule cell across the basolateral membrane. The actual impalement is performed by a piezo stepper, which accelerates the microelectrode to high speed and makes it possible to penetrate the rigid basal membrane. The simultaneous measurement of V_{te}, R_{te}, and basolateral membrane voltage (V_{bl}) allows for a complete analysis of voltages and resistances (Greger 1985; Ullrich and Greger 1985). Ion selective microelectrodes can also be used in impalement studies, and the cytosolic ion activities, for example Na^+, K^+, and Cl^-, can also be determined (Greger 1985). These methods are all rather difficult to perform. They are of high relevance for the understanding of the function of a given tubule segment and for the detailed description of the mechanism of action of a drug, which in preceding studies have been shown to act in a given tubule segment.

Patch clamp studies. The combination of in vitro perfusion of renal tubules and patch clamp analysis of ion channels in the luminal and basolateral membranes is described in section "Patch Clamp Technique in Kidney Cells."

Fluorescent dyes in the isolated perfused tubule. Several fluorescent dyes for the monitoring of Na^+, K^+, Cl^-, Ca^{2+}, and pH have become available during the past few years. These dyes can be used in the in vitro perfused tubule (Nitschke et al. 1991). The inverted microscope is equipped with an appropriate illumination and filter wheel for excitation. The emission is measured by photon counting or by a video camera. When compared with impalement methods, these techniques are probably easier for routine use.

Evaluation

For each of the above protocols, paired measurements of one or several given parameters of tubule transport are obtained under control conditions and in the presence of a substance under study. Also concentration response curves can be obtained in one single preparation (Schlatter et al. 1983; Wangemann et al. 1986; Wittner et al. 1987). Intracellular measurements are usually required to define the mechanism of action (Greger 1985). Especially, the electrical and optical measurements have a very high reproducibility. For screening, usually three preparations are sufficient. Approximately ten preparations are required for concentration response curves.

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Isolated Perfused Kidney

Purpose and Rationale

Isolated kidney is a good tool for studying proximal tubule, but of limited value for distal tubule function. The kidney can be perfused in situ and isolated in vitro. The isolated kidney can be perfused by a pump using blood or plasma-like solutions. One specific problem of the blood-perfused dog kidney in vitro is its instability. After only 1 h of perfusion, glomerular filtration and renal blood flow decline markedly. It was reported that in situ-perfused isolated dog kidney seems to be more stable. In isolated perfused rat, kidney plasma-like solutions are used for perfusion. This system, by inclusion of a dialyzing unit, provides optimal conditions for maintaining a constant electrolyte composition of the perfusate. However, function of distal tubule is also grossly impaired in this rat model. The isolated kidney does not acidify tubular fluid, and the concentrating ability is reduced.

Procedure

Kidneys are obtained from anaesthetized male rats with a body weight of 300–400 g. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a ventricular incision, the right ureter is cannulated with PE-50 polyethylene tubing and heparin is injected into the vena cava (500 U/kg body weight). The venous cannula is introduced into the vena cava below the right renal

vein. The right kidney is freed from the perirenal fat, not disrupting the renal capsule. The renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution fed from the gravity system situated 130 cm above the cannula. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. A perfusion pressure of 80–90 mmHg in the renal artery is maintained by adjusting the speed of the perfusion pump. For more details, see references.

Evaluation

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected, and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, ^{3}H-labelled polyethylene glycol is added to a modified Krebs-Henseleit bicarbonate buffer. Electrolytes are determined in urine by standard flame photometry. Fractional excretions of water, electrolytes, and test compounds are calculated.

Limitations of the Method

Isolated perfused dog kidneys have been reported to be less stable than those of other species, with glomerular filtration and renal flow markedly decreasing after only 1 h of perfusion. The in situ-perfused isolated dog kidney seems to be more stable. Distal tubule functions are impaired in isolated perfused kidneys in all species. Urine acidification, concentration, and dilution functions are also abnormal, and effects on these cannot be assessed in these models (Kirkpatrick and Gandolfi 2005).

Modifications of the Method

Tarako et al. (1991) evaluated oxygen supply and energy state in the isolated perfused rat kidney.

Metabolic activities of the isolated perfused rat kidney were described by Nishiitsutsuji-Uwo et al. (1967).

Cox et al. (1990) used the isolated perfused rat kidney as a tool in the investigation of renal handling and effects of nonsteroidal anti-inflammatory drugs.

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In Vivo Methods

Clearance Methods

Purpose and Rationale

Investigations of clearance represent indirect methods for the evaluation of renal function and provide information on the site of action of

diuretics and other pharmacological agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convolution, would augment the clearance of solute-free water (C_{H2O}) during water diuresis and the reabsorption of solute-free water (T_{CH2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both C_{H2O} and T_{CH2O}. On the other hand, drugs that act only in the distal tubule would reduce C_{H2O} but not T_{CH2O}.

Procedure

Clearance experiments are performed either in conscious or anaesthetized beagle dogs under conditions of water diuresis and hydropenia. The status of water diuresis and hydropenia may be accomplished as described by Suki et al. (1965). Water diuresis is induced by oral administration of 50 ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5 % glucose solution and 0.58 % NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter. Blood samples are obtained in the middle of each clearance period. After the control period, compounds to be tested are administered and further clearance tests are performed.

Hydropenia is induced by withdrawing the drinking water 48 h before experiment. On the day before the experiment, 0.5 U/kg body weight of vasopressin in oil is injected intramuscularly. On the day of the experiment, 20 mU/kg vasopressin is injected i.v., followed by infusion of 50 mU/kg per hour vasopressin. To accomplish constant urine flow, 5 % NaCl solution is infused at 1 ml/min per kg body weight up to i.v. administration of a compound to be tested, followed by i.v. infusion of 0.9 % NaCl solution at a rate equal to the urine flow. Glomerular filtration rate (GFR) and renal plasma flow (RPF) are measured by the clearance of inulin and *para*-aminohippurate, respectively. Therefore, appropriate infusion of inulin (bolus of 0.08 g/kg followed by infusion of 1.5 mg/kg per min) and *para*-aminohippurate (bolus 0.04 g/kg followed by infusion of 0.3 mg/kg per min) are initiated. Inulin and *para*-aminohippurate are measured according to Walser et al. (1955) and Smith et al. (1945), respectively.

Tracers are administered intravenously to achieve near steady-state concentrations; a priming dose loads the plasma and extracellular compartments, and subsequent infusion replaces renal losses. Once steady state plasma tracer levels are approached, a series of timed urine collections (clearance periods) are performed, with blood samples collected at the either the midpoint or beginning and end of the clearance periods. The urine and blood (plasma or serum) samples are analyzed for tracer(s) and the test compound.

Tracers for the determination of GFR must be freely filtered and then neither secreted nor reabsorbed. This allows the assumption that the amount of plasma cleared of the tracer per unit time represents that which has been filtered through the glomeruli (i.e., GFR). The fructose polysaccharide inulin (mw $\sim$ 5,200) is the most commonly used tracer in all species and serves as the "gold standard" to which others are compared (Finco 1983; Ragan and Weller 1999). Other indicators include isotopes of vitamin B_{12}, sodium iodothalamate, iohexol, and radiolabelled metal chelates of ethylenediamintetracetate (EDTA) and diethylaminotriaminepentaacetate (DPTA) (Sarkar et al. 1988, 1991; Gaspari et al. 1997; Ragan and Weller 1999).

Tracers for the assessment of renal plasma flow must be completely cleared (combination of filtration and efficient tubular secretion to the urine) on first pass through the kidney. This allows the assumption that the volume of plasma cleared per unit time represents that which was either filtered by the glomeruli or bypassed the glomeruli and perfused the tubules. *p*-Aminohippurate (PAH) is used for the assessment of RPF, because it is both freely filtered by the glomeruli and actively secreted by the organic acid transport

pathway of the proximal tubule. First-pass PAH extraction by the kidneys varies from about 70 % to 90 % in rats, dogs, and humans (Brenner et al. 1976), but for the purpose of estimating RBF it is assumed to be 100 %. Using this assumption, RPF is always slightly underestimated. Tetraethylammonium bromide (TEA), a substrate for the renal cation transporter, may also be used, and is subject to the same limitations (Ragan and Weller 1999).

Inulin is measured colorimetrically, either by acid hydrolysis to generate a green product or by a series of enzymatic reactions based on inulinase with subsequent reduction of NADH. HPLC methods are used for the remaining exogenous GFR tracers. PAH and TEA are measured colorimetrically (Newman and Price 1999).

Evaluation

Renal clearance (Cl) of any compound (X) can be determined by comparing the urinary excretion rate of compound X to the plasma concentration of compound X.

The urinary excretion rate is calculated as:

$$\text{Urinary excretion rate (mg/min)} = U_x(\text{mg/ml}) \times V(\text{ml/min})$$

where U_x represents the concentration of substance X in urine (in mg/ml) and V represents the volume of urine collected per unit time (in ml/min). Thus, the clearance equation may be constructed:

$$Cl_x(\text{ml/minute}) = U_x(\text{mg/ml}) \times V(\text{ml/min})/P_x(\text{mg/ml})$$

where P_x is the concentration of compound X in plasma (in mg/ml)

GFR is estimated by calculating the clearance of the tracer or endogenous substance. RPF estimated using PAH clearance is often designated effective renal plasma flow (ERPF). Renal plasma flow is converted to renal blood flow (RBF) by dividing ERPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct):

$$\text{RBF} = \text{ERPF}/(1 - \text{Hct})$$

The clearances of other compounds can be compared with inulin clearances to determine how the kidney functions in the elimination of the test compound. A clearance ratio is constructed by dividing the renal clearance of the test compound (X) by the renal clearance of inulin:

$$\text{Clearance Ratio} = CL_x, (\text{ml/min})/Cl_{\text{inulin}}, (\text{ml/min})$$

A clearance ratio <1.0 indicates reabsorption of the test substance following filtration, whereas active secretion will result in a clearance ratio of >1.0.

Modifications of the Method

Endogenous compounds can administered exogenously in place of tracer substances. This has been done for the dog using creatinine (Sapirstein et al. 1955; Finco et al. 2001) and for the rat using cystatin C (Tenstad et al. 1996). Either urine or plasma clearance methods can be used for the former. For the latter, plasma clearance was used to estimate GFR due to the fact that cystatin C is reabsorbed and degraded by the proximal tubule and does not appear in the urine.

Rönnhedh et al. (1996) described a simple method to perform serial renal clearance studies without urine collection in rats. This was applied to non-radiolabeled *para*-aminohippurate sodium and iothalamate sodium which were used respectively to estimate renal blood flow and glomerular filtration rate.

Gabel et al. (1996) described fast and accurate assays for measuring glomerular filtration rate and effective renal blood flow in conscious rats. An enzymatic method was developed for the determination of inulin and a colorimetric method was developed for determination of *p*-aminohippurate in the plasma and urine of rats.

Hropot et al. (1985) described clearance methods in monkeys. Chimpanzees weighing 30.7 ± 10.6 kg were anesthetized with 1 mg/kg Sernylan i.m. Food was withdrawn 24 h prior to the experiment and the animals received only tap

water ad libitum. In the morning before the experiment, the urinary bladder of the animals was emptied by catheterization. The urine was discarded. To determine the glomerular filtration rate (inulin clearance), a bolus injection of 50 mg/kg inulin i.v. was given and followed by a continuous infusion of 3 ml/min inulin dissolved in Ringer lactate solution. After an equilibrium of 60 min, urine and blood samples were collected for two control clearance periods of 30 min each. The control periods were followed by intravenous administration of the test preparation in a dose of 20 mg/kg. Thereafter, urine and blood samples were collected during six clearance periods. The following parameters were determined: urine excretion, inulin clearance and urate clearance [ml/kg/min], and fractional excretion of urate and plasma urate [mmol/l].

Tanaka et al. (1990) evaluated uricosuric and diuretic properties of diuretic agents using clearance studies in urate-loaded dogs and urate-loaded rabbits.

Limitations of the Method

First-pass extraction of PAH is highly variable both between species and between individuals within a species, which adds to the inherent inaccuracy of the estimate of RBF by this method. Furthermore, the test compound may interfere with the extraction of either PAH or TEA by competing for transport by the organic anion or cation transporters (Newman and Price 1999; Ragan and Weller 1999).

The limitations of the Jaffe method for creatinine determination have been discussed earlier in this chapter. Exogenous creatinine clearance compensates for the insensitivity of the method as well as the interference by endogenous chromagens by artificially increasing the plasma creatinine concentration (Finco 1997).

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Assessment of Glomerular Filtration Rate (GFR) and Renal Blood Flow (RBF)

Assessment of GFR by Plasma Chemistry

Purpose and Rationale

Estimation of the glomerular filtration rate (GFR) is considered a sensitive index of functional nephron mass (Newman and Price 1999). Point measurements of the plasma levels of several endogenous small molecules (urea, creatinine, 2-(a-mannopyranosyl)-L-tryptophan) or small (less than 66 kDa) proteins (cystatin C (γ-trace), prostaglandin D synthase (β-trace protein), α_1-microglobulin, β_2-microglobulin, and retinol binding protein) have been used to assess GFR in many species.

Procedure

Serum or plasma samples are collected from the test animals (if plasma is used, blood should be collected in heparinized tubes). Assay methods vary and are outlined below:

Urea is most commonly assayed by combined urease methods, in which the urea is first converted to two ammonium ions. The ammonium generated is then measured by either enzymatic or chemical methods. Urea nitrogen values determined by this method (mg/ml) are converted to urea values by the use of appropriate factors (2.14 for urea in mg/ml and 0.357 for urea in mmol/l) (Emeigh Hart and Kinter 2005).

Creatinine is most commonly measured by the Jaffe reaction of creatinine with picrate to generate an orange chromogen. Several enzymatic assays (based on reactions with creatinase or creatinine deaminase) have also been developed. These are equal in sensitivity to the Jaffe method but are considered less likely to be subject to interference by endogenous or exogenous chromogens (Finco et al. 1995; Finco 1997; Newman and Price 1999). Chromatography (HPLC or LC-MS/MS) assay methods are more sensitive than the Jaffe method and are not subject to interference by endogenous chromagens (Dunn et al. 2004; Owen and Keevil 2007).

2-(a-mannopyranosyl)-L-tryptophan (MPT) is measured by HPLC (Takahira et al. 2001). Cystatin C assays are all antibody based (nephelometric, agglutination or sandwich ELISA) (Pergande and Jung 1993; Finney et al. 1997; Jensen et al. 2001) and have been used successfully in dogs, rats, mice and cats (Hakansson et al. 1996; Boekenkamp et al. 2001; Braun et al. 2002; Martin et al. 2002). The other small molecular weight proteins are detected by immunoassays as well.

Evaluation

Analyte levels are compared either to those of a concomitant control group (using appropriate statistical methods of the group size is large enough) or to laboratory-specific reference intervals for the species, strain, age, and sex in question. Elevation of analyte levels outside of the reference range indicates a decrease in GFR, with the magnitude of the elevation being roughly proportional to the degree of the decrement.

Additionally, algorithms can be used to convert plasma creatinine to creatinine clearance, which provides a reasonably accurate estimate of GFR in humans (Cockroft and Gault 1976):

$$\text{GFR} \approx (140 - \text{age}) \times \text{weight (kg)} \times K72 \times \text{Serum creatinine (mg/dL)}$$

Where $K = 0.85$ for women, 1.00 for men

A similar algorithm has been generated for dogs of either gender (Finco et al. 1995):

$$\text{GFR} \approx 2.6/\text{Serum creatinine (mg/dL)}$$

Limitations of the Method

Effects on prerenal (dehydration, blood loss, altered vasomotor tone, age-related decreases in renal blood flow in rats) and/or postrenal factors (obstruction or extravasation of urine to the peritoneal cavity) may cause elevations of the commonly measured analytes that do not reflect effects on the GFR or loss of functional nephron mass (Baum et al. 1975; Corman and Michel 1987; Finco 1997; Newmann and Price 1999).

Urea and creatinine elevations in plasma are in general not sensitive enough to detect low-level alterations (less than 75 % loss) of GFR, due to the contribution of renal secretion and/or reabsorption to their overall excretion (which can compensate for their decreased filtration), to wide variations in baseline levels of some analytes, and to inherent imprecision in the assays used (Finn and Porter 1998; Price 2002; Starr et al. 2002; Shemesh et al. 1985). In particular, urea will underestimate GFR (due to extensive tubular reabsorption with decreased GFR) (Baum et al. 1975; Kaplan and Kohn 1992; Newman and Price 1999), and creatinine tends to overestimate GFR (because it is secreted by the tubule in many species and secretion increases with reduced GFR) (Shemesh et al. 1985; Andreev et al. 1999; Newman and Price 1999; Star et al. 2002). In addition, creatinine synthesis is regulated by feedback inhibition which limits the degree of elevation than can occur in plasma (Watson et al. 2002).

The Jaffe reaction for creatinine is subject to interference by numerous endogenous substrates and drugs or compounds (Schwendenwein and Gabler 2001; Sonntag and Scholer 2001; Dunn et al. 2004). This effect can be minimized by using appropriate substrate extraction or by the use of kinetic assessments. The urease assay is specific for urea, but increased circulating ammonia (such as occurs in aged plasma samples, metabolic disorders, and portocaval shunting) will react with the subsequent reaction and result in falsely elevated plasma levels (Newman and Price 1999).

Baseline levels of urea and creatinine are variable, as they are subject to influences on their extrarenal synthesis and release. Plasma urea reflects hepatic synthesis rate and will be elevated with increased protein catabolism (increased dietary protein intake, gastrointestinal hemorrhage, fever, severe burns, corticosteroid administration, sustained exercise, or muscle wasting) and decreased with low or poor quality protein diets, modest food restriction in rodents or hepatic insufficiency (Pickering and Pickering 1984; Finco 1997; Hamberg 1997; Tauson and Wamberg 1998; Newman and Price 1999). Baseline creatinine reflects muscle catabolism and will be elevated in individuals with higher muscle mass or following sustained exercise or acute muscle damage; it will be lower in individuals with loss of muscle mass (Finco 1997; Newman and Price 1999). Drugs which compete with creatinine for renal excretion may falsely elevate plasma levels in the absence of renal injury (Andreev et al. 1999).

2-(a-mannopyranosyl)-L-tryptophan (MPT) appears to be less likely to be affected by muscle mass than creatinine. Point-in-time measurements of this tryptophan glycoconjugate correlated extremely well with the inulin clearance, suggesting it may be a superior indicator of GFR. However, the renal handling of MPT has not been examined to determine if plasma levels may be influenced by either reabsorption or secretion (Horiuchi et al. 1994; Gutsche et al. 1999; Takahira et al. 2001).

Pitt et al. (2006) reported the pharmacological profile and toxicity of fluorescein-labelled sinsitrin, a novel marker for GFR measurements.

Cystatin C does not appear to be a sensitive index of GFR in the cat (Martin et al. 2002).

The other small protein markers of GFR are detected by immunoassays with reagents specific for the human proteins; the cross-reactivity of these reagents with other species and the usefulness of these markers in animal models have not been well established (Loeb 1998).

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Assessment of RBF by Intravascular Doppler Flow Probes

Purpose and Rationale

Probes utilizing electromagnetic or Doppler technology may be positioned around or within a renal artery to allow direct measurement of renal blood flow (Yagil 1990; Haywood et al. 1981). Detection of blood flow using Doppler systems is based on changes in the emitted ultrasonic frequency, a Doppler shift, caused by reflection of the signal of moving blood cells. The Doppler shift is proportional to the velocity of blood flow, as indicated by the following equation:

$$\Delta f = \frac{2 f_0 v \cos\theta}{c}$$

where Δf = Doppler peak frequency shift, f_0 = transmission frequency, v = instantaneous peak velocity, θ = angle of incidence of the beam to the bloodstream (assumed to be 0° for linear probes), and c = speed of sound in blood (1,570 m/s) (Chilian and Marcus 1982).

This technique is particularly useful when rapid or continuous assessment of effects on renal blood flow or assessment of renal vasoreactivity need to be assessed.

Procedure

Doppler probes have been applied to rats, rabbits, cats, dogs, pigs, monkeys, and humans; these must be appropriately calibrated prior to experimental use. Animals are anesthetized and the probes are placed within the renal artery via the abdominal aorta, which may be accessed via the femoral or carotid arteries. The diameter of the renal artery at the placement site of the Doppler probe must be determined simultaneously to correct velocity measurements for determination of renal blood flow (see Evaluation).

Evaluation

A number of parameters can be derived from the Doppler measurements, most commonly including average peak velocity (APV), pulsatility index (PI), and resistive index (RI). Renal blood flow (in mL/min) can be calculated as follows:

$$\mathrm{RBF} = \frac{\mathrm{APV} \times \pi \times \mathrm{D}^2 \times 60}{4}$$

where D = renal arterial diameter (Doucette et al. 1992).

Resistive index (RI) has been shown in several species to be positively correlated with tubular disfunction or postrenal obstruction and its return to normal may serve as a prognostic indicator of resolution of tubular disease (Rivers et al. 1997; Shokeir et al. 1996; Tsuji and Taira 2001).

Limitations of the Method

Dopper probe assessment of velocity is accurate primarily in straight blood vessels of small diameter (<4.76 mm) and at relatively low flow rates (<200 mL/min), both of which may be exceeded in normal renal arteries in many species (Lerman and Rodriguez-Porcel 2001).

In dogs, acute, severe normovolemic anemia significantly altered renal artery Doppler parameters without having an influence on Doppler assessment of splanchnic blood flow; the technique may thus be inaccurate in anemic animals (Koma et al. 2006). Furthermore, it is invasive and the size of the test species to which it can be applied depends on the availability of appropriately sized and calibrated probes (Lerman and Rodriguez-Porcel 2001).

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Assessment of GFR and RBF by Scintigraphic Imaging

Purpose and Rationale

Radioactive indicators (^{99c}Tc-DPTA, ^{113m}In-DPTA, and ^{99c}Tc-mercaptoacetyltriglycine) may be used to measure renal blood or plasma flows or glomerular filtration rate (Reba et al. 1968). These techniques require intraarterial or intravenous administration of the tracer followed by monitoring the amount of tracer in the kidneys with an external gamma camera (Fommei and Volterrani 1995).

In species where extrarenal clearance of tracers used in the determination of GFR or RPF is high or variable, external detection methods may actually be more accurate than plasma clearance methods because the extrarenal component of plasma removal is eliminated (Drost et al. 2003).

Procedure

The selected radiotracer is injected either intravenously or intraarterially. External measurements of radioactivity retained in the kidney are made either at timed intervals (rate of accumulation, or slope method) or at a selected interval, usually corresponding to either first pass or peak level of the tracer (percent accumulation, or integral method) (Kampa et al. 2002).

Limitations of the Method

These techniques do not yield absolute flow, but rather flow per unit volume or tissue mass. However, algorithms can be developed in the species of interest to allow conversion of tissue uptake to GFR or RBF expressed in standard units by simultaneous determination of plasma clearance of the tracer in preliminary experiments (Kelleher et al. 1991; Kampa et al. 2003), as the correlations between methods for any given tracer are in general high (Delpassand et al. 2000).

In general, assessment of GFR or RBF by external detection methods is less accurate than assessment by clearance methods, although the correction algorithm used for the external detection method can influence the degree of accuracy achievable (Pergande and Jung 1993; Itoh et al. 2000; Itoh 2003). Integral methods (calculated based on the percent of the dose accumulated) are in general more accurate because they eliminate variability resulting from variable durations of the uptake phase (Kampa et al. 2003). Manual selection of the region of interest (ROI) for the collection of the scintigraphy data also improves the accuracy (Kampa et al. 2002).

Modifications of the Method

Depending on the tracer used, either GFR or RBF can be estimated from the results. The table below lists different tracers that can be used for the assessment of either GFR or RBF (Emeigh Hart and Kinter 2005) (Table 1).

Table 1 Radiopharmaceuticals Used for Estimating

Glomerular Filtration Rate	Renal Blood or Plasma Flow
^{3}H-inulin	^{3}H-*para*-aminohippuric acid (PAH)
^{14}C-inulin	^{14}C-PAH
^{14}C-carboxy inulin	S^{99m}Tc-hippuran analogs
^{14}C-hydroxy-methyl inulin	^{99m}Tc-iminodiacetic PAH (PAHIDA)
^{131}I-chloroiodopropyl inulin	^{99m}Tc-mercaptoacetyltriglycine (^{99m}Tc-Mag3)
^{131}I-propargyl inulin	^{99m}Tc-mercaptosuccinyltriglycine (^{99m}Tc-MSG3)
^{125}I-diatrizoate	^{99m}Tc-N,N′bis (mercaptoacetyl)-2,3-diaminopropanoate (CO2-DADS-A)
^{125,131}I-iothalamate (Conray – 60)	^{125,131}I-iodopyracet (Diodrast)
^{131}I-diatrizoate (Hypaque, Renografin)	^{131}I-orthoiodohippurate (Hippuran, OIH)
^{51}Cr-, ^{99m}Tc-, ^{111,113m}In-, ^{140}La-, ^{169}Yb-EDTA	^{67,68}Ga-*N*-succinyl desferioxamine
^{51}Cr-, ^{99m}Tc-, ^{111,113m}In-, ^{140}La-, ^{169}Yb-DTPA	^{97}Ru-ruthenocenyl-glycine (Ruppuran)
^{57,58}Co-hydroxycobalamin	^{99m}Tc-thiodiglycolic acid
^{57,58}Co-cyanocobalamin	

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Assessment of Renal Tubule Functions

Urinalysis

Purpose and Rationale

More than 99 % of the reabsorption of glucose, protein, and electrolytes occurs in the proximal tubule. Electolyte concentrations can be affected by other tubule segments, but even low-level changes in proximal tubular function will be reflected by increased urinary excretion of protein and glucose (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price 1999; Aleo et al. 2002). Standard and modified urinalysis techniques can be successfully used to assess the effects of compounds on proximal tubule uptake processes (Katsuno et al. 2007).

Procedure

Collection of a good quality sample from the test species is paramount to obtaining high quality data from the urinalysis. Samples must be collected in clean containers and must be kept free of contamination from food, drinking water, feces, blood, and bacteria. For best results, samples should be analyzed promptly (ideally within 1 h) after collection, but where analysis must be delayed, the sample must be protected from chemical degradation and evaporation, best accomplished by using collection containers that are appropriately sized for the species in question, tightly sealing the containers when possible, and keeping the specimen cold (4 °C) until analysis. If chilled or frozen, samples must be allowed to slowly equilibrate to room temperature before analysis.

Point-in-time samples can easily be collected from most species, either by free-catch, urethral catheterization, manual compression of the bladder or cystocentesis (withdrawal of urine from directly from the bladder with a needle and syringe). Catheterization may require sedation or anesthesia, especially in females of all species and in male pigs whose urethral recess makes this process difficult (Van Metre and Angelos 1999). Manual compression of the bladder can also be performed on rodents, and cystocentesis may also be performed by a skilled operator using a small (25 gauge) needle and syringe (Loeb and Quimby 1999).

Timed urine collections in all species (including large animals) can also be obtained by the use of specially designed metabolism cages. A metabolism cage consists of an animal chamber mounted above an excrement collection system. The animal chamber must be equipped with feeder and waterer units, if an animal is to be housed in the metabolism cage for more than a few hours. These need to be sized appropriately to the test species of interest (especially regarding the collection container, which must minimize surface area available for evaporation) and designed to eliminate contamination of the urine by food, drinking water, or feces. In selecting the size of the collection container, the anticipated urine volume should be considered: a mouse will produce 0.25–1 ml in 24 h of urine; a rat, 10 ml; a hamster, 5–8 ml; a rabbit, 600 ml; and a dog or minipig, 500 ml or more (Loeb 1998; McClure

1999; Van Metre and Angelos 1999). Whatever type of metabolism cage is used, the following general precautions are offered:

1. For studies of >24 h duration, test animals should be acclimated to the metabolism cage for several days prior to study initiation. During this period, animals should be monitored frequently to insure that they learn to use the feeder and waterer systems properly. Test animals should be maintaining or gaining weight prior to study initiation.
2. Feeder and waterer systems should provide ample food and water to meet the animal's needs for the duration of urine collection and all separator systems should function properly. For chronic studies (>5–7 days duration), it is useful to have a complete exchange of feeder, waterer, and urine/feces collector and separator systems so that soiled units may be rapidly exchanged with clean, dry/filled units at regular intervals.
3. Cages should be decontaminated, cleaned, rinsed with distilled/deionized water, and thoroughly dried prior to use. Surfaces used to collect urine may be siliconized or sprayed with a suitable hydrophobic material (PAM, General Foods) to facilitate urine collection.
4. All surfaces contacting urine should be rinsed with distilled/deionized water or appropriate solvents to collect any residuals at appropriate intervals.
5. To preserve the quality of the specimens during prolonged collection times, the opening of the collection vial needs to be small to prevent evaporation and the vial should be surrounded either with wet ice or frozen cold packs to chill the sample promptly once it is deposited (Loeb 1998; Loeb and Quimby 1999). Urine may also be collected under mineral oil to prevent evaporative losses. For very small or antidiuretic animals (e.g., hamsters, gerbils) placing the cage over a shallow pan of oil and skim feces from the surface may be necessary while collecting urine with a pipette from under the oil. To maximize the volume of the sample collected in rodents in metabolism cages, food should be withheld (this also reduces the risk of contamination of the sample) and water provided (Lee et al. 1998).

Routine urinalysis consists of visual assessment (color, clarity), volume, specific gravity or osmolality, pH, and quantitative or semiquantitative determination of total protein and glucose and microscopic evaluation of urine sediment (Weing and et al. 1996). Urine constituents can be measured semiquantitatively using commercially available "dipstick" test strips (Chemstrips, Roche Diagnostics or Multistix, Bayer Heath Care Diagnostics), which contain reagents for the determination of specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, hemoglobin, nitrite, and leukocyte esterases (Newman and Price 1999). These strips may be read manually or using automated analyzers specific to each product.

If quantitative assessment of urine analytes is required (see under "Evaluation"), either urine volume or urine creatinine concentration must be measured and used to "normalize" the concentration of the measured analyte. This will negate the effects of differences in urine concentration between animals and allow for accurate comparison of the results. Creatinine can be measured by the same methods used for plasma.

Evaluation

Urine volume combined with assessment of urine concentration (specific gravity or osmolality) can serve as an index to renal function. With severe acute loss of functional nephron mass, urine output is decreased (oliguria) or absent (anuria), while loss of the ability of the kidney to adequately concentrate urine results in the excretion of large volumes of dilute urine.

The commercially available dipstick reagents do not detect the low levels of glucose found in normal urine and thus positive results indicate significant glucosuria in most species (the exception is the gerbil, where dipstick positive glucosuria is normal) (McClure 1999). If glucosuria is detected, time-matched plasma glucose levels are needed to rule out that this result has not resulted from an increase in the filtered glucose load. If plasma glucose is normal, the

appearance of glucose in the urine may indicate a functional deficit in the proximal tubule (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price 1999; Aleo et al. 2002).

Because of inherent inaccuracies in the dipsticks when applied to animal urine (see below under "Limitations of the Method"), positive results by dipstick for protein must be followed by detailed qualitative and quantitative assessment.

Quantitative assessment of urinary protein excretion is necessary to rule out a contribution from glomerular malfunction. In general, excretion of markedly elevated levels of protein is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/lowgrade glomerular injury (Peterson et al. 1969; Finco 1997).

A number of different methods have been developed for quantitative assessment, including turbidometric, colorimetric (Biuret and Lowry assays as examples), and dye binding assays. All have their advantages and limitations; in general, biuret assays tend to detect all types of proteins with equal sensitivity but require fairly large sample volumes, turbidometric assays can suffer from lack of precision with variations in urine ionic strength, and dye-based methods may suffer from interference with exogenous and endogenous urine substances. The Folin phenol (Lowry), Coomassie brilliant blue, and Ponceau S methods have been recommended as being particularly precise for urine samples (Petersen et al. 1969; Dilena et al. 1983; Finco 1997; Newman and Price 1999).

Qualitative assessment (identification of the proteins excreted) is necessary to determine if low-level proteinuria has resulted from glomerular or tubular malfunction. Concomitant elevation of albumin and one or more of the low molecular weight proteins (which are freely filtered by the normal glomerulus) indicates that the proteinuria has resulted from decreased tubular reabsorption of proteins, while albumin elevation alone or concurrently with a high molecular weight protein (normally excluded from the filtrate by the glomerulus) indicates primary glomerular injury (Peterson et al. 1969; Finn and Porter 1998; Guder et al. 1998; Umbreit and Wiedemann 2000). Commonly used filtered low molecular weight proteins include retinol binding protein (Price 2000; 2002; Aleo et al. 2002, 2003), α_2- or β_2-microglobulin (Viau et al. 1986; Loeb 1998; Finn and Porter 1998; Price 2000; 2002) or cystatin C (Finn and Porter 1998; Herget-Rosenthal et al. 2001; Uchida and Gotoh 2002).

Limitations of the Method

Because the commercially available dipsticks are designed for clinical use, they are inaccurate for a number of parameters in animal urine. The specific gravity reagents are completely inaccurate in all species. Dipsticks which use a glucose oxidase method for urine glucose can show a false positive result in species, such as the dog and mouse, with high urinary ascorbate levels or in urine contaminated with hypochlorite used as a disinfectant (Finco 1997; Loeb and Quimby 1999). For all glucose detection methods, test article formulations that contain glucose or other metabolizable sugars in quantities, should be avoided, as they may transiently overwhelm tubule reabsorption mechanisms and generate false positive test results. Protein detection is based on a bromphenol blue method that is most sensitive for albumin (Newman and Price 1999), which means that proteinuria that does not result primarily from an increased albumin excretion may not be detected by this method. Furthermore, since these tests are designed for human urine, false positives are frequent in species like the dog, whose normal urine protein levels are just above the lower limits for humans (Finco 1997), and dipstick tests are invariably positive in male rats and mice which have normal high proteinuria (Loeb and Quimby 1999).

The sensitivity of the quantitative protein assays to the protein(s) of interest depends in large measure on which protein is used to generate the standard curve. Albumin is most commonly used because it is the most abundant protein in urine and while it is adequate for most methods (Dilena et al. 1983), it will in general underestimate the abundance of many other proteins of interest in urine (Guder and Hofman 1992).

Qualitative assessment of urine proteins other than albumin is hampered in many species by the lack of suitable immunoassays; with the exception of cystatin C, commerically available antibodies do not cross react with the animal proteins (Loeb and Quimby 1999; Uchida and Gotoh 2002). Assessment of urinary retinol excretion has been shown to be a sensitive index to retinol binding protein excretion in the rat (Aleo et al. 2002, 2003).

Modifications of the Method

Point-in-time samples from laboratory rodents may be obtained by taking advantage of the fact that these animals frequently urinate when they are handled or shortly after being removed from their home cage. A skilled, quick, and prepared operator with a ready small container or plain microcapillary tube may be able to obtain a small sample in the first instance (Loeb and Quimby 1999); in the second, the animal can be placed in a small confined space on plastic food wrap and observed carefully for urination, as it has been shown that most rodents will urinate within 20 min after removal from their home cages. The sample thus generated can be collected by micropipette (Kurien and Scofield 1999).

For repeated point-in-time urine samples from rats over short periods of time (1–2 weeks after the surgery), the urethra or the ureter can be cannulated. Cannulated rats can also be used to collect accurate and complete timed urine samples (Mandavilli et al. 1991; Horst et al. 1988).

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Electrolyte Excretion

Since one of the kidney's primary functions is maintaining electrolyte and mineral homeostasis in the face of fluctuating dietary intake and body needs, examination of plasma and urine electrolyte levels will provide some insight into renal function. Because of the large functional mass of the kidney, alteration of plasma electrolyte levels are usually not detected until the effect on renal functional is significant (pathologic). In contrast, urine electrolyte levels examined with knowledge of plasma levels and dietary intake can serve as an extremely sensitive index to the effect of drugs or chemicals on the functional state of the kidney.

In animal studies, the diet can be carefully controlled and thus the intake (and hence the plasma electrolyte levels) can be assumed to be fairly constant. Provided there are no sources of significant electrolyte loss resulting from the experimental manipulations (e.g., vomiting, diarrhea, salivation), urine electrolyte levels will reflect the effects of the compound on either GFR (determines the filtered load) or tubular secretion or reabsorption (determines the final urine electrolyte composition).

Fractional Excretion Methods

Purpose and Rationale

Fractional excretion (FE) is the proportion of the filtered load of any analyte that is excreted from the plasma. If both tubular function and plasma electrolyte values are normal, increases in electrolyte FE values clearly reflect a decrement in GFR. With tubular malfunction, the direction of the change in FE values depends on the net direction of electrolyte transport (i.e., FE will increase for electrolytes that are primarily reabsorbed and will decrease for secreted electrolytes) (Finco 1997; Stockham and Scott 2002).

Procedure

FE assessments can be performed in any species, as it requires only carefully timed complete urine collections and a concurrent assessment

of GFR. FE will be unitless if the urine collection period is expressed in minutes, thus (Finco 1997):

$$\mathrm{FE} = (\text{urine electrolyte concentration (mmol/l)} \times \text{urine (ml/collection period)}) / (\text{GFR (ml/min)} \times \text{plasma electrolyte concentration (mmol/l)})$$

To eliminate the need for both complete timed urine collections (difficult to do in most animals) and concurrent assessment of GFR, FE values are usually calculated based on point-in-time urine collections by using creatinine excretion during the same time period as an estimator of GFR (Finco 1997).

Animals are placed in appropriately sized metabolism cages for an appropriate period of time to allow collection of an adequate volume of urine. At the midpoint of the collection period, a blood sample is obtained under appropriate anesthesia (note: the use of CO_2 will falsely elevate plasma potassium levels and render the method inaccurate) for the determination of electrolyte and creatinine levels.

Plasma and urine electrolytes and creatinine are determined by standard methods (Durst and Siggard-Andersen 1999; Newman and Price 1999; Scott et al. 1999). FE is calculated from the results as outlined below (Stockham and Scott 2002):

$$\mathrm{FE} = (\text{urine electrolyte concentration} \times \text{plasma creatinine concentration urine}) / (\text{creatinine concentration} \times \text{plasma electrolyte concentration})$$

Limitations of the Method

If the Jaffe method for creatinine is used, the investigator must be aware of potential interference due to endogenous chromogens (the error is magnified in species where these chromogens are present in higher concentration in the plasma than in the urine (dog, mouse) (Finco 1997; Dunn et al. 2004) or possibly the test compound (Sonntag and Scholer 2001). Additionally, plasma electrolyte levels may fluctuate as the result of eating or due to diurnal rhythms (Finco 1997). The impact of inaccuracies can be minimized by consistent timing of urine collection, fasting of animals before and during urine collection and the inclusion of a concurrent untreated (vehicle control) group in all studies.

Modifications of the Method

Although FE of sodium is most commonly used to assess tubular function, FE of magnesium has been shown to be the most sensitive index in detecting low-level tubular injury in humans (Barton et al. 1987; Futrakul et al. 1999; Kang et al. 2000; Oladipo et al. 2003). Increased FE of calcium has also been shown to serve as a sensitive index of effects on renal function unrelated to overt renal injury (Lam and Adelstein 1986; Tuso and Nortman 1992; Eliott et al. 2000)

FE of urea has been recently shown to be more useful than the FE of sodium in distinguishing between prerenal and renal azotemia in humans (Carvounis et al. 2002) and thus may also be useful in making this distinction in animal models. Changes in the FE of urea also reflect changes in urine flow rates (in general, these values move parallel to each other) and can be used as an estimate of this parameter (Finco 1997). The methods used to detect urea in urine are the same as those used in serum (Newmann and Price 1999).

FE of anions (ammonium, bicarbonate) can be used to determine the potential mechanism underlying systemic acid base imbalances (Rothstein et al. 1990; Kim et al. 2001).

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Quantitative Electrolyte Excretion

Purpose and Rationale

Fractional excretion methods are useful in that they can identify alterations in analyte excretion with relative ease, as they require only point-in-time assessment of analyte concentration and don't require complete and timed urine collection. Greater sensitivity, however, is obtained with quantitative evaluation of electrolyte excretion. This method is more difficult, requiring carefully timed complete urine collection, and because plasma levels are not evaluated there needs to be reasonable assurance that the changes in excretion

seen do not result from alteration of the filtered load of the analyte in question (e.g., plasma levels and GFR remain consistent). In animal studies where the diet is carefully controlled, these factors are not usually of concern.

Procedure

The method can be used on any species, although rats are most commonly used. Animals are usually fasted but allowed access to water; if anesthetized animals are used, intravenous administration of balanced electrolyte solutions (e.g., lactated Ringer's solution) or 0.9 % NaCl solution can be administered by intravenous infusion to ensure that plasma volume (and thus GFR) and electrolyte levels are maintained.

Urine is collected by any appropriate method over a specified period of time. If possible, the urinary bladder should be emptied before collection of test urine is initiatied and at the end of the collection period; this can be accomplished in dogs by catheterization and in rodents by handling or manual expression of the bladder. If metabolism cages are used, special care must be taken to avoid evaporation of urine and contamination of the vials with feces or drinking water, as accurate determination of volume is important. The volume of urine collected is determined gravimetrically, and urine electrolytes are measured by standard methods (Durst and Siggard-Andersen 1999; Scott et al. 1999).

Evaluation

The volume of urine collected over the time period is defined as the urine flow rate:

$$V = \text{volume collected/time period} \quad (\text{expressed in mL/min})$$

and electrolyte excretion (U_XV) is defined as:

$$U_X V = \{[(\text{concentration of } X \text{ in urine}) \times V]/\text{body weight in g}\} \times 100 (\text{expressed in mmol/min/100 g})$$

As a general rule, $U_{Na}V + U_KV = U_{Cl}V$. If it does not, the assumption must be made that there are additional anions in the urine. Carbonic anhydrase inhibition (i.e., excess urinary bicarbonate) may be the culprit if the ratio:

$$\frac{U_{Cl}V}{U_{Na}V + U_KV}$$

is less than 0.8.

Net natriuretic activity can be assessed by evaluation of the ratio:

$$\frac{U_{Na}V}{U_KV}.$$

Values greater than 2.0 indicate a favorable natriuretic effect, and values greater than 10.0 indicate a potassium-sparing effect.

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Assessment of Tubular Transport Processes using Transport Substrates and Inhibitors

Purpose and Rationale

A number of transporters for organic anions and cations are present on both the lumenal and basolateral surfaces of the proximal tubules. These are essential to the secretion and/or reabsorption of a number of endogenous substrates as well as functioning in the elimination of xenobiotics. These transporters frequently function as ion exchangers and their activity is thus bidirectional, so that the net direction of transport (i.e., secretion or reabsorption) for any given substrate depends not only on the numbers

Table 2 Transport substrates and inhibitors

Compound	Most common use	Transporter affected	Comments
Tetraethylammonium (TEA)	Substrate	OCT1, OCT2, OCT3	Most commonly used substrate
1-methyl-4-phenylpyridinium (MPP^+)	Substrate	OCT1, OCT2	Can be used to differentiate OCT3-mediated transport
Tributylmethylammonium (TBuMA)	Substrate	OCT3	Can be used to differentiate OCT3-mediated transport
Urate	Substrate	URAT1, OAT1, OAT3	URAT1 is selective for urate in human kidney but has other OA transport functions in other species
p-aminohippurate (PAH)	Substrate	OAT1, OAT2, OAT3(?), OAT4	Most commonly used substrate. Questionable substrate for OAT3
Estrone sulfate	Substrate	OAT3, OAT4, Oatp1	Oatps are homologs of bile acid transporters in the liver
Lucifer yellow	Substrate	OAT1	Can be detected spectrofluorometrically
Phenolsulfonphthalein (Phenol red)	Substrate	OAT3	Clearance has traditionally been used as a surrogate for urate transport
Probenecid	Inhibitor	OAT1, OAT2, OAT3, URAT1	Used therapeutically and experimentally
Cimetidine	Inhibitor	OAT3	Used to correct creatinine clearance for the contribution of tubular secretion
Benzbromarone	Inhibitor	URAT1	

and activity of the transporters on either side of the tubule but also on the presence and concentration of competing or cotransported substrates. This feature of tubular transport has been one of the major challenges in the development of uricosuric agents for the treatment of hyperuricemia (Terkeltaub et al. 2006). Compounds that affect either the expression or activity of transporters, are competitive substrates for them, or act as inhibitors of transport thus may affect not only the clearance of therapeutic agents but also that of endogenous metabolites (Wright and Danzler 2003) (See also Table 2).

Procedure

The choice of substrate or inhibitor used depends on the transport process or specific transporter of interest. The table below lists the most commonly used substrates and their transporters (note: most inhibitors act competitively and are themselves substrates for the transporter in question).

Transport studies can be performed in any animal model from which urine and plasma may be collected readily. Inhibitors are administered 30 min to 1 h before the administration of the test substance. Substrates are usually administered intravenously and are detected in urine and plasma most commonly by colorimetric methods.

Evaluation

Standard clearance or excretion methods are used to assess the effects.

Limitations of the Method

Significant sex differences exist in renal transporter expression and activity in rodents and rabbits (Sekine et al. 2006). Many of the substrates, and all of the inhibitors, are not specific enough for accurate determination of the exact transporter affected by the test compound.

Modifications of the Method

The listed substrates and inhibitors may also be used in in vitro modles such as isolated tubules or kidney slices. In the latter model, net transport is usually assessed by measuring either the disappearance of the substrate from the medium or, more commonly, the degree or rate of accumulation of the substrate over time in the tissue slice (Kirkpatrick and Gandolfi 2005).

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Assessment of Renal Concentrating Ability

Solute-Free Water Excretion and Reabsorption

Purpose and Rationale

Investigations of the clearance of solute-free water represent indirect methods for the evaluation of several aspects of renal function and provide information on the site and mechanism of action of agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convoluted tubule, would augment the clearance of electrolyte-free water (EC_{H2O}) during water diuresis and the reabsorption of electrolyte-free water (ETC_{H2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both EC_{H2O} and ETC_{H2O}. On the other hand, drugs that act only in the distal tubule would reduce EC_{H2O} but not ETC_{H2O}.

Procedure

These tests may be performed in any species from which urine and plasma can be readily collected, although low-level changes in concentrating ability may be more readily manifest in rats than in dogs (Sharrat and Frazer 1963; Osbourne et al. 1983). The general procedure involves initially placing the animals in a metabolism cage with unlimited access to water. After collection of a urine sample (16–24 h is best), the water is withdrawn and the urine is collected for the next 12–16 h. The urine specific gravity (or preferentially, urine osmolality) is measured and compared both with the value from the hydrated animal and the mean values from the water-deprived control group (Ragan and Weller 1999).

Assessment of renal urine diluting ability is more cumbersome but can be accomplished in rats by administration of an oral dose of water by gavage representing 5 % of the animal's body weight. The animals are then placed in metabolic cages, and the urine is collected every 30 min for the next 2 h (Sharrat and Frazer 1963). In dogs, water diuresis can be induced by oral administration of 50 ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5 % glucose solution and 0.58 % NaCl solution at 0.5 ml/min per kg body weight. When

water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter (Suki et al. 1965). The volume and specific gravity (or osmolality) of the collected urine is measured and the results expressed as the percent of the administered dose of water excreted during the time period. Time-matched plasma samples are collected for the determination of plasma osmolality.

Evaluation

In addition to gross assessment of renal dilution or concentrating ability (as described above), quantitative evaluation of either osmolar clearance, freewater clearance (C_{H2O}), or solute-free water clearance (EC_{H2O}) may add additional sensitivity to the evaluation of these functions.

Osmolar clearance can be calculated as follows:

$$C_{osm} = (U_{osm} X\, V)/P_{osm}$$

where C_{osm} = osmolar clearance, U_{osm} = urine osmolality (in mosm/kg water), V = urine flow (measured in ml/min), and P_{osm} = plasma osmolality (measured in msom/kg water). C_{osm} is expressed in ml/min. C_{osm} less than V indicates excretion of a dilute urine (i.e., excess water is being excreted), but C_{osm} greater than V indicates excretion of concentrated urine (i.e., excretion of excess solute).

Freewater clearance (C_{H2O}) provides an estimate of the amount of urine being excreted in excess that needed to clear solutes and is calculated traditionally as follows:

$$C_{H2O} = VX[(1 - U_{osm})/P_{osm}]$$
(Wesson and Anslow, 1952)

More accurate assessment of the ability of the kidney to appropriately regulate plasma tonicity and/or respond appropriately to antidiuretic hormone (ADH) can be made by calculation of electrolyte-free water clearance (EC_{H2O}). This formula takes into account only water needed to excrete excess "effective" osmolytes (in general, monovalent electrolytes and their associated anions) and is calculated as follows:

$$EC_{H_2O} = V\,X\{1 - ([Na^+ + K^+]_{urine}/[Na^+ + K^+]_{plasma})\}$$ (Shoker, 1994)

Limitations of the Method

In general, the sensitivity of either urine concentration or dilution tests to detect the effects of test compounds on renal function is quite low, regardless of species (Ragan and Weller 1999). Furthermore, these tests (especially the urine dilution test) may be significantly altered by extrarenal effects (e.g., vomiting, diarrhea, delayed GI absorption, altered adrenal cortical function). Calculation of osmolar, freewater, and electrolyte-free water clearances require accurate assessment of urine flow rates, which will require accurate and complete collection of all urine produced during the time interval, not always easily accomplished in small animal species. Catheterized models should be utilized in this circumstance. In addition, calculated electrolyte-free water clearances may be influenced by the presence of excess effective osmolytes in either urine or plasma (e. g., in circumstances of hyperglycemia or metabolic acidosis/alkalosis) and the formula must be corrected to account for these if they are known to be present (Shoker 1994; Nguyen and Kurtz 2005).

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Assessment of Medullary Osmolarity and Blood Flow

Purpose and Rationale

The ability of the kidney to concentrate urine depends entirely on the maintenance of the renal medullary interstitium in a hypertonic state. Hypertonicity reflects two phenomena: water-uncoupled transport of osmotically active substances (sodium and urea, primarily) into the medullary interstitium and the trapping of those solutes in the interstitium by the vasa recta countercurrent multiplier system. The hypertonic gradient is created by the active transport of sodium into the interstitium in the ascending loop of Henle, and a combination of passive and facilitated transport of urea located in the collecting duct; the magnitude and effectiveness of the gradient is determined by the rate of plasma flow through the vasa recta (Pallone et al. 2003; Sadowski and Dobrowolski 2003). The method described allows simultaneous assessment of both parameters.

Procedure

Male Wistar rats have been used in these experiments. Under appropriate anesthesia, the femoral artery and vein are cannulated for measurement of aortic blood pressure and infusion of fluids and test compounds, respectively. The left kidney is exposed and placed in a plastic cup as for micropuncture experiments (see above), except that the dorsal surface is placed at the top of the cup, facing the operator. The ureter is catheterized and a platinum-iridium admittance electrode is inserted along the corticopapillary axis from the dorsal surface of the kidney (the probe is sized to ensure correct placement of the recording surfaces within the inner renal medulla). The capsule is incised and a needle laser-Doppler (LD) probe is inserted adjacent to the admittance probe. Animals are maintained in intravenous infusions of balanced electrolyte solution to maintain plasma volume and ensure adequate renal perfusion.

Medullary blood flow (MBF) is assessed by the LD probe, which is connected to a perfusion monitor (Periflux 4001, Perimed). The number and velocity of erythrocytes moving between the two optical fibers of the LD probe is determined. Admittance (the reciprocal of impedence) is measured between the tip of the LD probe and the admittance electrode by a conductance meter connected to both of these, following the application of a measuring current at a frequency of 24 Hz. Following the experiment, the kidney is dissected to verify that the LD and electrode have been correctly placed.

Evaluation

Medullary blood flow (MBF) is expressed in arbitrary perfusion units (PU), based on the voltage generated by the Doppler flux of the blood cells moving beneath the LD probe (roughly, the product of cell number X velocity). By definition, a 10 V signal from the detector is considered 1,000 PU. Admittance (Y) is expressed in millisiemens (mS) and is directly related to the ionic tonicity of the tissue (primarily due to the NaCl content). Effects in treated animals are expressed relative to those in untreated controls. If desired, urine may be collected from the ureteral cannula for excretion determinations.

Limitations of the Method

Because MBF is expressed in arbitrary units, only relative changes within a single animal can be detected, thus each animal must serve as its own control (although results between animals in the same study can be compared with the use of a calibration algorithm). Significant tissue damage, resulting in non-functional mephrons, does result

from the placement of the equipment and thus the technique can only be applied to the innermost medulla (where the glomeruli originate below the depth of the damaged zone). The admittance recordings reflect only the concentration of NaCl in the medulla and thus changes in tonicity resulting strictly from changes in urea concentration cannot be detected by this method.

Modification of the Method

Regional changes in medullary tonicity and the steepness of the osmotic gradient can be detected using a system of three needle electrodes that allows assessment of admittance in the outer and inner medulla separately. This arrangement cannot be coupled with the LD probe because excessive tissue damage would result (Sadowski and Portalska 1983).

An admittance electrode surrounded by a steel cannula fitted to syringe pumps may be used in place of the needle electrode. This setup allows for direct infusion of test substances into the medulla so that local effects may be monitored (Dowbrowolski and Sadowski 2004).

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Micropuncture Techniques in the Rat (Contribution by M. Hropot)

Purpose and Rationale

Micropuncture techniques have been applied to the direct investigation of the effect of diuretics on single nephron function. The observed changes in tubular fluid reabsorptive rates and electrolyte concentrations can be used to asses the mechanism of action. The rat is the model of choice since proximal and distal tubules as well as collecting ducts are accessible for micropuncture.

Procedure

Clearance and free-flow micropuncture studies are performed in rats with a body weight of about 250 g, anaesthetized by the intraperitoneal injection of thiopentone (Trapanal 50 mg/kg). The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia, the animals are placed on a thermostatically heated table. Thereafter, rats are tracheotomized and carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and infusion of compounds, respectively. The left kidney is carefully exposed by a flank incision, embedded in a small plastic vessel with cotton wool, and bathed with paraffin oil at 37 °C. The ureter is cannulated and rectal temperature monitored continuously. A bolus injection of 75 μCi inulin ^{3}H in 0.7 ml NaCl solution is given, followed by 0.85 % NaCl solution at a rate of 2.5 ml/min per 100 g body weight. The sustained infusion delivers 75 μCi inulin ^{3}H per hour. The control puncture of tubules is performed 45 min after beginning of the intravenous infusion. The direct collection of tubular fluid samples from proximal and distal tubules is carried out with glass capillaries of 8–10 μm external diameter using a micromanipulator and microscopic observation. Distal tubules are identified by intravenous injection of lissamine green.

The control period is followed by the test period. After an equilibration period of 30 min with the compound to be tested, micropuncture is performed again and tubular fluid is collected. The uretral urine is collected and blood sampling is performed in the middle of each clearance period.

The infusion rate/volume is determined by the size of the animal. Distal collection is done by identification of the perfused segment by its dye content; a second micropipette is then inserted, a distal oil or wax block is placed and the infusion fluid can be collected for subsequent analysis (Knox and Marchand 1976; Ramsey and Knox 1996; Lorenz et al. 1999; Wang et al. 1993, 1999).

Evaluation

The following parameters may be determined: inulin clearance (GFR), single nephron GFR, and fractional delivery of water, sodium, and potassium in proximal and distal tubules and in urine.

Fluid reabsorption (J_v) is assessed by comparing the inulin concentration in the perfusate to that in the collected sample, thus (Wang et al. 1993):

$$J_v = V_0 - VL,$$

where VL is the collected fluid volume (normalized for the length of the tubular segment over which it was collected), and:

$$V_0 = VL(INL/IN_0),$$

where INL = concentration of inulin in the collected sample, and IN_0 is the inulin concentration in the perfusate.

The net flux of the desired analyte (J_X, where X is the anlyte of interest – Na, K, HCO_3, glucose, etc.), is calculated thus:

$$J_X = V_0[X]_0 - VL[X]_L,$$

where $[X]_0$ is the concentration of the analyte in the perfusion fluid, and $[X]_L$ is the concentration of the analyte in the collected fluid.

All data are expressed as mean values ± SEM. Comparison of the effects of compounds to be tested with controls is performed by one way analysis of variance and by Student's *t*-test for paired and unpaired data.

Modification of the Method

Inulin conjugated to fluorescein isothiocyanate (FITC) may be used in place of the radiolabelled inulin. The inulin concentration in the sample is assessed by comparing the fluorescence in the sample to a standard curve (Lorenz and Gruenstein 1999).

This technique can be applied to distal tubules as well as to proximal tubules. Distal tubules are identified at the surface of the isolated kidney following micropuncture and perfusion of a proximal tubule as described above. Once the dye reaches the superficial portion of the distal tubule, it is blocked with oil or wax and accessed both proximally and distally as described above (Levine 1985).

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Stop-Flow Technique (Contribution by M. Hropot)

Purpose and Rationale

This procedure is of considerable value in the localization of transport processes along the length of the nephron. During clamping of the ureter, glomerular filtration is grossly reduced. The contact time for the tubular fluid in the respective nephron segments increases, and the concentration of the constituents of tubular fluid should approximate the static-head situation. After release of the clamp, the rapid passage of the tubular fluid should modify the composition of the fluid only slightly. The first samples should correspond to the distal nephron segment, the latest to glomerular fluid. However, with introduction of the micropuncture technique, the stop-flow method appears less attractive.

Procedure

This method can be performed in different animals during anesthesia and was originally described by Malvin et al. (1958). The ureter of an animal undergoing intense osmotic diuresis is clamped for several minutes allowing a relatively static column of urine to remain in contact with the various tubular segments for longer than the usual periods of time. Thus, the operation of each segment on the tubular fluid is exaggerated. Then the clamp is released, and the urine is sampled sequentially. Small serial samples are collected rapidly, the earliest sample representing fluid which had been in contact with the most distal nephron segment. Substances examined are administered along with inulin before the application of uretral occlusion. However, tubular segments downstream from the proximal segments may modify the tubular fluid during its egress.

Evaluation

In each sample, the concentration of a glomerular marker, such as inulin, and the concentration of the substance under study are measured. Fractional excretion of the substance and the glomerular marker are plotted versus the cumulative urinary volume.

Modifications of the Method

Shinosaki and Yonetani (1989) and Shinosaki et al. (1994) performed stop-flow studies on tubular transport of uric acid in rats treated with pyrazinoic acid, an inhibitor of tubular urate secretion.

Tanaka et al. (1990) used stop-flow experiments to test uricosuric and diuretic activities of new compounds in dogs.

Stop flow techniques can be performed in isolated perfused tubular segments using micropuncture techniques. The proximal and distal micropunctures are performed as described, but instead of immediately collecting fluid from the distal micropipette, pressure is applied equal to that of the perfusion pressure at the proximal pipette, until flow ceases at the infusing pipette. Sampling occurs following a period in which the perfusate remains static in the perfused tubule segment. Care must be taken, especially in mice, to prevent tubular leaking with excessive stop-flow pressures or infusion rates (Schnermann 1999).

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Impaired Renal Function

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Contents

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Chronic Renal Failure in the Rat

Purpose and Rationale

Chronic renal failure is a frequent pathological condition in man. An animal model as described by Acott et al. (1987) is of value to test new diuretics under these conditions.

Procedure

Sprague–Dawley rats weighing 150–200 are anesthetized by i.m. injection of ketamine (40 mg/kg) and droperidol/fentanyl (Inovar) 0.25 mg/kg. Through a 6-cm midline incision in the abdominal wall, the small bowel and cecum are lifted and placed on saline-soaked gauze sponges. The exposed right kidney is dissected from the retroperitoneal area and the vascular and ureteric pedicles ligated with 2–0 silk sutures, transected, and the kidney removed. The renal artery of the left kidney is dissected into the hilum to expose the three main segmental renal arteries. The kidney is not dissected out of the peritoneum. The anterior caudal branch of the artery is then temporarily ligated to establish the volume of renal tissue supplied. The area of ischemia becomes demarcated within 10–15 s. If this approximates 1/4–1/3 of the kidney, a permanent ligature is placed. The viscera are then carefully replaced in the abdomen and peritoneum

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and linea alba closed with a continuous suture. The skin is closed with stainless-steel clips.

Blood for serum creatinine is collected by retro-orbital puncture under anesthesia at various time intervals up to 12 months. In association with this, 24-h urines are collected for measurement of creatinine, protein, and specific gravity.

Evaluation

Serum creatinine increases up to 500 μM/l after 12 months, whereas creatinine clearance decreases. Significantly increased urine volumes are accompanied by decreased urine specific gravity indicating a decreased concentrating ability. Proteinuria is significantly increased. Terminal uremia occurs after 14–15 months.

Critical Assessment of the Method

The method may be used for special pharmacological studies as well as for evaluation of renal toxicity of new chemicals.

Modifications of the Method

Sancho et al. (1989) used a similar procedure in rats ligating two of the three terminal branches of the left renal artery, followed by right nephrectomy.

Freeman (1971) induced azotemia combined with hypothermia in rats by ligation of the urinary bladder at the base.

Williams et al. (1997) described renal ischemia-reperfusion injury in rats. The animals were anesthetized and subjected to 45 min of bilateral renal occlusion using atraumatic vascular clamps before renal perfusion was reestablished. After various time interval (up to 1 week) blood urea nitrogen, creatinine and myeloperoxidase activities in the kidney were determined. The protective effects of an intracellular adhesion molecule monoclonal antibody were tested.

Ishidoya et al. (1995), Klahr and Morrissey (1997) induced interstitial renal fibrosis by unilateral ureteral obstruction in Sprague Dawley rats and tested the effect of ACE inhibitors and angiotensin II receptor antagonists.

Hartenbower and Coburn (1972) described a method for producing chronic renal insufficiency in the *chick*. By urethral ligation, the function of one kidney was completely eliminated, and the functional mass of the other was reduced by two-thirds. The method resulted in elevation of plasma concentration of uric acid, the major product of protein catabolism in avian plasma, to levels two to four times normal for periods as long as 3 weeks.

Two to three weeks old White Leghorn cockerels are anesthetized with ether, and the abdominal feathers are clipped. The chick is placed supine on a small operating board with hips flexed and legs extended over the head. An incision is made along the left side of the abdomen extending into the peritoneal cavity. Self-retaining retractors are used to maintain exposure. The right ureter is identified and ligated just proximal to its junction with the cloaca. The left ureter and renal vein are ligated with a single suture near the middle of the left kidney.

The degree of azotemia is assessed by measuring uric acid levels in blood samples 0.2–0.3 ml, obtained by cardiac puncture at intervals of 2–6 days after surgery.

Uric acid blood levels are compared between operated and sham-operated animals. Histological examination is performed after sacrifice of the animals.

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Chronic Renal Failure After Subtotal (Five-Sixths) Nephrectomy in Rats

Purpose and Rationale

Subtotal (five-sixths) nephrectomy in rats has been used by many authors as model for chronic renal failure.Levine et al. (1997) used this model to evaluate the hypothesis that under these conditions, endogenous angiotensin II modulates in vivo bicarbonate reabsorption (J_{tCO2}) in distal tubules via H^+-adenosinetriphosphatase and Na^+H^+-exchange. Bicarbonate reabsorption (J_{tCO2}) in distal tubules is significantly increased in five-sixths nephrectomized rats.

Procedure

Surgical Procedure

Male Sprague–Dawley rats weighing 230–280 g are anesthetized with halothane via a face mask. Prior to the first incision, the animal is given 20 ml/kg normal saline subcutaneously. On a heated table, a midline laparatomy is performed 2 cm below the xiphoid bone cartilage and the abdomen opened. The right kidney is isolated and brought out of the abdomen by grasping the fat at the lower pole of the kidney. This fat is blunt dissected (sparing the adrenal gland), and three silk 4–0 ligatures are passed under the ureter, renal artery, and renal vein, tying one distally and two proximally. The ureter, renal artery, and renal vein are then cut between the ligatures, the proximal ties are cut short, and the distal tie is pulled out, removing the kidney. After the area is checked for bleeding, the left kidney is similarly isolated and brought out of the abdomen. The fat around the kidney is blunt dissected, avoiding excessive handling of the kidney or damage to the ureter, and the left and right renal poles are removed (two-thirds nephrectomy). The left remnant kidney is then returned to the abdomen and moistened with 5 ml of normal saline. The abdomen is closed with sutures and the skin with autoclips. Sham rats undergo the same procedure, except that the kidneys are only touched by the instruments. All rats are allowed 13–16 days recovery prior to microperfusion.

Microperfusion Experiments

The rats are anesthetized with 100 mg/kg thiobutabarbital sodium and placed on a heated operating table. After tracheostomy, using a PE-240 tubing, the left carotid artery is cannulated for continuous blood pressure measurement and collection of blood for acid–base and electrolyte analyses while the left jugular vein is cannulated with three lines for infusion of fluid, pentobarbital sodium anesthetic, and 10 % Lissamine green (Levine et al. 1996). The left kidney is exposed by flank incision, carefully dissected from the adrenal gland, and immobilized in a stainless steel cup covered with mineral oil. The ureter is catheterized with PE-50 tubing to ensure proper urine flow.

To replace surgical fluid losses, the rats are infused at 1 % body wt/h for 30 min via the jugular vein with donor plasma from control rats. The animals are then maintained on 0.9 % saline at 1 % body wt/h for the remainder of the experiment.

Two-loop perfusable surface distal tubules are identified by injecting a bolus of 1 % Lissamine green into surface proximal loops and observing its passage through the nephron. The distal tubules are perfused at 15 nl/min with a hypotonic solution containing (in mM) 28 HCO_3, 26 Cl, 56 Na, 2K, 1.8 Ca, 22 urea, and 4 gluconate as well as 0.05 %

FD and C green dye no. 3 (Keystone, Chicago, IL) and 0.1 % albumin. The perfused bicarbonate load, higher than in free flow, is chosen to more easily reveal the effect of inhibitors. Sample collections are quantitative and timed. A 10-min preperfusion period precedes all collections.

Groups of five-sixths nephrectomized rats are treated with various agents, e.g., angiotensin II, angiotensin$_1$ receptor antagonists, or Na^+/H^+ antiporter inhibitors.

Analyses

Whole blood and urine pH and P_{CO2} are measured quantitatively by an electrode blood-gas system and HCO_3 concentrations calculated. Plasma and urine Na^+ and K^+ concentrations are measured by flame photometry and Cl^- concentrations by electrotitration. Plasma total protein concentrations and urine specific gravity are measured by refractometry, and hematocrits are determined by a microcapillary reader. Urine osmolalities are determined by freezing-point osmometry. Plasma creatinine concentrations are determined by the Jaffé method without deproteinization.

Perfusate and sample total CO_2 concentrations are measured by microcalorimetry (Levine et al. 1996).A standard curve is run before sample analysis, and standard samples bracket the determination of sample and perfusate CO_2 determination.

Evaluation

The perfusion rate (R_P) is calculated as the product of the measured collection rate (R_C) and the ratio of inulin concentration in collected tubular fluid and perfusate. Water absorption (J_V) is calculated as the difference between the calculated perfusion rate and the measured collection rate (R_P minus R_C). J_{tCO2} is calculated as

$$J_{tCO_2} = [(R_P C_P) - (R_C C_C)/L$$

where C_P and C_C are the measured CO_2 concentrations in perfusate and collected fluid, respectively, and L is the tubular length in millimeters, measured by dissection after latex injection.

Data are expressed as the means ± SE. Statistical significance is assessed by two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by either Dunnett's test for multiple comparisons versus control or the Newman-Keuls test for all pairwise comparisons. Tests indicating a value of $P < 0.05$ indicate a statistically significant difference between groups.

Modifications of the Method

Function of the remnant kidney after subtotal (5/6) nephrectomy in **rats** has been used for many purposes:

- To test the potential benefit of calcium antagonists (Tolins and Raij 1990; Jarusiripipat et al. 1992; Van den Branden et al. 1997) or antioxidants (Vaziri et al. 1998)
- To verify the effect of ACE inhibitors (Pelayo et al. 1990; Kakinuma et al. 1992; Ashab et al. 1995; Liu et al. 1996; Ali et al. 1998; Cohen et al. 1998; MacLaughlin et al. 1998), angiotensin antagonists (Kohzuki et al. 1994, 1995; Brooks et al. 1995; Barreto-Chaves and Mello-Aires 1996; Noda et al. 1997; Lariviere et al. 1998; Roczniak et al. 1999) and endothelin receptor antagonists (Nabokov et al. 1996; Potter et al. 1997; Wolf et al. 1999; Brochu et al. 1999; Shimizu et al. 1999), or Na^+/H^+ antiporter inhibitors (Fernadez et al. 1994)
- To study the influence of hormones, such as parathyroid hormone (Fukagawa et al. 1991; Urena et al. 1994; Yi et al. 1995; Schaefer et al. 1996), growth hormone (Santos et al. 1992; Garcia de Boto et al. 1996), insulin-like growth factor I (Hazel et al. 1994; Mak and Pak 1996; Tonshoff et al. 1997), vasopressin (Bardoux et al. 1999), atrial natriuretic factor (Wong and Wong 1991, 1992), Luk et al. 1995), or erythropoietin (Poux et al. 1995; Zhou et al. 1997), during development of chronic renal failure.

Kimura et al. (1999) reported a model of progressive chronic renal failure in *rats*, produced by

a single injection of microspheres (20–30 μm in diameter) into the left renal artery after right nephrectomy.

Cowley et al. (1996) described the **Han:SRPD rat** strain, which develops autosomal dominant polycystic kidney disease with chronic renal failure that resembles human autosomal dominant polycystic kidney disease.

Chronic renal failure can be induced by feeding a lithium-containing diet (40–50 mmol/kg) to newborn *rats* until an age of 55–65 weeks (Christensen et al. 1992, 1997; Nyengaard et al. 1994).

Stockelman et al. (1998) described chronic renal failure in a **mouse** model of human adenine phosphoribosyltransferase deficiency. Hamilton and Cotes (1994) used a partial nephrectomy model in *mice* with two-thirds of total renal mass excised to evaluate erythropoiesis and erythropoietin production from extrarenal sources such as the submandibulary salivary gland. Koumegawa et al. (1991) suggested the DBA/2FG-pcy *mouse*, which develops numerous cysts in kidney cortex and medulla, a progressive anemia, and an elevation of blood urea nitrogen, as useful spontaneous model of progressive renal failure.

Brown et al. (1990) studied the metabolism of erythropoietin in normal and uremic **rabbits** with 5/6 nephrectomy. Bonilla-Felix used *rabbits* after 75 % nephrectomy to study the response of cortical collecting ducts from remnant kidneys to arginine vasopressin.

Fine et al. (1990), and Vaneerdeweg et al. (1992) described surgical techniques for kidney resection to produce chronic renal failure in **dogs**.

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Experimental Nephritis

General Considerations

Various experimental procedures were described as models for glomerulonephritis in human beings. Most of them were developed in rats and rabbits. They involve the reactions of antibodies against renal components, such as Masugi nephritis (Masugi and Sato 1934), Heymann nephritis (Heymann 1959), nephrotoxic serum nephritis (Unanue and Dixon 1967), crescentic type antiglomerular membrane nephritis (Nagoe et al. 1994, 1998), and anti-Thy1 nephritis (Chen et al. 1999).

Moreover, MRL Mpf lpr/lpr (MRL/lpr)-mice were described which spontaneously develop a severe disease with many symptoms very similar to human systemic lupus erythematodes, i.e., hypergammaglobulinemia and glomerulonephritis (Theofilopoulos and Dixon 1981; see also chapter "▶ Methods for Testing Immunological Factors").

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Nephrotoxic Serum Nephritis

Purpose and Rationale

Nephrotoxic serum nephritis is produced in animals by administration of heterologous antibody against glomerular basement membrane. This is regarded as an experimental model of human glomerular immune injury resulting in glomerulonephritis (Unanue and Dixon 1967). The glomerular lesions induced by nephrotoxic serum nephritis vary with species. The Wistar–Kyoto rat is susceptible to the induction of a crescentic

glomerulonephritis following small doses of nephrotoxic serum (Kushiro et al. 1998; Suzuki et al. 1998).

Procedure

Preparation of Nephrotoxic Serum

Normal Wistar rat kidneys are fully perfused with physiological saline through a catheter placed in the aorta. Renal cortical tissue is removed, homogenized, and diluted with physiological saline at about 20 % suspension. Two ml of renal cortical homogenate are emulsified with an equal volume of Freund's complete adjuvant. This emulsion is injected subcutaneously into rabbits twice a month for 2 months. Seven days after the last injection, the rabbits are bled from the carotid artery under anesthesia. The sera are decomplemented for 30 min at 56 °C and absorbed with freshly harvested rat erythrocytes.

Experimental Protocol

Male Wistar–Kyoto rats weighing 150 g receive either continuous administration of the test drug by an osmotic pump (ALZA Co., Palo Alto, USA) or saline. Twenty-four hours later, the rats are injected with 1 ml of nephrotoxic serum. At 9, 12, and 14 days, urine samples are collected and urinary protein levels measured using the Lowry method. At 14 days, the rats are sacrificed under ether anesthesia, and both kidneys are removed. Portions of these tissues are processed for light microscopy, immunofluorescence staining, and immunoperoxidase staining.

For light microscopy, tissues are fixed and embedded in paraffin. Sections are stained with hematoxylin and eosin and periodic acid Schiff's reagent. Twenty glomeruli are examined per rat and number of glomeruli which forms crescent counted.

Indirect immunofluorescence studies are performed on 3 μm-thick cryostat sections which are air dried and incubated with antirat intercellular adhesion molecule-1 (ICAM-1) antibody (Tamatani and Miyasaka 1990) for 60 min at room temperature. After washing the antibody binding is visualized by incubating the sections for 30 min with fluorescein isothiocyanate-labeled goat antimouse IgG.

Direct immunofluorescence studies are performed on 4 μm thick cryostat sections, which are incubated with fluorescein isothiocyanate-labeled goat antirat IgG, goat antirat C3, goat antirat fibrinogen, and goat antirabbit IgG.

The staining intensity of 20 glomeruli per rat is semiquantitatively assessed into four grades.

For **immunoperoxidase staining,** the distribution of leukocytes is examined using an immunoperoxidase ABC kit (Vector Lab, Burlingame, USA). Nonspecific protein binding is blocked by incubating the cryostat sections with 10 % bovine serum in Tris-buffered saline for 20 min. Nonspecific staining is blocked by 15 min incubation with avidin and then biotin using the avidin-biotin blocking kit (Vector Lab). Endogenous peroxidase activity is inhibited by incubating the sections in methanol containing 0.3 % H_2O_2 for 20 min. Sections are first incubated for 60 min with primary antibodies at room temperature, incubating monoclonal antibodies against rat monocytes/macrophages (ED-1), rat CD4 and rat CD8. Then, the sections are incubated with biotinylated donkey antimouse IgG for 30 min at room temperature. Biotinylated horseradish peroxidase is applied for 30 min at room temperature. Peroxidase activity is developed in 3,3-diaminobenzidine and hydrogen peroxide. The sections are then counterstained with Mayer's hematoxylin. The number of ED-1 positive cells, CD4 positive cells, and CD8 positive cells per glomerular cross section is counted in 20 glomeruli per rat.

Evaluation

All data are expressed as mean ± SEM. Significance of differences between groups is determined using Wilcoxon's test.

Modifications of the Method

Masugi and Sato (1934), Krakower and Greenspon (1951), Heyman et al. (1959, 1965), Eddington et al. (1968) already described experimental allergic glomerulonephritis in rats.

Ito et al. (1983) and Nagao et al. (1994, 1998) induced crescentic-type antiglomerular basement membrane nephritis in male Sprague Dawley rats by injecting 6.5 mg rabbit gamma-globulin in 0.25 ml Freund's complete adjuvant into the hind foot pads, following the injection of 0.6 ml of rabbit antirat glomerular basement membrane serum into the tail vein.

Couser et al. (1978) studied the development of immune deposits on the subepithelial surface of the glomerular capillary wall in isolated rat kidneys perfused at controlled perfusion pressure, pH, temperature, and flow rates with recirculating oxygenated perfusate containing bovine serum albumin in buffer and sheep antibody to rat proximal tubular epithelial cell brush border antigen.

Hayashi et al. (1996) tested the effects of a flavonoid in original-type antiglomerular basement membrane antibody-associated glomerulonephritis in male Sprague Dawley rats on upregulation of intracellular adhesion molecule expression and on increase in leukocyte function-associated antigen positive cells in nephritic glomeruli.

Nagamatsu et al. (1999) found beneficial effects of an angiotensin II type I receptor antagonist in antiglomerular basement membrane antibody-associated nephritis in rats.

Sanaka et al. (1997) evaluated the effects of a free radical scavenger on the progression of nephrotoxic serum nephritis in male Sprague Dawley rats. The rat glomerular basement membrane was prepared according to the method of Krakower and Greenspon (1951), who localized the nephrotoxic antigen within the isolated renal glomerulus.

Kawasaki et al. (1992) induced crescentic glomerulonephritis with a small dose of nephrotoxic serum in WKY rats, which was characterized by the early infiltration of CD8 positive cells in glomeruli. In vivo depletion of CD8 positive cells completely prevented proteinuria and crescent formation.

Okuda et al. (1990) provided evidence of an elevated expression of transforming growth factor-β, proteoglycans, and fibronectin in glomerulonephritis induced in rats by injection of antithymocyte serum.

Hamada and Nagase (1996), and Chen et al. (1999) induced anti-Thy1 nephritis with the antibody to the Thy-1 antigen which is present in the mesangial cells of the glomeruli. The early pathobiological cellular events are characterized by invasion of platelets, polymorphonuclear leukocytes, and monocytes into the glomerulus which occurs within hours after induction of nephritis. Complement-dependent mesangiolysis then ensues between day 1 and 3.

Liebler et al. (2004) induced acute anti-Thy1.1-mesangioproliferative glomerulonephritis by administering 500 μg of Moab 1-22-3 dissolved in PBS as a single-shot injection into the tail vein of the rats on day 0. Moab 1-22-3 is a monoclonal antibody directed against the Thy1.1-like antigen on the surface of rat mesangial cells.

Passive Heymann nephritis (Heymann 1959) was used a model by Hara et al. (1991), Nagao et al. (1996), and Heise et al. (1998).The disease is induced in rats by heterologous antibody to crude renal border antigen Fx1A. The model is characterized by granular deposition of heterologous and homologous antibody and complement along the glomerular capillary wall, and as a counterpart, extensive electron-dense subepithelial deposits are seen at the ultrastructural level. Massive proteinuria develops after a latent period of 2–4 days in the absence of glomerular hypercellularity.

Kawasaki et al. (1995) studied the therapeutic effect of combined treatment with monoclonal antibodies against intercellular adhesion molecule 1 (ICAM-1) and lymphocyte-function-associated antigen 1 (LFA-1) in **Masugi nephritis** of Wistar–Kyoto rats.

Thaiss et al. (1989) evaluated the effect of the immunosuppressant cyclosporin A on an active model of in situ immune complex glomerulonephritis. Wistar rats were preimmunized with human IgG, and 2 weeks after the last antigen injection, the left kidney was perfused with cationized human IgG in order to induce unilateral in situ immune complex glomerulonephritis.

Okubo et al. (1990) studied the immunosuppressive effects of FK506 on active Heymann's nephritis and the autologous phase of Masugi nephritis.

Rennke et al. (1994) developed a model system of acute nephritis in the rat whereby a chemically reactive form of the hapten azobenzenearsonate is introduced directly into the left kidney of preimmunized Brown Norway rats.

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Experimental Nephrosis

Purpose and Rationale

Some compounds used in antineoplastic therapy induce nephrosis in animals, like the antibiotic adriamycin (=doxorubicin) (Milner et al. 1991, 1994; Chagnac et al. 1994; Wapstra et al. 1996; De Boer et al. 1999; Mutti et al. 1999) or daunomycin (Kimura et al. 1993), the aminonucleoside puromycin (Yayama et al. 1993; Guoji et al. 1994; Magil 1996; Ebihara et al. 1997; Nosaka et al. 1997; Park et al. 1998; Asami et al. 1999; Pedraza-Chaverri et al. 1999), or cisplatin (Abdel-Gayoum et al. 1999).

These models allow to test the protective effects of drugs even after the renal disease in established, i.e., mimic the clinical situation (Wapstra et al. 1996).

Procedure

Male Wistar rats with an initial weight of about 300 g receive a single intravenous dose of 2 mg/kg adriamycin. Twice a week during a 12-weeks period, the animals are weighed, 24-h urine is collected, and blood pressure is measured by the tail-cuff method.

During the first 5 weeks, all animals are kept on a low-sodium diet with tap water ad libitum. After stabilization of proteinuria (5 weeks), animals are divided into two groups receiving either low-sodium or normal-sodium diet. After a week stabilization on these diets, animals receive different doses of treatment in their drinking water. These regimen is continued until the end of the study (week 12), at which time all animals are sacrificed and blood samples and kidney tissue are obtained.

During each blood pressure measurement session, five measurements are recorded for each animal. The blood pressure is taken as the mean of the last three recordings. Urinary protein is determined by the Pyrogallol Red-molybdate method (RA-1000 Technicon). Urinary sodium, creatinine and urea and serum electrolytes, creatinine, albumin, cholesterol, and triacylglycerols are measured by a standard autoanalyzer technique. Kidney samples are fixed in paraformaldehyde and embedded in paraffin. Sections are stained with the periodic acid/Schiff technique. Focal glomerular sclerosis is scored semiquantitatively by light microscopy.

Evaluation

Analysis of covariance is used to compare the experimental groups (defined by diet or dose of drug) after 2 weeks of treatment (week 8) and the end of the study (week 12) with the baseline value (week 6) as a covariable. Tuckey's method is used for comparison of groups receiving different doses of drug. The other statistical comparisons are performed using Student's *t*-test.

Modifications of the Method

Fawn-Hooded rats develop systemic hypertension and spontaneous age-dependent glomerulosclerosis with proteinuria (Mackenzie et al. 1997).

Spontaneous nephrosis with proteinuria occurs in Dahl salt-sensitive rats fed on a normal-sodium diet (Yoneda et al. 1998).

Mizuno et al. (1999) studied the ICGN mouse strain as a unique model for naturally occurring nephrotic syndrome.

Focal segmental glomerulosclerosis with heavy proteinuria has been found in mice in which the Mpv17 gene was inactivated ($Mpv17^{-/-}$ mice). Binder et al. (1999) recommended these animals as model of steroid-resistant nephrosis sensitive to radical scavenger therapy.

Kimura et al. (1993) described strain specificity in the susceptibility of mice to daunomycin-induced nephrosis.

Klahr and Morrissey (1997) described the effects of ACE inhibitors and angiotensin II receptor antagonists on various parameters associated with renal interstitial fibrosis induced by unilateral ureteral obstruction in rats.

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Uricosuric and Hypo-Uricemic Activity

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Contents

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In Vitro Methods

Inhibition of Xanthine Oxidase In Vitro Indicating Hypouricemic Activity

Purpose and Rationale

Synthesis of uric acid primarily occurs in the liver, but the kidney has an important role in the pathophysiology of hyperuricemic syndromes. Because uric acid is poorly soluble, excessive amounts in the circulation may precipitate out into the tissues, particularly the joints, resulting in a painful arthropathy ("gout"). In humans, this condition is usually the result of faulty tubular transport of urate, resulting in increased net reabsorption. Attempts to treat hyperuricemia with tubular transport inhibitors (theoretically increasing urinary excretion of urate) frequently exacerbate the condition because tubular transport is bi-directional; reduction of net uric acid synthesis by the inhibition of xanthine oxidase is the preferred therapeutic approach. Furthermore, in renal hypoxic conditions, xanthine oxidase contributes to renal injury by the generation of oxygen free radicals and xanthine oxidase inhibition has been shown to be useful in such conditions (Shinosaki and Yonetani 1991; Rhoden et al. 2000; Terkeltaub et al. 2006).

Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Xanthine oxidase is a complex metalloflavoprotein containing one molybdenum, one FAD, and two iron-sulfur centers of the ferredoxine type in each

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of its two independent subunits. Usually, the enzyme is isolated from cow's milk. The enzyme is inhibited by allopurinol and related compounds. The production of uric acid from the substrate (xanthine) can be determined by measuring the change in optical density in the UV range.

Procedure

The test compound is incubated with xanthine oxidase (usually derived from milk, sometimes derived from rat liver or small intestine), EDTA, and phosphate buffer solution (pH 7.8) at 37 °C. Control solutions without test compound are incubated under identical conditions. Following addition of xanthine, the change in absorbance is determined.

Assay conditions:

- Wavelength: 293 nm
- Line path: 10 mm
- Final volume: 1.0 ml

Evaluation

The percent inhibition of xanthine oxidase is determined relative to control solutions.

IC_{50} values of test compounds are calculated. Standard data:

- Allopurinol: IC_{50}: ca 10^{-8} mol/l

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Urate Uptake in Brush Border Membrane Vesicles

Purpose and Rationale

The urate-anion exchanger system in brush border membrane vesicle, which mediates hydroxyl ion gradient-dependent urate uptake, is the most likely route for the mediation of urate transport in the first step of urate reabsorption in the proximal tubules. Luminal drugs which inhibit urate reabsorption are inhibiting the transport of urate by blocking the urate/anion exchanger.

Procedure

Male Sprague Dawley rats weighing 320–380 g are sacrificed by decapitation, and the kidneys are removed immediately. All steps for the preparation of brush border membrane vesicles are carried out at 4 °C. Renal cortex is homogenized for 2 min in a medium containing 250 mmol/l mannitol, 10 mmol/l Tris, and 16 mmol/l HEPES buffer (pH 7.5) using a Physcotron homogenizer. The homogenate is centrifuged at 2,400 *g* for 10 min, and the supernatant is centrifuged at 2,800 *g* for 20 min. Subsequently, the supernatant is discarded and the loosely packed membrane-rich layer is flushed off from the bottom densely packed brown pellet. The membrane-rich layer is resuspended manually in 250 mmol/l mannitol containing 10 mmol/l Tris-HEPES (pH 7.5) using a Dounce homogenizer and $MgSO_4$ is added to a final concentration of 10 mmol/l. After standing for 20 min, the suspension is centrifuged at 2,400 *g* for 20 min and the supernatant containing brush border membranes is recentrifuged two more times at 2,400 *g* for 20 min. The final supernatant is centrifuged at 28,000 *g* for 20 min, and the pellet is suspended in a small amount of medium containing 150 mmol/l mannitol, 50 mmol/l potassium phosphate buffer (pH 7.5), and 2 mmol/l $MgSO_4$ to a final protein concentration of 4–8 mg/ml.

The brush border membrane vesicle preparation is frozen and stored at −80 °C until use.

After preincubation of the brush border membrane vesicle preparation for 2 h, [2-^{14}C]urate uptake is initiated by adding 200 µl of incubation medium to 20 µl of the membrane suspension. The incubation medium has the following composition (mmol/l): 150 mannitol, 2 $MgSO_4$, 50 potassium phosphate buffer, pH 6.0 or 7.5, 0.02[2-^{14}C]urate, and various concentrations of the inhibitor. At 10 s after the addition of the incubation medium, 200 µl portions of the suspension are pipetted onto the center of prewetted cellulose acetate filters kept under suction. The vesicles retaining on the filter are washed immediately with 5 ml of an ice-cold solution containing 150 mmol/l mannitol and 50 mmol/l potassium phosphate buffer, pH 6.0 or 7.5, which is used at the same pH as the incubation medium. Preincubations and incubations are performed at 23 ± 1 °C. Each experiment is performed in triplicate. Corrections are made for the radioactivity bound to the filters in the absence of membrane vesicles. The term of the OH^- gradient-dependent urate uptake is defined as the difference between the uptakes in the incubation medium at pH 6.0 and that at pH 7.5. The OH^- gradient-dependent urate uptake at 10 s is assumed to present an initial velocity.

Evaluation

From a concentration-response curve relating log concentration of drug to the logit activity of the OH^- gradient-dependent urate uptake for 10 s, IC_{50} (concentration producing 50 % of inhibition) is determined by least-squares regression analysis.

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In Vivo Methods

Diuretic and Uricosuric Activity in Mice

Purpose and Rationale

Renal excretion of uric acid consists of three components: complete filterability of uric acid in the glomerulus, subsequent tubular reabsorption, and tubular secretion (Gutman and Yü 1961). Pronounced species differences have been described in uric acid metabolism including man. Mice were recommended for primary screening of uricosuric drugs.

Procedure

Male NMRI mice weighing 25–30 g are used. On the evening prior to the experiment, food but not water is withheld. In the morning, the mice are orally loaded with 50 ml/kg 0.9 % NaCl-solution. Together with the sodium load, the test compound is applied by gavage in 2 % starch suspension. Controls receive saline and starch suspension only. Groups of five mice are placed into metabolism cages. Urine is collected over 4 h. In the urine, sodium and potassium are determined by flame photometry, chloride by argentometrically with potentiometrical end point titration (Chloride titrator, Aminco), uric acid by the Uriquant-method, creatinine by the Jaffé-reaction, as well as pH and osmolality.

Evaluation

Urine excretion is calculated in ml/kg. Uric acid-, creatinine-, and ion-excretions are calculated in mmol/kg and expressed as percent changes versus controls. The changes are evaluated statistically using Student's *t*-test.

Critical Assessment of the Method

Some saluretic-diuretic agents, like ethacrynic acid, are inactive in the rat, when given orally.

Moreover, uricosuric activity in mice is less reliable than that in primates.

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Hypouricemic Activity After Allantoxanamide Treatment in Rats

Purpose and Rationale

Most species used for pharmacological experiments have rather low blood levels of uric acid. Experimental hyperuricemia can by induced by inhibition of the enzyme uricase. In most species uricase metabolizes uric acid to allantoin. Allantoxanamide blocks uricase and increases endogenously synthesized uric acid. This increase is blocked by compounds like allopurinol.

Procedure

Non-fasted male Sprague Dawley rats weighing 230–280 g are treated by intraperitoneal injection of 250 mg/kg allantoxanamide suspended in 5 ml/kg sesame oil. The test compound is applied orally in a dose of 50 mg/kg in 40 ml/kg water. Likewise, the standard compound allopurinol is given in a dose of 50 mg/kg. Eight rats are used for the each dose of test drugs and standard. The animals are placed individually into metabolism cages with free access to food and water. Urine is collected during the periods of 1–6 and 7–24 h. Blood is withdrawn by retroorbital puncture prior and 2, 6, and 24 h after compound administration. Uric acid is determined with the Uric-aquant-method in plasma [mmol/l] and urine [mmol/l].

Evaluation

Mean values of uric acid concentrations in plasma at the different time intervals and mean values of uric acid excretion after 6 and 24 h of the test group are compared with the control group (allantoxanamide treated only) using Student's *t*-test.

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Hypouricemic and Uricosuric Activity After Potassium Oxonate Treatment in Rats

Purpose and Rationale

Increase of uric acid in serum of rats is induced by a special diet and the uricase inhibitor potassium oxonate. Uric acid concentration in serum and uric acid excretion in urine prior and after three experimental days are determined.

Procedure

Male Wistar rats weighing 250 g are placed individually in metabolic cages. They are offered a special diet containing 5 % fructose, 3 % uric acid, 2 % potassium oxonate (2,4-dihydroxy-1,3-triazine-6-carbonic acid), and 0.001 % artificial sweetener. Drinking water consists of a 0.5 % solution of potassium oxonate. The animals are treated orally with 50 or 100 mg/kg of the test compound or the standard (allopurinol) dissolved in 5 ml/100 g body weight of 0.5 % potassium oxonate solution. The treatment is repeated on the second day. On the third day, 24 h urine is collected and the animals are sacrificed by exsanguination. Concentrations of uric acid and electrolytes (Na^+, K^+, Cl^-) are determined in blood and urine.

Evaluation

Concentrations of uric acid and electrolytes in blood and urine of animals treated with the test compound are compared statistically with control and standard drug-treated animals.

Modifications of the Method

Clearance techniques in oxonate-treated rats were used by Yonetani et al. (1987), Shinosaki et al. (1991), Dan et al. (1994).

Sugino and Shimada (1995) tested uricosuric effects in oxonate-loaded rats, in the pyrazinoic acid suppression test, and in the phenolsulfonphthalein test.

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Phenol Red Excretion in Rats

Purpose and Rationale

Phenol red (= phenolsulfonphthalein) excretion is an indirect test for uricosuric activity. After

intravenous injection, phenol red is mainly eliminated by active secretion in the proximal tubulus of the kidney. Treatment with uricosuric agents decreases the secretory activity of tubulus cells resulting in a delayed excretion of phenol red. Plasma values of phenol red are increased in treated animals as compared to controls.

Procedure

Male Wistar rats weighing 120–150 g are treated orally with the test compound or the standard 30 min prior to intravenous injection via the tail vein with 2.5 ml/kg of a 3 % aqueous solution of phenolsulfonphthalein. For intravenous application, 5.0 ml/kg of the test drug solution are injected immediately after the phenolsulfon-phthalein injection followed by flushing with 2.5 ml/kg saline. By retro-orbital puncture, blood samples are withdrawn after 30, 60, and 180 min. Blood (0.2 ml) is diluted with 2 ml 0.9 % NaCl-solution and centrifuged. To 1 ml of the supernatant, 1 ml of 1 % sodium carbonate solution and 8 ml of saline are added. Using a spectrophotometer (Eppendorf, Hamburg) extinction at 546 nm is determined.

Evaluation

Extinction values are calculated for total blood. At each time interval, the values in treated rats are compared statistically with those of controls.

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Uricosuric Activity in Dalmatian Dogs

Purpose and Rationale

Most species have low plasma levels and low renal excretion of uric acid. The Dalmatian dog is an exception with excessive uric acid excretion, but with relatively high plasma levels. Explanations are a genetically determined defect in tubular reabsorption of the filtered urate (Friedman and Byers 1948; Kessler et al. 1959) and a defective hepatic uricase activity (Yü et al. 1971). The Dalmatian dog is being used for studies of uricosuric agents.

Procedure

Conscious male Dalmatian dogs with a body weight of about 20 kg are used. Food but not water is withheld 24 h prior to the experiment. The animals are placed individually in metabolic cages. The urinary bladder is emptied by catheterization. Twenty ml/kg drinking water is applied by gavage. Every 2 h, the animals are catheterized again, blood is withdrawn from a jugular vein, and additional 8 ml/kg drinking water is applied by gavage. The urine and blood values obtained after the first 2 h serve as control. Then, the test compound is applied either i.v. or orally. Up to 8 h, blood and urine samples are collected every 2 h. No water is given after the last sampling. The dogs stay over night in the metabolic cages. Twenty-four hours after beginning of the experiment, venous puncture and catheterization is performed once more. Blood and urine samples are analyzed for uric acid (Uricaquant-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

Evaluation

The values after application of the drug are compared with predrug values.

Critical Assessment of the Method

Dalmatian dogs bred by commercial breeders are not always homozygous. Therefore, not every dog is suitable for experiments on uric acid excretion.

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Uricosuric Activity in Cebus Monkeys

Purpose and Rationale

The Cebus monkey is described not to possess uricase (Simkin 1971) and to have different metabolic conditions for uric acid than other experimental animals. This species is chosen for special studies of anti-uricopathic drugs since it resembles more closely human uric acid metabolism.

Procedure

Cebus monkeys (*Cebus albifrons*) of either sex weighing 3.0–5.0 kg are used. Twenty-four hours prior to the test, food is withheld, but water is available ad libitum. On the morning of the experiment, the animals receive 20 ml/kg drinking water by gavage, followed by oral application of the test compound. Control animals receive water only. The animals are placed in individual metabolism cages and the spontaneously voided urine is collected after 2, 6, and 24 h. After 2 and 6 h, additional 4 ml/kg water is given by gavage. From a cubital vein, blood is withdrawn prior to the experiment and 2, 6, and 24 h after application. Urine and serum samples are analyzed for uric acid (Uricaquant-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

Evaluation

Allopurinol and probenicide are used as standard drugs and are compared with test compounds.

Critical Assessment of the Method

The use of the Cebus monkey as animal model has been proven to be the most valuable method to test putative hypouricemic compounds (Hropot 1988).

Modifications of the Method

Onuma et al. (1988) used Cebus monkeys for evaluation of uricosuric effects of an aryloxyacetic derivative.

Yonetani et al. (1987) performed clearance experiments with uricosuric drugs in anesthetized chimpanzees.

Dan et al. (1989) tested the activity of AA-193, an uricosuric agent in rats, mice, and Cebus monkeys.

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Drug Influence on Lower Urinary Tract

Susan Emeigh Hart

Contents

Drug Influence on Lower Urinary Tract

In Vivo Studies

Micturition Studies

Purpose and Rationale

Urinary incontinence is a major psychosocial, medical, and economic problem. The most common condition to be treated pharmacologically is incontinence due to detrusor instability. The response of the urinary bladder to filling with increasing volumes of fluid (cystometrogram) is a common procedure for evaluating bladder function in both animals and humans. The response of the vesicourethral complex can be arbitrarily divided into the collection and expulsion phases. The nervous control of the detrusor and the internal and the external sphincter has been reviewed by Kuro (1965). A detailed description of the nervous control of the urinary bladder of the cat has been given by de Groat (1975). The pharmacology of lower urinary tract muscles and penile erectile tissues has been reviewed by Anderson (1993). Ferguson and Christopher (1996) reviewed urine bladder function and drug development. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of reflexes involving cholinergic, sympathetic, and, possibly, purinergic, serotonergic, and peptidergic innervation. In view of this complexity, in vivo models were developed for the quantitative analysis of the

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effects of drugs on the function of the vesicourethral complex (Maggi et al. 1983, 1985, 1986, 1987a, b, 1992).

Procedure

Male Wistar rats weighing 340–360 g are anesthetized by subcutaneous injection of 1.2 g/kg urethane. The left jugular vein is cannulated for drug injection. Body temperature is kept constant by means of a heating pad maintained at 37 °C. Through a midline incision of the abdomen, the urinary bladder is exposed and emptied of urine by application of a slight manual pressure. A 20-gauge needle is inserted through the apex of the bladder dome for 3–4 mm into its lumen. The needle is connected to a pressure transducer by means of a polyethylene tubing (1.5-mm OD and 1.0-mm ID) and the whole system filled with saline. The tubing is provided with an internal coaxial polyethylene tubing (0.6-mm OD and 0.3-mm ID) inserted through a side hole and sealed by a drop of epoxy resin. The second tubing serves for intravesical infusion of fluid and is connected, through a peristaltic pump, to a saline reservoir.

Intraluminal pressure signals are delivered to an amplifier and displayed on a four-channel polygraph. Warm saline-soaked cotton wool swabs are laid around the exteriorized organ to maintain its temperature and to keep it moist in experiments involving the topical application of substances on the bladder dome.

After a 15-min equilibration period at zero volume, variations in intraluminal pressure are recorded in response to continuous infusion of saline at a rate of 2.8 ml/h at 37 °C for 30–40 min by means of a peristaltic pump connected to the polyethylene tubing inserted into the bladder. This infusion rate simulates the maximal hourly diuresis within the physiological range. In each preparation, the infusion is continued until micturition occurs. Micturition is referred as the emission of several drops of fluid during a sustained phasic contraction of the detrusor muscle which is followed by return to zero or, in any case, to a value lower than that recorded just before micturition.

For both intravenous and topical administration, substances are dissolved in saline.

Evaluation

In each experiment, the following parameters are evaluated:

1. Pressure threshold = intraluminal pressure value recorded just before micturition
2. Volume threshold = the volume of infused saline required to obtain micturition
3. Maximal amplitude of micturition contraction
4. Residual volume after micturition

The effect of substances on the compliance of the bladder wall is evaluated by comparing the volume–pressure relationship of treated animals with that of controls.

Statistical analysis of the data is performed by means of the Student's *t*-test for paired or unpaired data or by means of analysis of variance followed by Tukey's test. Statistical analysis of nonparametric data is made by the chi-square test.

Modifications of the Method

Either chemical (6-hydroxydopamine, reserpine) or surgical (section of hypogastric nerves) sympathectomy produces a picture of detrusor hyperreflexia and urine dropping, mimicking cystometric finding in human disease (Maggi et al. 1987a).

Postius and Szelenyi (1983) described a model for in vivo screening of spasmolytic compounds using the rat bladder.

Dray (1985) used the spontaneous, volume-induced contractions of the urinary bladder in the anesthetized rat to assess the central activity of substances with opioid properties.

Pietra et al. (1990) studied the effects of some antidepressants on the volume-induced reflex contractions of the rat urinary bladder. The urinary bladder of anesthetized rats was filled via the recording catheter by incremental volumes of warmed saline until bladder contractions occurred as a result of central activity. Volume-induced contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h. Drug activity was assessed in each animal against the

background frequency of bladder contractions, for a 15-min time period following intravenous administration of different doses.

Harada et al. (1992) proposed a method for rapid evaluation of the efficacy of pharmacologic agents and their analogues in enhancing bladder capacity and reducing the voiding frequency. Conscious rats were placed in a restrainer over a urine collector. The collector was secured to a Statham UC3 strain transducer, the output of which was amplified by a Gould bridge amplifier. Data were monitored on a polygraph. This method has been simplified by the use of an infrared photodiode sensor and matched phototransistor that can detect the appearance of urine flowing from the bottom of a metabolic cage, recording the frequency of these events. This method produces results comparable to the original cup-fore transducer methods (Argentieri and Argentieri 2002).

Conte et al. (1991) proposed a method for simultaneous recording of vesical and the external urethral sphincter pressure in urethane-anesthetized rats.

Angelico et al. (1992) reported in vivo effects of different antispasmodic drugs on the rat bladder contractions induced by topically applied KCl.

Oyasu et al. (1994) measured spontaneous bladder contractions caused by raising the intravesical volume in anesthetized rats.

Yaksh et al. (1986) described a chronic model for study of micturition in unanesthetized rats. A bladder catheter was implanted chronically through laparotomy and externalized percutaneously.

Horváth et al. (1994) reported an ultrasonic method to study the influence of drugs on micturition in intact rats.

Tillig and Constantinou (1996) described videomicroscopic imaging of urethral peristaltic function in anesthetized rats. Cystometrograms were performed by recording continuously the bladder pressures while detecting micturition using a sensor placed at the orifice of the urethra. Renal pelvic pressure was measured during continuous perfusion using a nephrostomy inserted through the parenchyma. A catheter was placed in the femoral vein for intravenous drug administration. The left pyelo-ureteric junction and the upper part of the ureter were visualized using a stereomicroscope equipped with a video camera and a tape recorder. One syringe pump was used for filling the bladder to perform continuous cystometrograms. Another syringe pump was used for the infusion of indigo carmine to assist the visualization of the bladder pressure.

Conte et al. (1988) developed a cystometric technique for quantitative studies on physiopharmacology of micturition in conscious, freely moving rats.

Seif et al. (2004) performed urinary bladder volumetry by means of a single retrosymphysically implantable ultrasound unit.

Peterson et al. (1989) and Noronha-Blob et al. (1991) described in vivo cystometrogram studies in urethane-anesthetized and conscious **guinea pigs**.

Moreau et al. (1983) described simultaneous cystometry and uroflowmetry for the evaluation of the caudal part of the urinary tract in **dogs**.

Imagawa et al. (1989) reported an in vivo procedure for functional evaluation of sympathetically mediated responses in lower urinary tract of dogs.

Häbler et al. (1990, 1992) examined the functional properties of unmyelinated and myelinated primary afferent neurons innervating the pelvic viscera in anesthetized **cats**. The axons were isolated from the intact dorsal root and the intact or chronically de-efferented ventral root of segment S2. The responses of the neurons were studied with natural stimulation of the urinary bladder using innocuous or noxious increases of intravesical pressure.

Tai et al. (2004) described bladder and urethral sphincter responses evoked by microstimulation of S2 sacral spinal cord in spinal cord intact and chronic spinal cord injured **cats**.

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Studies in Isolated Organs

Studies on Renal Pelvis

Purpose and Rationale

Periodic contraction of the renal pelvic wall is important to the process of concentration of urine in the renal medulla as well as the propulsion of urine from the kidney into the bladder (Santicioli and Maggi 1997, 1998; Knepper et al. 2003). The isolated renal pelvis of the guinea pig has been used to examine the effects of compounds in this activity (Maggi and Giuliani 1991, 1992; Maggi et al. 1992a, b, c, 1994, 1995; Giuliani and Maggi 1996; Santicioli et al. 1995; Santicioli and Maggi 1997; Patacchini et al. 1998; Bigoni et al. 1999).

Procedure

Male albino guinea pigs weighing 250–300 g are used. Following appropriate anesthesia and euthanasia, one whole kidney and attached ureter are removed and placed in oxygenated Krebs solution. The renal pelvis is carefully dissected from the renal parenchyma, separated from the ureter, cut, and connected to threads to record motility along the circular axis. The preparation is suspended in a 5-ml organ bath and mechanical activity recorded by means of an isotonic transducer (load 1 mN). Transmural electrical field stimulation is made by means of platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S 88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) are delivered in trains of 10 s duration at frequencies of 5–10 Hz.

Experiments commence after a 60–90-min equilibrium period after which the amplitude and frequency of spontaneous activity have reached a steady state. In vitro capsaicin desensitization is made by exposure of the preparation to 10 μM for 15 min, followed by washing out and further equilibration for 30–60 min.

Concentration–response curves to noradrenaline and acetylcholine are performed by noncumulative addition to the bath at 20-min intervals. Contact time of drugs is 15 min.

Evaluation

The amplitude and frequency of spontaneous contractions are assessed, and the motility index (MI) is calculated as follows:

$$\mathrm{MI} = (\Sigma\,\mathrm{amp}/5) \times F$$

where Σ amp/5 is the mean amplitude of five contractions (in mN) and F is the frequency (in min^{-1}) of those five contractions. Concentration–response curves are generated by plotting the concentration versus the MI (either raw or expressed as a percent of control, using the parallel incubation as the control) (Davidson and Lang 2000).

Modifications of the Method

Zhang and Lang (1994), Lang et al. (1995), Lang and Zhang (1996), and Teele and Lang (1998) recommended circumferentially cut strips from the proximal renal pelvis of guinea pigs since these strips contract more frequently than strips cut from the mid region.

Kimoto and Constantinou (1990, 1991) studied contractility of smooth muscle strips from the pacemaker regions and pelviureteric junction of renal pelvis from **rabbits**.

Kondo et al. (1992) determined the effects of dobutamine and terbutaline on adenylate cyclase activity and cyclic AMP content in the renal pelvis of rabbits.

Seki and Suzuki (1990) made intracellular recordings to study the electrical properties of smooth muscle cells in the rabbit renal pelvis.

Zwergel et al. (1991) developed an intact **canine** model to measure renal pelvic pressure after complete ureteral obstruction with a balloon catheter inflated in the distal ureter.

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Propagation of Impulses in the Guinea Pig Ureter

Purpose and Rationale

The mammalian ureter provides one of the clearest examples of electromechanical coupling in smooth muscle. The ureter smooth muscles are electrically and mechanically quiescent, but, when depolarized to threshold, they fire an action potential characterized by an unusually long-lasting potential and generate a phasic contraction (Shuba 1977; Brading et al. 1983; Meini et al. 1995). Both the action potential and the accompanying contraction critically depend upon the influx of extracellular calcium through voltage-sensitive L-type channels, which are enhanced and blocked by dihydropyridine drugs, Bay K 8644, and nifedipine, respectively. The model predicts that suppression of action potentials at any site of the ureter will suppress the propagation of contraction and peristalsis (Weiss 1992).

Procedure

A number of species (most commonly rats and guinea pigs) have been used for whole-mount or strip preparations. Following appropriate anesthesia, the whole kidney and ureter are excised and placed in a Petri dish containing oxygenated Krebs solution for dissection. A 4–5-cm long piece of ureter is dissected from the inferior renal pole and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex

partitions are used to separate the renal, middle, and bladder sites. They include a window covered with condom rubber: a small hole (about 300 μm) is made in the rubber to enable the passage of the ureter. Proximal to each partition, the renal and bladder ends are pinned to a Sylgard support. The distal portions of the renal and bladder ends are connected via a pulley to isotonic transducers (Basile 7006, load 2 mN) for recording of mechanical activity on a two-channel polygraph. Each compartment is perfused by means of a peristaltic pump at a rate of 1 ml/min with oxygenated Krebs solution at 34 °C.

The frequency and amplitude of spontaneous contractions are recorded. Alternatively, electrical field stimulation is applied to either compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter. Square wave pulses (5–25 ms pulse width, 20 V) are automatically delivered every 100 s by means of a GRASS S88 stimulator.

Drugs are applied by superfusion at the middle site. Amplitudes of contraction are recorded.

Evaluation

The amplitude and frequency of contractions are assessed and the motility index (MI) is calculated as follows:

$$\mathrm{MI} = (\Sigma\,\mathrm{amp}/5) \times F$$

where Σ amp/5 is the mean amplitude of five contractions (in mN) and F is the frequency (in min^{-1}) of those five contractions.

Data are expressed as a percent of the control value and appropriate statistical analysis is performed. Concentration–response curves may be plotted and pA_2 or pD_2 values may be calculated. Hill coefficients and EC_{50} values may also be calculated (Weiss et al. 2002).

Modifications of the Method

Ureter preparations have been made from pig or human tissues collected at slaughter or following surgery, respectively. Ideally, no more than 20 minutes should elapse between tissue collection and incubation. Tissues are placed into cold (4 °C) Krebs buffer or suitable balanced electrolyte solution and cut into rings of 0.5–1 cm in length. The rings are attached to a force transducer, suspended in an organ bath at 37 °C, and allowed to equilibrate for up to 1.5 h while the frequency and amplitude of spontaneous contraction are measured. If the preparation demonstrates acceptable and stable frequency and amplitude of spontaneous contraction within this time period, it is considered suitable for experimental use. In pigs, the washout period between treatments is variable and thus the preparation is monitored for return to pretreatment baseline before the next treatment is applied. In human tissue, spontaneous contraction seldom occurs, and electrical stimulation (trains of 300 ms at an interval of 200 s and impulses of 200 mA with a duration of 6 ms at a frequency of 50 H) is required. A washout period of 20 min between treatments is used (Weiss et al. 2002).

The evaluation of regional differences in response, and the effect of regional responses on adjacent regions, can be evaluated in a whole ureter preparation where each region remains physically attached but can be isolated for the application of test compounds or stimuli. The entire ureter is dissected and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the proximal (renal), middle, and distal (bladder) ends. They include a window covered with condom rubber: a small hole (about 300 mm) is made in the rubber to enable the passage of the ureter. The proximal portions of each segment are pinned to a Sylgard support, and the distal portions are connected to isotonic transducers for the recording of mechanical activity. Each compartment is perfused separately and electrical field stimulation can be applied to any compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter (Meini et al. 1995).

Tomiyama et al. (2003) characterized functional muscarinic cholinoceptors in the isolated ureter of **dogs**.

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Studies on Urinary Bladder and Internal Urethral Sphincter

Purpose and Rationale

Several authors investigated the influence of drugs on isolated parts of the lower urinary tract. Ueda et al. (1984) studied the effects on smooth muscle of the rabbit bladder dome, trigone, and proximal urethra.

Procedure

Male New Zealand rabbits weighing 2–3 kg are sacrificed and the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue are removed, the bladder and the urethra are dissected into dome, trigone, and proximal urethral preparations. All strips are cut transversely being approximately 2×6 mm unstretched. Ligatures are placed on both ends of the strips and one end is attached to a tissue holder and the other to a strain gauge force–displacement transducer connected to a polygraph on which isometric tension changes are recorded. Each of the strips is then placed into a 20-ml tissue bath containing Krebs–Ringer solution bubbled with 95 % O_2 + 5 % CO_2 at 37 °C. Resting tension is adjusted to 1 g during an equilibrium period of at least 2 h. The contractile and relaxant responses are measured as increases or decreases from the resting tension. Doses–response curves are performed in a cumulative matter. Tissues are pretreated with an antagonist 20 min before the addition of an agonist.

Evaluation

The values are expressed or plotted as the means ± SE and pA_2 values are calculated according to Arunlakshana and Schild (1959). Data are analyzed using the *t*-test, analysis of variance, Dunnett's test, and regression analysis.

Modifications of the Method

Rats and guinea pigs have been used for whole bladder preparations. Following appropriate anesthesia, the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue are removed, the preparation is placed en bloc into appropriate balanced electrolyte solution (Tyrode's or Krebs buffer) bubbled with 5 % CO_2 and 95% O_2. The urethra is cannulated and connected to a pressure transducer, and the bladder is filled with a volume of solution adequate to evoke a contractile response. The bladder preparation is allowed to equilibrate for 30 min before any experimental manipulations are performed. The amplitude and frequency of pressure changes are recorded (Birder et al. 1999; Drake et al. 2003).

Alternatively, contractility can be assessed visually. Surface blood vessels or externally applied markings (India ink or carbon particles)

are used as landmarks. Video image recording of the bladder preparations are made during the experimental manipulations. Still images from the video are evaluated, and the change in linear distance between pairs of landmarks is used as an index of contractility (Drake et al. 2003).

Pietra et al. (1990) studied the effects of some antidepressants by the in vitro inhibition of carbachol-induced contractions of rat detrusor strip preparations. The detrusor muscle tissue (bladder dome) was cut in a semicircular direction and further dissected into strip preparations measuring approximately 2 × 20 mm.

The preparation of the isolated, innervated urinary bladder of the rat was reported by Hukovic et al. (1965). Electrical stimulation was performed by a bipolar electrode from the nerves running close to the ureter.

Mapp et al. (1990) used the isolated rat urinary bladder to study the pharmacological modulation of the contractile response to toluene diisocyanate.

Maggi et al. (1985) used isolated detrusor strips of rat bladder connected to an isometric strain gauge and stimulated by field stimulation.

Anderson (1978) recommended the **rabbit** detrusor muscle a unique in vitro smooth muscle preparation. Rabbit detrusor muscles are thin and devoid of underlying submucosal tissue with parallel fiber orientation. The tissue exhibits autorhythmicity, characteristic of most single-unit type smooth muscle preparations, and can be employed in either isometric or isotonic organ bath recording systems.

Honda and Nakagawa (1986) studied the effects of the optical isomers of an alpha-1 adrenoceptor antagonist in rabbit lower urinary tract and prostate.

Khanna et al. (1977, 1981) evaluated the in vitro responses of three segments of rabbit lower urinary tract, e.g., the bladder body, the bladder base, and the proximal urethra.

Ferguson and Marchant (1995) studied the inhibitory actions of GABA on rabbit urinary bladder muscle strips.

Andersson et al. (1983) studied the electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. In rabbits, two circular transverse sections, each 4-mm long, were taken from the middle and upper parts of the urethra. Human urethral preparations were obtained from male patients undergoing cystourethrectomy en bloc because of bladder cancer. Rings of tissue were taken from the membranous and supra- and infra-collicular parts of the prostatic urethra.

Andersson et al. (1992) used transversal strips from the middle and upper part of rabbit urethra to study the involvement of nitric oxide in the electrically induced, nerve-mediated relaxation.

Contractile responses to nervous stimulation can be assessed in whole isolated bladder preparations. The pudendal nerve remnants can be identified near the ureter and can be attached to electrodes for stimulation of the bladder. Alterations in the contractile responses by the test compound are considered to result from effects at the neuromuscular junction (Hukovic et al. 1965; Weetman 1972; Dhattiwala and Dave 1975).

Inci et al. (2003) used a coaxial bioassay system to test the effect of inflammation on rat urinary bladder-dependent relaxation. In this bioassay model, the bladder was the donor organ for the assays, and rat anococcygeus muscle was the assay tissue. The rat bladders removed from control or treatment groups were used in its original shape after making small cuts at bladder neck and dome. The rat anococcygeus muscle was always isolated from control group of rats. The anococcygeus muscle was placed in the lumen of the bladder, so that freely enveloped by the bladder, and mounted under a resting tension of 1 g in a 20-ml organ bath filled with Krebs–Henseleit solution at 37 °C and gassed with 95 % O_2–5 % CO_2.

Denervated urinary bladder preparations (whole or strips) can be used to determine the degree of effect on nervous system inputs. Following appropriate anesthesia, the pelvic plexus is accessed in rats by a ventral midline incision and both pelvic ganglia are obliterated by electrocauterization. The animals are allowed to recover for at least four days before the urinary bladder is harvested as described above; during this period, the neurotransmitters downstream from the nervous system inputs become inactive and only the

autonomous inputs remain. Differences in response between the denervated urinary bladder preparation and one harvested from a sham-operated animal are considered to result from neural inputs. The urinary bladders of the denervated animals must be manually emptied on a daily basis during the 4-day recovery period (Birder et al. 1998; Brauerman et al. 2006).

Inputs from the urothelium can be assessed in isolated urinary bladder smooth muscle preparations. Following preparation of strips from the urinary bladder, the urothelium is gently peeled away from the cut surface and the strips are mounted as described above. Effects on the capsaicin-mediated nitric oxide pathways located in the urothelium can be assessed in these preparations (Birder et al. 1998). Effects on the release of neurotransmitter substances from the epithelial strips removed from these preparations can also be examined; following isolation, the epithelial strips are incubated in Krebs buffer of suitable balanced electrolyte solution. Following a 20-min equilibration period, the strips are stimulated either mechanically (by stoking with a glass rod or pinching with forceps) or electrically (using field stimulation of the incubation medium). The neurotransmitter substance of interest is analyzed in the bath solution and dose–response curves are generated (Downie and Karmazyn 1984).

Ukai et al. (2006) investigated the participation of endogenous endothelin and ET_A receptor in premicturation contractions in rats with bladder outlet obstruction, a model for bladder overactivity.

Weetman (1972) described the preparation of the isolated, innervated urinary bladder in **guinea pigs**. Contractions of the tissue induced by nerve stimulation could be blocked by local anesthetics and by tetrodotoxin.

Isolated innervated, rat and guinea pig hemi-urinary bladder preparations were described by Dhattiwala and Dave (1975).

Burnstock et al. (1978) used recorded isometric tension of mucosal-free strips of the detrusor of the bladder from guinea pigs in vitro after electrical field stimulation.

Von Heyden et al. (1997) tested urethral relaxation after electrostimulation in guinea pigs. Male Hartley guinea pigs weighing 350–450 g were sacrificed and the bladder, urethra, and penis were dissected out. From each animal 4–6 urethral rings 1–2-mm thick were cut. The urethral rings were mounted according to their anatomical order: in channel 1 the most proximal ring (near the bladder neck) and in channel 6 the most distal ring (near the penile crura). The urethral rings were stretched by two spring–wire clips (Harvard Apparatus, South Natick, MA) whose tips closed in the urethral lumen. The manner in which the urethral rings were cut and mounted ensured that only the circularly oriented, mostly striated fibers contributed to the tension measured. Detrusor muscle was cut as a horizontal ring proximal to the trigone and mounted in the same way. The clips were connected with 4-O silk to a glass tissue support hook on one side and to an isometric force transducer (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) on the other. A double-chambered bath (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) was used in which the working chamber was connected to a second chamber, in which the gas (95 % O_2 and 5 % CO_2) was fed. The gas flow induced fluid circulation. Forces lower than 0.1 g could be measured without bubble artifacts in the working chamber. The transducer signals were fed into a thermal array recorder (Dash 10, Astro-Med, 63110 Rodgau, Germany). For tissue stimulation, vertical, L-shaped custom-made platinum electrodes (20-mm long, 0.3-mm diameter) 10-mm apart were used with a custom-made stimulator. The tissue was mounted parallel with the electrodes. Bipolar, monophasic balance-charged rectangular pulses of 0.8-ms duration and 75-mA current were used.

Kunisawa et al. (1985) performed a pharmacological study of alpha adrenergic receptor subtypes in smooth muscle of **human** urinary bladder base and prostatic urethra.

Wuest et al. (2005) studied the effect of a potassium channel opener on contraction of detrusor strips from **mouse**, **pig**, and **man** in the presence and absence of urothelium.

Thornbury et al. (1992) reported on the mediation of nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in **sheep**. Urinary

bladders of sheep of either sex were obtained approximately 15 min after slaughter. Circularly oriented rings were cut from the region of the bladder just above the trigone. These were opened and the mucosa removed by sharp dissection to give strips with approximate dimensions of 10 × 4 × 4 mm. The strips were mounted in organ baths and perfused with Krebs solution. Tension was measured with isometric transducers after field stimulation via platinum ring electrodes.

Hills et al. (1984) used isolated strips of the bladder from female **pigs**. Bladder neck strips were cut longitudinally and horizontally from the region of the bladder just below the trigone. The preparations were stripped of mucosa and trimmed to give a muscle strip of about 2 × 15 mm.

Klarskov (1987) studied the non-cholinergic, non-adrenergic inhibitory nerve responses of bladder outlet smooth muscle from female Danish Landrace pigs in vitro. Trigone strips were taken in an oblique direction from the internal urethral orifice and medially to one of the ureteric orifices, bladder neck strips transversal from the posterior half of the borderline between bladder and urethra, and urethral strips longitudinal from the proximal posterior part.

Teramoto et al. (1997) examined the membrane potential in the proximal urethra of pigs by the use of the microelectrode technique.

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Effects on Isolated Urethra

Purpose and Rationale

The relaxation response in the urethra results in a combination of decreased intraurethral pressure, increased urethral diameter, and urethral shortening resulting in a flaring of the urethral orifice. A number of neurotransmitters and mediators are involved in this process, with each affecting a different stage of the process (Brading 1999: Andersson and Wein 2004). The urethra is difficult to study in situ because of the large amount of surrounding connective tissue, which can be overcome by the use of a whole-mount preparation (Jankowski et al. 2004).

Procedure

Female rats or guinea pigs have been used. Following appropriate anesthesia, the bladder and urethra are exposed via a lower midline incision, and a catheter is inserted in the urethral lumen, extending the entire axial length and exiting from a hole placed in the bladder dome. The urethra is secured to the tubing with sutures at both ends to maintain the correct in vivo length after dissection and is measured. The pubic bone is then cut at a position lateral to the urethra and then separated and resected. The exposed urethra was gently removed from the ventral vaginal wall and the whole bladder–urethra unit was immediately placed in cold, oxygenated suitable balanced electrolyte solution bubbled with 21 % O2-5 % CO2-74 % N2.

The catheter is removed so that the urethra may be secured inside the experimental apparatus, which provides a controlled fixed intraluminal pressure via an adjustable static fluid reservoir (the intraluminal pressure is controlled by adjusting the height of the reservoir). The mounted urethra is then enclosed in a bathing chamber filled with the same gassed, balanced electrolyte solution at 37 °C. The preparation is allowed to equilibrate for at least 30 min before testing. A laser micrometer is positioned to measure urethral outer diameter at chosen locations along the axial length. Proximal, mid, and distal regional measurements were performed by positioning the laser at axial positions 25 %, 50 %, and 75 %, respectively, from the apex of the bladder, based on in vivo length. Both pressure and outer diameter (OD) measurements are recorded simultaneously.

The test compound of interest is added to the bath and pressure and OD measurements are obtained following a 30-min equilibration period. Pressure–diameter (P–D) responses are generated by incrementally increasing the pressure, in 2-mmHg steps, from 0 to 18 mmHg, and OD data are collected at 10 Hz over a 1-min period for each 2-mmHg step. The OD data are then averaged for each incremental increase to obtain a discrete value for each value of applied pressure.

Contractile responses can be evaluated in this preparation as well. The urethra is exposed to a fixed intraluminal pressure of 8 mmHg, which causes the tissue to be predilated and allows for a contractile response to be generated. The OD resulting from this 8-mmHg applied pressure is measured at a single axial location (i.e., proximal, mid, or distal) 30 min after pressurization. The test chemical of interest is added to the bath and the preparation is exposed for 30 min; at the end of this time, a series of 100 OD measurements (taken at 1 Hz) are collected at the same location and are averaged.

Evaluation

The P–D response data are used to generate tissue compliance (C) as follows:

$$C = [D_{max} - D_{min}/D_{min}]X(P_{max} - P_{min})^{-1}$$

where D_{max} and D_{min} represent the OD at the maximum (P_{max}, 18 mmHg) and minimum (P_{min}, 0 mmHg) applied pressures, respectively.

Contractile responses are assessed by determining the percent change in mean OD before and after the addition of the test compound.

Modification of the Method

With careful dissection, the pudendal nerves can be identified and left intact during the dissection of the urethra from the guinea pig. These nerves can then be attached to electrodes and alterations of effects of neural inputs can be assessed. In this preparation, contractile responses were assessed by measuring changes in isometric tension, and intraluminal pressure changes were also recorded (Walters et al. 2006).

Triguero et al. (2003) studied the relaxation effects of scorpion venom in the isolated urethra of **sheep**.

Michel et al. (2006) performed in vivo studies on the effects of α_1-adrenoceptor antagonists on pupil diameter and urethral tone in **rabbits**.

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Effects on External Urethral Sphincter

Purpose and Rationale

In contrast to the smooth musculature of the internal urethral sphincter, the external urethral sphincter is a striate muscle and a part of the urogenital diaphragm (Kuro 1965). Judged from electromyographic studies, it is generally accepted that the preponderance of continence depends on the external urethral sphincter. The external urethral sphincter shows a steady tonic discharge under resting conditions. As the bladder fills, there is initially an increase in this activity. When the rise in tension in the bladder wall leads to reflex contraction, the activity in the external urethra sphincter ceases and it remains quiescent during voiding. Parlani et al. (1992) used the external urethral sphincter of the rat as an in vitro model to evaluate the activity of drugs on the smooth and striated components of the urinary bladder outlet.

Procedure

Male Wistar rats weighing 360–400 g are sacrificed by decapitation and exsanguination. Through a midline incision of the lower abdomen, the external urethral sphincter (Watanabe and Yamamoto 1979) is isolated from the perineal muscles and surrounding connective tissue and removed in toto. The preparation is placed in oxygenated Krebs solution, and a ring is taken from its middle region. In this area the urethra is encircled by bundles of striated muscle fibers partly interlaced with urethral smooth muscle. The rings are cut to obtain strips that are suspended in a 5-ml organ bath containing Krebs solution at 37 °C. A mixture of 96 % O_2 and 4 % CO_2 is bubbled into the organ bath. The preparations are connected by means of a silk thread to an isometric strain gauge under a constant load of 1 g. The contractile activity is recorded on a polygraph. Field stimulation is carried out by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The preparations are allowed to equilibrate for at least 60 min. Square wave pulses are delivered at an intensity between 10 and 60 V, a frequency between 0.1 and 3 Hz, and a duration between 0.1 and 1 s; trains are of 5 s every 5 min. After three consecutive reproducible responses are obtained, drugs are added to the organ bath. The effect of drugs is expressed as the percent of inhibition of contractile response before exposure to drugs and is evaluated as soon as the maximum effect is reached.

Evaluation

Means ± standard error of the mean are calculated. Statistical evaluation is performed by using Student's *t*-test for paired or unpaired data.

Modifications of the Method

In some studies, denervation of the external urethral sphincter was performed (Somma et al. 1989; Parlani et al. 1992). Rats were anesthetized with 30-mg/kg pentothal i.p., and then the major pelvic ganglia, known to provide both sympathetic and parasympathetic innervation to the urinary bladder and the external urethral sphincter (Hulsebosh and Goggeshall 1982; Purinton

et al. 1973; Watanabe and Yamamoto 1979), are isolated and bilaterally removed through a small incision of the lower abdomen.

In the same preparations, somatic denervation of the external urethral sphincter was obtained by cutting the pudendal nerves. The paravertebral muscles were carefully dissected through an incision of the skin to exteriorize the sacral plexus. The pudendal nerves were isolated, and 2–3 mm of the nerve were removed. An absorbable sponge soaked with amikacin solution was left in place to prevent bleeding and infection. The muscles and the skin were sutured with cat gut. The rats were allowed to recover for 10 to 15 days in individual cages with free access to water and food.

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Part IV

Respiratory Activity

Effects of Drugs on Air Ways

Kristy D. Bruse

Contents

Effects on Air Ways

Tests in Isolated Organs

Spasmolytic Activity in Isolated Guinea Pig Lung Strips

Purpose and Rationale

Several autacoids such as histamine and leukotrienes induce bronchoconstriction. Histamine is an important mediator of immediate allergic and inflammatory reactions. It causes bronchoconstriction by activating H_1-receptors. Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that appear to act on smooth muscles via specific receptors. In this method, drugs are tested for their capability of inhibiting bronchospasm induced by histamine or calcium ionophore. It is used to detect H_1- and leukotriene receptor blocking properties of test compounds.

Procedure

Albino guinea pigs of either sex weighing 300–450 g are sacrificed with an overdose of ether. The chest cavity is opened and the lungs removed. They are cut into strips of 5 cm and placed into a physiological saline solution. Thereafter, the lung strips are mounted in an organ bath containing a nutritive solution. The bath is bubbled with carbogen and maintained at 37 °C. Under a preload of 0.5–3 g, the tissue is left to

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equilibrate for 30–60 min. Prior to testing, carbachol is added to the bath to test the lung strips' ability of contraction. Twenty minutes later, two prevalues are obtained by adding the spasmogen

- Histamine dihydrochloride 10^{-6} g/ml for 5 min
- Ca – ionophore 5 × 10^{-6} g/ml for 5 min
- Leukotriene LTC_4 10^{-9}–10^{-8} g/ml for 10 min
- Leukotriene LTD_4 10^{-9}–10^{-8} g/ml for 10 min

to the bath and recording the contractile force at its maximal level. Following a 20 min equilibration period, the spasmogen is administered again. Five minutes thereafter, the test compound is added in cumulative doses from 10^{-8} to 10^{-4} g/ml at 5 or 10 min intervals. The contractile response is determined isometrically.

Test Modification: Inhibition of Prostaglandin Synthesis

This procedure is identical to the test described above with the exception that the prostaglandin synthesis is inhibited by addition of indomethacin at 10^{-6} g/ml prior to spasmogen administration.

Evaluation

The percent inhibition of spasmogen-induced contraction is calculated.

Modifications of the Method

Lung parenchyma strips from various species were used to measure bronchoactivity by Kleinstiver and Eyre (1979).

A descriptive model of the events occurring during an inflation-deflation cycle using excised rat lungs was proposed by Frazer et al. (1985).

Barrow (1986) measured volume-pressure cycles in air-filled or liquid-filled rabbit lungs ranging from intact lungs with the rib cage immobilized to isolated lungs.

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Spasmolytic Activity in Isolated Trachea

Purpose and Rationale

The isolated tracheal chain of guinea pigs can be used to test for β-blocking activity. In addition, this model can be used to test compounds which inhibit bronchospasms. It is used to detect β-sympathomimetic, H_1-receptor blocking, and leukotriene receptor blocking properties of test drugs.

Carbachol is a cholinergic agonist that produces contraction of bronchial smooth muscle by muscarinic stimulation.

Histamine is an important mediator of immediate allergic (type 1) and inflammatory reactions. It causes bronchoconstriction by activating H_1-receptors.

Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that

appear to act on smooth muscle via specific receptors.

To assess a compound's ability to inhibit carbachol-induced bronchospasm via β-receptor activation, a β-receptor blocking agent (e.g., propranolol) must be added. If relaxation of bronchial smooth muscle is brought about by β-receptor activation, the spasmolytic effect will decrease following propranolol administration.

The effect of bradykinin can be abolished by bradykinin antagonists (Hock et al. 1991).

The effects of potassium channel openers can also be studied in this test (Englert et al. 1992).

Procedure

Albino guinea pigs of either sex weighing 300–550 g are sacrificed by CO_2 narcosis. The entire trachea is dissected out and cut into individual rings (2–3 cartilaginous rings wide). Twelve–fifeteen rings are tied together with silk threads and mounted in the organ bath containing Krebs-Henseleit solution. The tissue is maintained at 37 °C under a tension of 0.5 g and gassed with carbogen. Isometric contractions are recorded via a strain gauge transduced on a polygraph. Forty-five minutes are allowed for equilibration before the addition of the spasmogen.

The following spasmogens are used:

- Carbachol (2×10^{-7} g/ml)
- Histamine (10^{-7} g/ml)
- Ca – ionophore for release of leukotrienes
- Leukotriene LTC_4 (10^{-9}–10^{-8} g/ml)
- Leukotriene LTD_4 (10^{-9}–10^{-8} g/ml)

When the contraction has reached its maximum (initial spasm) after 10–12 min, the standard drug, e.g., isoprenaline (1 ng/ml) or aminophylline (10 ng/ml), is administered. The bronchial responses are allowed to plateau and are recorded. The tissue is rinsed thoroughly and control contractions induced again by adding spasmogen. After obtaining the initial spasm again, the test drug is added and the contractile force recorded at its maximal level.

Determination of mechanism of action (testing for β-sympathomimetic effect). After obtaining the initial carbachol-induced spasm, propranolol is administered 5 min before the addition of the test drug. Three minutes later, the tissue is challenged by carbachol administration.

Evaluation

The percent inhibition of carbachol or other spasmogen-induced contractions is calculated. From dose–response curves ED_{50} values can be calculated.

Critical Assessment of the Method

The isolated guinea pig trachea has been proven to be a useful tool for several purposes, e.g., screening procedures and studies on mode of action, e.g., of potassium channel openers.

Modifications of the Method

The molecular mechanisms of β-adrenergic relaxation of airway smooth muscle were described by Kotlikoff and Kamm (1996).

Farmer et al. (1986) studied the effects of epithelium removal on the sensitivity of **guinea pig** isolated trachealis to bronchodilator drugs.

Wilkens et al. (1992) described a bioassay system for a tracheal smooth muscle-constricting factor using an isolated guinea pig trachea which was cannulated from both sides, mounted in an organ bath, and monitored by a TV camera attached to a microscope. The picture was digitized continuously and the diameter of the trachea calculated and displayed on a monitor throughout the experiment.

Coleman and Nials (1989) described a versatile, eight-chamber superfusion system for the evaluation of spasmogenic and spasmolytic agents using guinea pig isolated tracheal smooth muscle.

Goldie et al. (1986a) studied the influence of the epithelium on responsiveness of guinea pig isolated trachea to contractile and relaxant agonists.

Lee et al. (1997) studied the effects of bupivacaine and its isomers on guinea pig tracheal smooth muscle. The trachea from the larynx to the carina was removed and cut into single rings. Seven tracheal rings were tied together with the circular muscle running on the same side of the chain, placed into an organ bath containing

Krebs Ringer's solution, and connected to a force placement transducer for measurement of isometric tension.

Wong et al. (1997) tested the effects of tyrosine kinase inhibitors on antigen challenge of guinea pig lung in vitro. Guinea pigs were passively sensitized by a single i.p. injection of 1 mg/kg rabbit IgG antibody against ovalbumin. Bronchial rings, 3 mm in length, were obtained from the hilar bronchi of sacrificed animals and suspended isometrically in an organ bath in Krebs bicarbonate buffer with a resting load of 2 g. To determine maximum antigen-induced contractions, bronchial rings were exposed to increasing concentrations of ovalbumin. To evaluate the role of protein tyrosine kinase in mediating smooth muscle anaphylactic contraction, protein tyrosine kinase inhibitors were preincubated with bronchial rings 30 min before addition of ovalbumin.

Eltze and Galvan (1994) compared the inhibition of preganglionic and postganglionic contraction of the **rabbit** isolated bronchus/trachea by antagonists with selectivity for different muscarinic receptor subtypes with their affinities at M_1, M_2, M_3, and M_4 receptors (Barnes 1993).

For **experiments with vagus nerve stimulation**, the vagi were isolated with rings of the proximal mainstem bronchi and a small portion of the distal trachea. The tissues were hung on stainless steel hooks, which passed through the lumen, and were placed in a water-jacketed organ bath filled with Krebs solution plus 2×10^{-5} M choline chloride to promote resynthesis of acetylcholine, 10^{-5} M indomethacin to prevent generation of cyclo-oxygenase products, and 10^{-6} M DL-propranolol to block possible β-adrenoceptor-mediated effects. The preparation was fixed under a resting tension of 1 g for isometric contraction measurement using a force transducer. The vagi were passed around bipolar platinum electrodes held at the surface of the bath. Electrical stimulation was performed with trains (20 Hz, 0.3 ms at 20 V) elicited every 20 min.

For experiments with **field stimulation**, two-ring preparations from the distal trachea of the rabbit were suspended in organ bath in Krebs solution with the abovementioned additions under a resting tension of 1 g. Isometric contractions were elicited at 1 h intervals by continuous electrical field stimulation via platinum electrodes (20 Hz, 0.3 ms at 20 V) for an average 3–7 min to reach a stable plateau.

Vaali et al. (1996) studied in isolated tracheal rings of **rats** and guinea pigs the bronchorelaxing effects of nitric oxide donors. The epithelium of some rings was removed by gentle rubbing. Rat mesenteric rings were cut from the same animals as the bronchi and similarly prepared by removing the endothelium by gently rubbing the intimate surface.

Farmer et al. (1994) used the isolated trachea from male **ferrets** weighing 1.5–2.5 kg to study the effects of bradykinin receptor agonists.

Toews et al. (1997) assessed the effects of the phospholipid mediator lysophosphatidic acid on the contractile responsiveness of isolated tracheal rings from **rabbits** and **cats**.

Tamaoki et al. (1993) used isolated rings of segmental bronchi from **dogs** to study atypical β-adrenoceptor (β_3-adrenoceptor)-mediated relaxation.

The preparation of **bovine** tracheal smooth muscle for measuring airway responsiveness in vitro was described by Hashjin et al. (1995).

Wali (1987) used tracheal strips from 2- to 4-week-old **chicks** to study inhibition of cholinergic and noncholinergic neural and muscular contractions by local anesthetics.

Lulich and Paterson (1980) used **human** isolated bronchial muscle preparations and compared the effects of histamine and other drugs with the effects observed on the central and peripheral airways of the rat.

Goldie et al. (1986b) measured the responses of human bronchial strip preparations to contractile and relaxant agonists in preparations from nondiseased and from asthmatic lung obtained 3–15 h post mortem.

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Reactivity of the Isolated Perfused Trachea

Purpose and Rationale

The mechanism by which the epithelium affects the reactivity of tracheal musculature can be studied using the isolated perfused trachea preparation. Contractile agonists can be added either to the serosal (extraluminal) or to the mucosal (intraluminal) surface (Fedan and Frazer 1992).

Procedure

A 4-cm segment of the trachea of male guinea pigs is removed after sacrifice of the animal and placed for cleaning in modified Krebs-Henseleit solution at 37 °C containing (millimolar) NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, and glucose 5.7 (pH 7.4) being gassed with 95 % O_2/5 % CO_2. The trachea is then attached to a stainless-steel perfusion holder (Munakata et al. 1988), extended to its in situ length, and placed in an organ chamber (the serosal compartment) at 37 °C containing 25 ml of gassed modified Krebs-Henseleit solution. This solution is also pumped at a constant rate of 30 ml/min through the lumen (mucosal compartment). Responses of the tracheal musculature are obtained by measuring changes in inlet–outlet ΔP between the side holes of indwelling catheters, while the trachea is perfused at a constant rate of modified Krebs-Henseleit solution. The inlet and outlet catheters are connected to the positive and negative sides, respectively, of a differential pressure transducer.

Agonists are added in stepwise increasing, cumulative concentrations. Two consecutive dose–response curves are obtained after the addition either to the serosal or mucosal compartment. The second dose–response curve is obtained 1.5 h after the end of the first, the preparation being washed every 15 min during the intervening period.

Evaluation

Responses are quantified as ΔP in centimeters of H_2O. Geometric EC_{50} values are determined from least squares analysis of a logit model and are presented along with 95 % confidence intervals.

Modifications of the Method

Baersch and Frölich (1996) measured continuously the changes of the diameter of isolated guinea pig tracheal tubes by a newly developed imaging bioassay system. The tracheal tube between larynx and bifurcation was prepared to a length of 1.8–2.5 cm, cannulated at both ends, and mounted in a 15 ml organ bath filled and perfused with oxygenated Krebs solution at 37 °C. The lumen of the trachea was perfused with warm (37 °C) Krebs solution from a reservoir that was isolated from the tissue bath. Changes in diameter of the trachea were assessed by computerized video microscopy. The tracheal diameter was used as marker for airway size, thus allowing calculation of muscle contractions under experimental conditions. Following a 30-min equilibrium period, a contraction was induced by intraluminal application of methacholine (0.1 mmol/l) as reference contraction. After washout and return to a stable baseline, cumulative concentration–response curves were obtained by luminal or extraluminal drug application.

Yang et al. (1991) studied the role of epithelium in airway smooth muscle responses to relaxant agents. The results suggested that the epithelium is a relatively weak barrier for

lipophilic agents but has a major role as a diffusion barrier to hydrophilic substances.

Munakata et al. (1988, 1989) developed an in vitro system to assess the role of epithelium in regulating airway tone using the intact **guinea pig** trachea. The responses to histamine, acetylcholine, and hypertonic KCl when stimulated from the epithelial or serosal site were first examined in tracheae with intact epithelium. Then the responses to these agonists were registered after epithelial denudation.

Pavlovic et al. (1989) studied the role of airway epithelium in the modulation of bronchomotor tone in the isolated trachea of **rats**. An organ bath was constructed that permitted independent circulation of fluid within the lumen or around the exterior of the tracheal segment. In one-half of the preparations the epithelium was mechanically removed.

Fernandes et al. (1989) described a coaxial bioassay system, whereby rat cross-cut aorta strip preparations were set up in coaxial assemblies under 500 mg resting tension within a guinea pig tracheal segment serving as donor of the smooth muscle relaxant factor from guinea pig tracheal epithelium.

Lewis and Broadley (1995) investigated the influence of spasmogen inhalation by guinea pigs upon subsequent demonstration of ovalbumin-induced hyperreactivity in isolated airway tissues. Guinea pigs were sensitized with ovalbumin (i.p.) 14 days before use. In vitro airway hyperreactivity induced by ovalbumin inhalation was determined by challenging with aerosolized spasmogen (5-HT, methacholine, the thromboxane-mimetic U-46619, or adenosine) 24 h before and again 18–24 h after the ovalbumin inhalation. One hour later, the animals were sacrificed and isolated airways perfused lung halves and tracheal spirals were set up for determination of tissue sensitivity to carbachol, histamine, and adenosine.

Sparrow and Mitchell (1991) used bronchial segments obtained from the lungs of Large White/Landrace cross **pigs** to study the modulation by the epithelium of the extent of bronchial narrowing produced by substances perfused through the lumen.

Mitchell et al. (1989) compared the reactions of perfused bronchial segments and bronchial strips of *pigs* to histamine and carbachol.

Hulsman et al. (1992) recommended the perfused **human** bronchiolar tube as a suitable model.

Omari et al. (1993) studied the responsiveness of human isolated bronchial segments and its relationship to epithelial loss.

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Bronchial Perfusion of Isolated Lung

Purpose and Rationale

Bronchial perfusion of the isolated lung was described by Sollmann and von Oettingen (1928) as a simple method for studying pharmacological reactions of bronchiolar muscle. The method consists in perfusing fluid down the trachea through the bronchi and allowing it to escape from the alveoli through scratches on the surface of the lungs. Bronchoconstriction results in a reduced rate of flow; bronchodilatation is indicated by an increased flow. The method has been used to evaluate sympathomimetic drugs by Tainter et al. (1934) and by Luduena et al. (1957).

Procedure

Guinea pigs weighing about 200 g are sacrificed by a head blow. The chest is opened, the trachea cut at the upper end, and removed with the lung. The trachea is attached to the cannula of a perfusion apparatus. Only one lung is perfused, the other being tied off. The lower part of the lower lobe is cut off and the rest of the lung surface scratched deeply assuring maximal premedication flow.

The perfusion fluid has the following composition in percentage of anhydrous salts: NaCl 0.659, $NaHCO_3$ 0.252, KCl 0.046, $CaCl_2$ 0.005, $MgCl_2$ 0.0135, NaH_2PO_4 0.01, Na_2HPO_4 0.008, glucose 5 %, pH 8.0. The temperature of the perfusion medium is 37.5 °C, and the lung is enclosed in a glass cylinder to be protected from variations in the environmental temperature.

The trachea is attached to the cannula of a perfusion apparatus which pumps the solution at a constant rate into a manometric tube connected with the perfused organ. Resistance to the flow (bronchoconstriction) results in an increase in the height of the column of fluid in the manometer. The intensity of bronchodilator effect is measured by the fall of the column in the manometer.

After the lung is attached to a T-shaped cannula, the pump is set in motion, and the fluid, after filling the lung, flows out of the system through the third opening of the cannula. By gentle pressure air bubbles are forced out of the lung into the overflow. The lung is then treated in the aforesaid manner and the upper outlet of the cannula closed. Histamine HCl is added in a concentration of 1:2,500,000 as soon as the perfusion starts and the flow adjusted to obtain a constant progressive increase in pressure.

The drugs are injected near the cannula when the perfusion pressure reaches a level of 500–650 ml of water. The volume injected is always 0.1 ml.

Each drug is tested for bronchodilating activity against the bronchoconstriction induced by histamine in parallel with L-arterenol following a Latin square, including three doses of each drug and three doses of L-arterenol graded at 0.5 log intervals.

Evaluation

Activity ratios of bronchodilating agents versus the standard can be calculated with a 3 + 3 point assay including confidence limits.

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Vascular and Airway Responses in the Isolated Lung

Purpose and Rationale

The isolated perfused rat lung allows the simultaneous registration of pulmonary vascular and airway responses to various drugs.

Procedure

Male Sprague Dawley rats weighing 300–350 g are intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg). The trachea is cannulated with a short section of polyethylene tubing, connected to a rodent ventilator, and ventilated with room air enriched with 95 % O_2/5 % CO_2, with a tidal volume of 4–5 ml/kg and 2 cm H_2O positive end-respiratory pressure. The rats are heparinized with 1,000 units of intravenous heparin and rapidly exsanguinated by withdrawing blood from the carotid artery.

The lung is exposed by median sternotomy, and a ligature is placed around the aorta to prevent systemic loss of blood. The main pulmonary artery is catheterized, and the lung is removed en bloc and suspended in a warmed (39 °C), humidified (100 %) water-jacketed chamber. An external heat exchanger is used to maintain the temperature of the perfusate and the isolated lung chamber constant throughout the experiment. The perfusate solution (15 ml heparinized blood and 5 ml modified Krebs-Henseleit solution) is placed in a reservoir and mixed constantly by a magnetic stirrer. The lungs are perfused with a peristaltic roller pump at a flow rate of 8–14 ml/min to maintain a physiological baseline pulmonary arterial perfusion pressure of 15 ± 0.5 mmHg. Pulmonary arterial perfusion pressure, airway pressure, and reservoir blood level are continuously monitored, electronically averaged, and recorded with a polygraph.

Evaluation

Changes (increase or decrease) in pulmonary arterial pressure and in airway pressure after injection of test compounds are measured in mm Hg and compared with baseline values.

Modifications of the Method

Bernard et al. (1997) described an isolated perfused lung model with real-time data collection and analysis of lung function. Male Sprague Dawley rats were anesthetized with 130 mg/kg pentobarbital i.p. The trachea was cannulated and then ventilated with 5 % CO_2 and 95 % air at a rate of 60 breaths/min and a tidal volume of 2.5 ml. An injection of 650–700 units/kg of heparin was made into the right ventricle. A cannula was placed into the main pulmonary artery. The left ventricle was incised and the lungs washed free of blood with warmed Krebs-Henseleit bicarbonate buffer with 4.5 % BSA and 0.1 % glucose. The left atrium was then cannulated to allow outflow of the perfusate. The lung was then removed and suspended in a chamber for perfusion. The flow rate of the perfusate was adjusted to 8–10 ml/min/kg. Ventilation was maintained at 60 breaths/min with humidified and warmed gas. The lung was allowed to recover for 15 min at which time the lung mechanic parameters of flow, volume, transpulmonary pressure, pulmonary artery pressure, weight, resistance, elastance, and positive end-diastolic pressure were measured.

Hauge (1968) studied the conditions governing the pressor responses to ventilation hypoxia in isolated perfused rat lungs.

Uhlig and Heiny (1995) measured the weight of the isolated perfused rat lung during negative pressure ventilation for quantitating edema formation in the isolated lung.

Uhlig and Wollin (1994) described an improved setup for the isolated perfused rat lung. Breathing mechanics, such as tidal volume, pulmonary compliance, and pulmonary resistance, as well as perfusate characteristics, such as pulmonary vascular resistance, pulmonary pre- and postcapillary resistance, perfusate pH, P_{O2}, and P_{CO2}, and the capillary filtration coefficient were determined.

Byron et al. (1986) used the isolated perfused rat lung preparation for the study of aerosolized drug deposition and absorption.

Hendriks et al. (1999) published a modified technique of isolated left lung perfusion in the rat.

Riley et al. (1981) determined the tissue elastic properties of saline-filled isolated hamster lungs by measuring the pressure-volume relationships and studied the prevention of bleomycin-induced pulmonary fibrosis by *cis*-4-hydroxy-L-proline.

Lewis and Broadley (1995) tested the influence of spasmogen inhalation by **guinea pigs** upon subsequent demonstration of albumin-induced hyperreactivity in isolated airway tissues, such as perfused lung halves and tracheal spirals.

Corboz et al. (2000) used the isolated guinea pig lung to study the inhibition of capsaicin-induced bronchoconstriction by nociceptin. The lungs and the heart were removed en bloc from guinea pigs euthanized with an intraperitoneal overdose of sodium pentobarbital. The trachea and pulmonary artery were rapidly cannulated and half of the heart cut to facilitate drainage. The lungs were then placed inside a warmed (37 °C) glass chamber and suspended from a force displacement (Grass FT-03). They were mechanically ventilated with room air using a small animal ventilator. The respiratory rate was set at 60 strokes/min with a volume of 2.0 ml/stroke. Pulmonary inflation pressure was continuously monitored with a pressure transducer connected to a sidearm of the tracheal cannula. Perfusion pressure was maintained with a peristaltic pump at a rate of 4.5–5.0 ml/min to produce a baseline pulmonary arterial pressure of between 6 and 14 cmH_2O. The pulmonary artery pressure was continuously monitored using a pressure transducer connected to the sidearm of the pulmonary artery cannula. Transducers were connected to a polygraph for continuous monitoring of variables. The lungs were perfused with Tyrode's solution maintained at 37 °C. Cumulative dose–response curves were constructed by adding increasing doses of capsaicin or the tachykinin NK_2 receptor agonist neurokinin A directly into the pulmonary artery. Test drugs were perfused 30 min before the addition of increasing doses of capsaicin or neurokinin A.

Anglade et al. (1998) measured the pulmonary capillary filtration coefficient in isolated **rabbit** lungs which were suspended by a string tied around the tracheal cannula from a counterbalanced force transducer to perform continuous weight measurement. To measure the pulmonary capillary filtration coefficient, pulmonary venous pressure was raised stepwise which results in an initial large weight gain for a few seconds followed by a slower rate of weight gain. The pulmonary capillary filtration coefficient was calculated on the slow phase of the weight curve by a time zero extrapolation or from the slope of the curve.

Nakamura et al. (1987) studied neurogenic pulmonary edema in lung perfusion preparations in situ in the **dog**.

Allen et al. (1993) studied the cardiovascular effects of a continuous prostacycline administration into an isolated in situ lung preparation in the dog.

Pogrebniak et al. (1994) investigated the influence of tumor necrosis factor in an isolated lung perfusion model in **pigs**.

The fluid filtration coefficient before and after infusion of *Escherichia coli* endotoxin was measured in excised **goat** lungs by Winn et al. (1988).

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In Vivo Tests

Bronchospasmolytic Activity in Anesthetized Guinea Pigs (Konzett–Rössler method)

Purpose and Rationale

The principle war first described by Kiese (1935). Konzett and Rössler (1940) published a method suitable for screening procedures which found worldwide acceptance. A survey on the history and further modifications was given by Döring and Dehnert (1997).

The method is based on registration of air volume changes of a living animal in a closed system consisting of the respiration pump, of the trachea and the bronchi as well as, of a reservoir permitting measurement of volume or pressure of excess air. Bronchospasm decreases the volume of inspired air and increases the volume of excess air. Thus, the degree of bronchospasm can be quantified by recording the volume of excess air. Administration of spasmogens like acetylcholine, histamine, bradykinin, serotonin, ovalbumin, PAF, substance P, methacholine, or leukotrienes results in contraction of bronchial smooth muscle.

The method permits the evaluation of a drug's bronchospasmolytic effect by measuring the volume of air, which is not taken up by the lungs after bronchospasm.

Procedure

Guinea pigs of either sex weighing 250–500 g are anaesthetized with 1.25 g/kg i.p. urethane. Pentobarbital (60 mg/kg s.c.) and alcuronium chloride (1 mg/kg s.c.) are to be preferred when the bronchospasm is elicited by PAF or substance P. Anesthesia has to be deep enough in order to prevent influence of spontaneous respiration. The trachea is cannulated by means of a two-way cannula, one arm of which is connected to the respiratory pump and the other to a Statham

P23 Db transducer. The animal is artificially respired using a Starling pump with an inspiratory pressure set at 90–120 mm of water, an adequate tidal volume of 3 ml/100 g body weight, and a frequency of 60 strokes per minute. Excess air, not taken up by the lungs, is measured and recorded on a polygraph. The internal jugular vein is cannulated for the administration of spasmogens and test compounds. The carotid artery is cannulated for measuring blood pressure.

Testing

Guinea pigs receive the following spasmogens by i.v. administration:

- Acetylcholine hydrochloride (20–40 μg/kg)
- Methacholine (20–40 μg/kg)
- Histamine dihydrochloride (5–20 μg/kg)
- Bradykinin triacetate (10–20 μg/kg)
- Ovalbumin (1 mg/kg)
- PAF (25–50 ng/ml)
- Leukotrienes LTC_4, LTD_4 (about 1 μg/kg)
- Substance P (0.5 μg/kg)

After obtaining two bronchospasms of equal intensity, test compounds are administered i.v., p.o., s.c., or intraduodenally.

The spasmogen is given again at the following time intervals:

- 5, 15, and 30 min after i.v. administration of the drug
- 15, 30, and 60 min after intraduodenal administration of the drug
- 30 and 60 (sometimes also 120) min after p.o. administration of the drug

The following standard compounds are used:

- Atropine sulfate (0.01 mg/kg, i.v.) to inhibit acetylcholine- or methacholine-induced spasms
- Aminophylline (6 mg/kg, i.v.) to inhibit bradykinin-induced spasms
- Tolpropamine-HCl (0.2 mg/kg) to inhibit histamine induced spasms
- Imipramine-HCl (3–5 mg/kg) to inhibit serotonin-kreatinin-sulphate induced spasms.

Evaluation

Results are expressed as percent inhibition of induced bronchospasm over the control agonistic responses. The ED_{50} value is calculated.

Critical Assessment of the Method

The "Konzett-Roessler"-method has been proven to be a standard procedure in respiratory pharmacology being modified by several authors (Rosenthale and Dervinis 1968).

Modifications of the Method

Forced insufflation was proposed as a simple but accurate inhalation procedure for investigating the activity of antiasthmatic drugs in guinea pigs by Schiantarelli et al. (1982).

Lundberg et al. (1983), Belvisi et al. (1989) and Miura et al. (1994) determined airway opening pressure (P_{ao}) as an index for tracheobronchial resistance to air flow.

Orr and Blair (1969) and Riley et al. (1987) sensitized rats intravenously with a potent antiserum to ovalbumin, obtained by infecting ovalbumin-sensitized donor rats with the parasitic nematode *Nippostrongylus brasiliensis* in order to boost IgE antibody production. The anesthetized animals were challenged with ovalbumin 48 h after sensitization and the subsequent increase in tracheal pressure recorded.

Collier et al. (1963) and Collier and James (1967) published a modification of the Konzett-Rössler method using the forced reinflation to overcome the severe bronchoconstriction occurring in sensitized guinea pigs.

Further modifications are by Schliep et al. (1986) and Marano and Doria (1993).

Groeben and Brown (1996) measured changes in the cross-sectional area of conducting airways by cumulative doses of ipratropium with and without gallamine, a selective M_2 muscarinic receptor blocker, and after metaproterenol in anesthetized **dogs** using high-resolution computed tomography. Using a Somatom Plus scanner (Siemens), 50–55 contiguous scans were obtained, starting approximately 5 mm above the origin of the right upper lobe bronchus from the trachea and proceeding caudally using 1-mm table feed and 2-mm slice thickness. The dogs were anesthetized

with thiopental. After paralysis was induced by succinylcholine, the trachea was intubated and the lungs ventilated with a volume-cycled ventilator with 100 % oxygen. During the scans, the dogs were apneic at function residual capacity (approximately 2 min). Images were reconstructed using a high–spatial frequency algorithm.

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Effect of Arachidonic Acid or PAF on Respiratory Function In Vivo

Purpose and Rationale

Based on the classical method of Konzett and Rössler (1940), Lefort and Vargafting (1978) and Vargaftig et al. (1979) studied the effects of arachidonic acid and PAF on respiratory function of guinea pigs in vivo.

Arachidonic acid is metabolized into thromboxane (TXA_2) and prostacyclin (PGI_2). TXA_2 produced in the lung leads to bronchoconstriction, which is independent from circulating platelets and leukotrienes; TXA_2 produced intracellularly in platelets induces a reversible thrombocytopenia. PGI_2 produced in the vessel wall leads to the reduction of systolic and diastolic blood pressure. All three effects are inhibited by drugs which block cyclo-oxygenase. In contrast, agents which block thromboxane synthetase inhibit bronchoconstriction and thrombocytopenia, but lead to a potentiation of blood pressure reduction.

In contrast to arachidonic acid, PAF as inducer leads to bronchoconstriction, which is platelet dependent. In addition, PAF induces thrombocytopenia, leukocytopenia, reduction of blood pressure, and increase of hematocrit. These effects are also reversible, but more persistent than those induced by arachidonic acid, and quickly result in tachyphylaxis. The test allows to evaluate the sites of action of drugs, which interfere with the mechanisms of bronchoconstriction and thrombocytopenia; in an in vivo model guinea pigs are challenged with the spasmogens and platelet-aggregating substances arachidonic acid or PAF (platelet-activating factor).

Procedure

Male guinea pigs (Pirbright White) weighing 300–600 g are anesthetized with 60 mg/kg pentobarbital sodium (i.p.). One of the jugular veins is cannulated for the administration of spasmogen and test compound. Both external carotid arteries are cannulated; one is connected to a pressure transducer to register blood pressure; the other is used for blood withdrawal. The trachea is connected to a Starling pump with an inspiratory pressure set of 80 mm H_2O, an adequate tidal volume of approx. 10 ml/kg body weight, and a frequency of 70–75 strokes/min. Spontaneous respiration is inhibited by intravenous injection of pancuronium (4 mg/kg) or gallamine (2 mg/kg) on time.

In some experiments, pulmonal β-receptors are blocked by intraperitoneal administration of propranolol (2 mg/kg).

Excess air, not taken up by the lungs, is conducted to a transducer with bronchotimer (Rhema, Germany) which translates changes in air flow to an electrical signal. Changes in air flow and arterial blood pressure are recorded continuously.

Animals receive multiple intravenous injections of the same dose of arachidonic acid (Sigma, 250–600 µg/kg prepared from a stock solution 10 mg/ml ethanol, 1:20 dilution with Na_2CO_3) until two bronchospasms of equal intensity are obtained. The test compound is administered intravenously and the spasmogen given again at the following intervals: 2, 10, 20, and, if necessary, 30 min after administration of the drug.

Ordinarily, the lung has to be passively dilated (bronchotimer) after each of the bronchoconstrictions. Immediately before and 30–45 s after each of the arachidonic acid applications, approx. 50 µl blood are collected into Na-EDTA-coated tubes. The number of thrombocytes is determined with a platelet analyzer (Becton Dickinson Ultra-Flo-100 or Baker 810) in 10 µl samples of whole blood.

PAF (Paf-acether C16, Bachem, 0.03–0.04 µg/kg in 0.9 % saline + 0.1 % human serum albumin) as inducer is injected intravenously 60 min before, 5 min, and, if necessary, 60 min after intravenous drug administration. Blood samples are collected 30 s before and 15 s after each of the PAF applications. The number of leukocytes and hematocrit values are determined automatically (TOA-microcell counter CC 108, Colora Meßtechnik).

Standard Compounds

- Dazoxiben HCl (inhibitor of thromboxane synthetase, TSI)
- Acetylsalicylic acid (inhibitor of cyclo-oxygenase, COI)

Evaluation

Percent inhibition or increase of bronchospasm, reduction of blood pressure, thrombocytopenia, leukocytopenia, and hematocrit following test drug administration are calculated in comparison to control values before drug treatment. For the reduction of blood pressure, both the magnitude [mm Hg, systolic and diastolic] and the duration [min] are determined. Even a sole increase in duration of blood pressure reduction is considered as an increase of the effect. From the pattern profile of the influence on bronchoconstriction, thrombocytopenia, and blood pressure reduction, the mechanism of action of a test drug is concluded:

- Inhibitor of thromboxane synthetase
- Inhibitor of cyclo-oxygenase
- Other effect = no profile

In addition, the inherent action of the test substance on blood pressure is determined before arachidonic acid or PAF administration.

Modifications of the Methods

Kagoshima et al. (1997) used a modification of the Konzett-Rössler method to test the suppressive effects of a PAF antagonist on asthmatic responses in guinea pigs actively sensitized with ovalbumin. The immediate and the late asthmatic response were measured by the oscillation method according to Mead (1960).

Pauluhn (1994, 2004) described inhalation systems, mainly for toxicological studies, by which large numbers of animals can be studied simultaneously.

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Bronchial Hyperreactivity

Purpose and Rationale

Symptoms like asphyctic convulsions resembling bronchial asthma in patients can be induced by inhalation of histamine or other bronchospasm-inducing agents in guinea pigs. The challenging agents are applied as aerosols produced by an ultrasound nebulizer. The first symptoms are increased breathing frequency, forced inspiration, and finally asphyctic convulsions. The occurrence of these symptoms can be delayed by antagonistic drugs. Preconvulsion time, i.e., time until asphyctic convulsions, can be measured.

Procedure

Ten male albino guinea pigs weighing 300–400 g per group are used. The inhalation cages consist of three boxes each ventilated with an air flow of 1.5 l/min. The animal is placed into box A to which the test drug or the standard is applied using an ultrasound nebulizer LKB NB108 which provides an aerosol of 0.2 ml solution of the test drug injected by an infusion pump within 1 min. Alternatively, the animal is treated orally or subcutaneously with the test drug or the standard. Box B serves as a sluice through which the animal is passed into box C. There, the guinea pig is exposed to an aerosol of a 0.1 % solution of histamine hydrochloride provided by an ultrasound nebulizer (De Vilbiss, Model 35 A). Time until appearance of asphyctic convulsions is

measured. Then, the animal is immediately withdrawn from the inhalation box. The aerosols are removed from the back wall of the boxes by applying low pressure.

Evaluation

Percent of increase of preconvulsion time is calculated versus controls. ED_{50} values can be found, i. e., 50 % of increase of preconvulsion time.

Critical Assessment of the Method

The "guinea pig asthma" has been applied as useful method in various modifications by many laboratories.

Modifications of the Methods

Simple methods to test bronchospasmolytic activity in conscious animals called "thoracography" were described by Herxheimer (1956), Olsson (1971), and Beume et al. (1985). A silicon tube with a diameter of 1 mm is filled with mercury and serves as strain gauge applied as belt around the thorax of conscious guinea pigs. Changes in electrical resistance due to breathing movements of the thorax are registered. The animals are exposed in a Plexiglas chamber to acetylcholine nebulized by an ultrasonic device. Time until onset of coughing indicated by an increase of signal amplitude and of severe asthmatic dyspnea is registered.

Immunological factors are involved in bronchial hyperreactivity (Reynolds 1991).

Harris et al. (1976) immunized rabbits with thermophilic actinomyces antigen (Micropolyspora faeni). Lesions resembling hypersensitivity in man were found, characterized by a mononuclear cell interstitial reaction and a marked increase in the number of intra-alveolar cells.

Ufkes et al. (1983) induced bronchial and cardiovascular anaphylaxis in Brown-Norway rats, sensitized with trinitrophenyl-haptenized ovalbumin and $AlPO_4$ as adjuvant 12 days prior to challenge with trinitrophenyl-haptenized bovine serum albumin intravenously.

Raeburn et al. (1992) gave a survey on techniques for drug delivery to the airways and the assessment of lung functions in animal models including parameters such as lung compliance and airway resistance.

Elwood et al. (1992) studied the effects of dexamethasone and cyclosporin A on the airway hyperresponsiveness and the influx of inflammatory cells into bronchoalveolar lavage fluid seen 18–24 h after exposure to aerosolized ovalbumin in actively ovalbumin-sensitized Brown-Norway rats.

Schmiedl et al. (2003) found an increase of inactive intra-alveolar surfactant subtypes in lungs of asthmatic Brown Norway rats. The volume fractions of surfactant subtypes and the epithelial surface fraction covered with alveolar edema were determined by point and intersection counting. The surface activity of surfactant from bronchoalveolar lavage was determined as the minimum surface tension at minimal bubble size with a pulsating bubble surfactometer.

Pahl et al. (2002) and Kuss et al. (2003) exposed ovalbumin-sensitized Brown Norway rats to an ovalbumin-containing aerosol in a nose-only inhalation system (TSE, Bad Homburg, Germany) for 1 h to provoke an influx of inflammatory cells into the airways. At the time of maximal influx of eosinophilic granulocytes into the airways (48 h later), the animals were sacrificed and a bronchoalveolar lavage performed.

A model of bronchial hyperreactivity after active anaphylactic shock in conscious guinea pigs has been described by Tarayre et al. (1990). The guinea pigs were sensitized by an intramuscular injection of a large dose of ovalbumin in Freund's adjuvant. The administration of ovalbumin to induce anaphylactic shock was by aerosol. Bronchial hyperreactivity to histamine was observed 3–6 h after the anaphylactic shock.

Bolser et al. (1995) sensitized guinea pigs by intraperitoneal injection of 200 μg/kg ovalbumin mixed with 200 mg/kg aluminum hydroxide. The animals were placed 28 days later in a transparent plastic chamber and exposed to aerosols of ovalbumin (0.1–1 %) at an air flow of 4 l/min to elicit coughing. Coughs were detected by a microphone placed in the chamber and connected to an audio monitor and chart recorder. The number of coughs

elicited during a 4-min exposure was counted by visual inspection of the chart record.

Pons et al. (2000) described a guinea pig model of asthma. Animals were sensitized by two intraperitoneal injections of 20 μg ovalbumin and 100 mg aluminum hydroxide given 24 h apart. The sensitized animals were anesthetized and mechanically ventilated. Air flow was measured with a pneumotachograph, along with transpulmonary pressure and arterial pressure. Lung resistance and dynamic compliance were calculated. Then, 30 min after administration of test drug, the animals were challenged by inhaled ovalbumin (5 mg/ml).

The importance of eosinophil activation for the development of allergen-induced bronchial hyperreactivity was underlined by Santing et al. (1994). A significant increase in bronchoreactivity to histamine was observed 6 h after allergen exposure, which was associated with an increase of eosinophils in the bronchoalveolar lavage and an increase in the eosinophil peroxidase activity.

The increased pulmonary vascular permeability may be related to the adult respiratory distress syndrome in man (Snapper and Christman 1989).

The infusion of small amounts of *Escherichia coli* endotoxin into chronically instrumented awake **sheep** results in well-characterized pulmonary dysfunction (Brigham and Meyrick 1986).

One animal model associated with both increased airway responsiveness and pulmonary inflammation is endotoxemia in sheep (Hutchinson et al. 1983).

The effect of a platelet-activating factor receptor antagonist on the sheep's response to endotoxin was studied by Christman et al. (1987).

Chiba and Misawa (1995) characterized muscarinic cholinoceptors in airways of antigen-induced airway hyperresponsive rats. The animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract together with *Bordetella pertussis* (2×10^6) as an adjuvant and were boosted with 2,4-dinitrophenylated *Ascaris suum* extract 5 days later. Isometric contractions of the circular muscle of isolated bronchial rings after addition of increasing doses of acetylcholine were measured with a force displacement transducer.

Laboratory infection of **primates** with *Ascaris suum* may provide a model of allergic bronchoconstriction (Patterson et al. 1983; Pritchard et al. 1983; Eady 1986).

Richards et al. (1986) used various models of airway hypersensitivity, such as inhalation of *Ascaris suum* antigen in monkeys and dogs.

Rylander and Marchat (1988) studied the effect of a corticosteroid on an acute inflammation in the lungs of guinea pigs exposed to an aerosol of bacterial endotoxin. The subsequent inflammatory response was evaluated counting the number of cells obtained from airway lavage and in the lung interstitium as well as the chemotactic effect of alveolar macrophages.

Hatzelmann et al. (1996) reported on automatic leukocyte differentiation in bronchoalveolar lavage fluids of guinea pigs and Brown-Norway rats using an automatic cell analyzing system (Cobas Helios 5Diff; Hoffmann-La Roche; Grenzach-Wyhlen, Germany).

Minshall et al. (1993) demonstrated that neonatal immunization of **rabbits** with *Alternia tenuis* can lead to the development of persistent airway hyperresponsiveness.

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Body Plethysmography and Respiratory Parameters After Histamine-Induced Bronchoconstriction in Anesthetized Guinea Pigs

Purpose and Rationale

Guinea pigs can be placed in a plethysmograph for measurement of respiratory parameters. Respiratory frequency and respiratory amplitude are recorded. The decrease of respiratory amplitude (diminished respiratory volume due to bronchoconstriction) and the reflectory increase of respiratory frequency after histamine inhalation are attenuated by bronchodilatatory drugs. Additional respiratory parameters can be recorded using a Fleisch tube and a catheter inserted into the pleural cavity (Englert et al. 1992). The method can be used for various purposes, e.g., to evaluate the antagonism against bradykinin-induced bronchoconstriction (Wirth et al. 1991, 1993) or the bronchodilator effects of potassium channel openers (Englert et al. 1992) or to measure the effect of morphine on respiration in rats (Kokka et al. 1965).

Procedure

Guinea pigs of either sex weighing 400–600 g are anesthetized with 70 mg/kg pentobarbital i.p. The trachea, pleural cavity, jugular vein, and carotid artery are prepared and cannulated. The animals are mechanically ventilated with a Starling respiratory pump which delivers an inspiration volume

that represents a tracheal pressure of 8 cm water at a rate of 60 strokes/min. Succinylcholine chloride at a dose of 1 mg/kg is given i.v. to prevent interference from spontaneous respiration. The guinea pigs are placed inside a whole body plethysmograph and tracheal, pleural, venous, and arterial catheters connected to onset ports in the wall of the plethysmograph box. The tracheal port is then connected with the respiration pump. **Airflow rate** into and out of the plethysmograph are measured as pressure difference with a No. 000 Fleisch tube and a differential pressure transducer (Fa. Hellige, PM 97 TC). Airflow is calibrated by passing compressed air through a rotameter. The **tidal volume** (V_T) is calculated from the flow signal. **Transpulmonary pressure** (P_{TP}) is measured with a differential pressure transducer (Hellige, PM 97 TC), with one side attached to a catheter inserted into the right pleural cavity and the other side connected to a side port of the tracheal cannula. P_{TP} is calibrated with a water manometer. Signals from airflow, tidal volume, and transpulmonary pressure are fed into an online computer system (PO-NE-MAH, Model PF-1, Storrs) for calculation of **pulmonary resistance** (R_L) and **dynamic lung compliance** (C_{DYN}). These parameters are calculated for each breath with a sampling rate of 100 s^{-1} for each circle. Flow and pressure signals for computation are obtained from a PLUGYS measuring system (Fa. Hugo Sachs Elektronik, Freiburg, Germany). Systemic arterial pressure is measured using a Statham pressure transducer (P 23 Db). Heart rate is computed from pressure pulses (Döring and Dehnert 1997).

Three doses of test compound or standard are injected intravenously. Saline injections serve as controls. Intravenous injections of histamine (0.5–2 μg/kg) lead to a short decrease in C_{DYN} and to a short increase in R_L by approximately 200 % compared with baseline. Challenges are repeated at 5-min intervals, yielding the same increase in R_L during the whole 1-h experimental period. After three reproducible responses the test agent is administered intravenously 1 min before the histamine injection.

To evaluate test compounds for inhalation route, aerosols are generated with an ultrasonic nebulizer (LKB, model NB 108) and are administered to the animals through a shunt in the afferent limb of the respiratory pump, allowing the inspired air to pass through the nebulizer chamber before entering the animals' lungs.

Evaluation

Inhibition of histamine-induced bronchoconstriction by various doses of test compound and standard is recorded. ED_{50} values for inhibition in R_L are calculated. Furthermore, the time course of histamine antagonism can be evaluated. Compounds can be tested either after i.v. injection of histamine (prevention) or during intravenous infusion of histamine (intervention).

Critical Assessment of the Method

Whole body plethysmography has been proven to be a useful tool in respiratory pharmacology for studies on the antagonism against various bronchoconstrictors, such as histamine and bradykinin, as well as for airway pharmacology of potassium channel openers.

Modifications of the Method

Several authors use body plethysmography to study respiratory functions in animals (Amdur and Mead 1958; Blümcke et al. 1967), Pennock et al. 1979; Agarwal (1981), James and Infiesto (1983), Kisagawa et al. (1984), Griffith-Johnson (1988), Danko and Chapman (1988), Ball et al. (1991), Chand et al. (1993).

The effect of β-blockers on pulmonary function and bronchoconstrictor responsiveness in **guinea pigs** and rats has been studied by Chapman et al. (1985).

Finney and Forsberg (1994) developed a technique for quantification of nasal involvement in a guinea pig plethysmograph. Nasal and lower respiratory system conductance could be measured simultaneously in anesthetized animals.

A whole body plethysmograph for conscious animals has been described by Elliott et al. (1991) and improvements by Linton (1991).

Studies of bronchospasmolytic agents with aerosol challenge in conscious guinea pigs using a double-chamber plethysmograph box have been reported by Schlegelmilch (1991).

Ball et al. (1991) described a method for the evaluation of bronchoactive agents in the conscious guinea pig. The method involves the use of "head out" whole body plethysmographs from which respiratory rate can be recorded by monitoring respiration-related changes in pressure within the body chamber.

Hey et al. (1995) used a head-out whole body plethysmograph to examine the effects of $GABA_B$ receptor agonists on minute ventilation, tidal volume, and respiratory rate due to room air and carbon dioxide-enriched gas hyperventilation in conscious guinea pigs.

Murphy et al. (1998) developed a method for chronic measurement of pleural pressure in conscious **rats**. Pleural pressures were measured by surgically implanting a fluid-filled polyurethane catheter attached to a pressure-sensitive radiotelemetry transmitter (Model TA11PA-C40, Data Sciences Int., St. Paul, MN) beneath the pleural surface. A compatible receiver (Model RLA0120) and a data acquisition and analysis software system (LabPRO, Version 3) sampling at a rate of 500 Hz were used to analyze the telemetric signals.

Sinnett et al. (1981) described a fast integrated flow plethysmograph for small animals (**mice**).

Glaab et al. (2001) used tidal mid-expiratory flow in a head-out body plethysmograph as a measure of airway hyperresponsiveness in allergic mice.

Simple methods to determine respiratory parameters in small animals were described by Schütz (1960) and Höbel et al. (1971).

Schlenker (1984) and Schlenker and Metz (1989) evaluated ventilatory parameters in dystrophic **Syrian hamsters**.

Wasserman and Griffin (1977) studied bronchoactivity in the intact anesthetized **dog**.

Paré et al. (1976) determined pulmonary resistance and dynamic lung compliance in **rhesus monkeys**.

Wegner et al. (1984) measured dynamic respiratory mechanics in monkeys by forced oscillations generated by a loudspeaker in an airtight chamber.

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Pneumotachography in Anesthetized Guinea Pigs

Purpose and Rationale

The use of a pneumotachograph based on the principle of the Fleisch tube and of additional pressure transducers allows simultaneous measurements of several respiratory and circulatory parameters in anesthetized guinea pigs (de la Motta 1991).

Procedure

Guinea pigs (Pirbright white) weighing 300–400 g are anesthetized with 1.5 g/kg urethane i.p. The animals are shaved ventrally at the neck, placed on a heated operating table, and fixed at the upper extremities. A metal cannula with a blunted tip is inserted into the trachea and secured with a loop within the caudal section of the trachea. A thin plastic catheter is inserted into the esophagus and the tip located inside the thorax in order to register intrathoracic pressure. Furthermore, the cephalic vein at one side and the carotid artery on the opposite side are cannulated. The tracheal cannula is connected with pieces of tubing to a Fleisch tube (pneumotachograph), size 0000. In order to avoid water condensation, the Fleisch tube is heated. The Fleisch tube is connected with a sensitive differential pressure transducer with a range of 2 cm H_2O (Validyne, model MP 45 -xx-871). One side of another pressure differential transducer with a range of 20 ml H_2O is connected with the esophageal catheter, the other side remaining open to room air. Both Validyne pressure transducers are connected to a separate preamplifier. For recording of the arterial blood pressure a Gould pressure transducer, Type P23Gb, is used. The signals for airflow and esophageal pressure are monitored at the output of the preamplifier with a digital two-channel oscilloscope. To obtain various respiratory and circulatory parameters from the three primary signals they are calculated by certain formulas by an analog computer (Buxco Pulmonary Mechanics Analyzer, Model 6). The following parameters are presented at the output of the instrument as analog electrical signals:

Circulation
Systolic blood pressure, diastolic blood pressure, mean blood pressure

Respiration
Tidal volume, respiratory volume per minute, respiratory rate

Pulmonary Mechanics
Airway resistance, dynamic compliance, end-respiratory work

A multichannel recorder (Graphtec Linearcorder Mark VII) serves a functional check on the Buxco Analyzer and as analog presentation of the calculated parameters. Data processing is performed by a 12-channel A/D converter (Buxco Data Logger, Model D/C-12 F/V) which digitizes the analog output signals of the Buxco Analyzer and sends them through a serial interface (RS232) to an IBM PC. A special software program (Lomask 1987; Hastings 1990a, b) provides a flexible facility for data reduction and statistical evaluation.

Evaluation

For each individual experiment the data of the last 5 min before the first substance application are averaged and used as controls. The response values after substance application are then expressed as percentages of the controls. In this way each animal serves as its own control. For an analysis of the results the response values are averaged over certain time intervals.

Critical Assessment of the Method

The method described in great detail by de la Motta (1991) may be modified using different equipment according to individual needs.

Modifications of the Method

Measurement of respiratory parameters is based on earlier studies to be mentioned for historical reasons (Gad 1880; Pflüger 1882; Zwaardemaker and Ouwehand 1904; Jaquet 1908; Rohrer 1915; Gildemeister 1922; Fleisch 1925; v. Neergaard and Wirz 1927; survey by Döring 1991).

Lorino et al. (1988) assessed respiratory mechanics of histamine bronchopulmonary reactivity in guinea pigs.

O'Neil et al. (1981) published a comparative study of respiratory responses to bronchoactive agents in rhesus and cynomolgus monkeys.

Rayburn et al. (1989) described a computer-controlled pulmonary function system for studies in large animals.

Five methods of analyzing respiratory pressure-volume curves have been compared by Lai and Diamond (1986).

A specially designed pneumotachograph that is placed inside the trachea of guinea pigs was described by Santing et al. (1992) allowing the evaluation of airway functions in conscious, unstressed animals.

Lorino et al. (1993) estimated the changes in end-respiratory lung volume-accompanied histamine-induced bronchoconstriction in anesthetized, paralyzed, and mechanically ventilated guinea pigs from measurements of thoracic cross-sectional area, assessed from the voltage induced by an external uniform magnetic field in a pickup coil encircling the rib cage.

Gozzard et al. (1996) evaluated the effects of PDE-inhibitors in **New Zealand White rabbits** which were immunized within 24 h of birth with *Alternaria tenuis* antigen. Spontaneously breathing rabbits were intubated in neuroleptanalgesia with a cuffed endotracheal tube connected to a thermoregulated Fleisch pneumotachograph to allow measurement of tidal air flow. An esophageal balloon catheter was inserted to provide a measure of intrapleural pressure and transpulmonary pressure. Antigen challenge was performed with inhaled *Alternaria tenuis* extract.

An excellent survey on various methods and equipment to measure air flow is given by Döring and Dehnert (1997).

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Airway Microvascular Leakage

Purpose and Rationale

Plasma exudation in guinea pig airways in vivo can be determined by Evans Blue dye and is fairly correlated with radiolabeled albumin (Rogers et al. 1989). This method can be used to study the antagonism against bradykinin- and platelet-activating factor-induced airway microvascular leakage and vagal stimulation-induced airway responses (Sakamoto et al. 1992, 1994).

Procedure

Female Dunkin-Hartley guinea pigs weighing 380–600 g are anesthetized with an initial dose of 1.5 g/kg urethane injected i.p. Additional urethane is given i.v. 30 min later to achieve an appropriate level of anesthesia. A tracheal cannula is inserted into the lumen of the cervical trachea, a polyethylene catheter into the left carotid artery to monitor blood pressure and heart rate, and another polyethylene catheter into the external jugular vein for administration of drugs. The animals are connected to a constant-volume mechanical ventilator and then given an injection of 1.0–1.5 mg/kg suxamethonium i.v. to prevent interference with spontaneous respiration. A tidal volume of 10 ml/kg and a frequency of 60 strokes/min are used.

Lung resistance is measured as an index of airway function and monitored throughout the experiment. Transpulmonary pressure is measured with a pressure transducer with one side attached to a catheter inserted into the right pleural cavity and the other side attached to the side port of the intratracheal cannula. Airflow is measured by a pneumotachograph connected to a pressure transducer. The signals of the transducers are used for instantaneous calculation of lung resistance by an appropriate computer program.

The test compound (bradykinin receptor antagonist) is given intravenously. Ten minutes later, Evans Blue dye (20 mg/ml) is injected i.v. for 1 min. After 1 min, bronchoconstriction and microvascular leakage is induced by injection of bradykinin or by inhalation of bradykinin or PAF or vagal stimulation.

Six minutes after induction of leakage, the thoracic cavity is opened, and a cannula is inserted into the aorta through a ventriculotomy. Perfusion is performed with 100–150 ml 0.9 % saline at a pressure of 100–120 mm Hg in order to remove the intravascular dye from the systematic circulation. Blood and perfusion liquid are expelled through an incision in the right and left atrium. Subsequently, the right ventricle is opened, and the pulmonary circulation is perfused with 30 ml

of 0.9 % saline. The lungs are then removed, and the connective tissue, vasculature, and parenchyma are gently scraped. The airways are divided into four components: lower part of the trachea, main bronchi, the proximal 5 mm portion, and the distal intrapulmonary airways. The tissues are blotted dry and then weighed. Evans Blue dye is extracted in 2 ml of formamide at 40 °C for 24 h and measured in a spectrophotometer at 620 nm.

Evaluation

Evans Blue dye concentration, expressed as ng/mg tissue, as well as lung resistance are compared by statistical means (unpaired Student's *t*-test or Mann–Whitney *U* test) between treated groups and controls receiving the challenge only.

Modifications of the Method

Boschetto et al. (1989) tested the effect of antiasthma drugs on microvascular leakage in guinea pig airways. Microvascular leakage was induced by intravenous injection of platelet-activating factor (50 ng/kg) which acts directly on venular endothelial cells and measured by quantifying extravasation of Evans Blue dye.

Xu et al. (1998) induced pulmonary edema in rats by injection of 20 μg/kg angiotensin I and studied the suppression by ACE-inhibitors, ATII antagonists, and α-adrenergic receptor blockers.

Rapidly developing pulmonary edema was induced by intravenous injection of 1.2 mg/kg of the GABA agonist bicuculline in rats and the role of endogenous endothelin examined by Herbst et al. (1995).

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Isolated Larynx In Situ

Purpose and Rationale

The in situ isolated larynx of rats has been recommended by Willette et al. (1987) for evaluation peripheral opiate receptor antagonists. Peripheral opioid-induced laryngospasm and central opioid-induced respiratory depression can be measured simultaneously. Fentanyl citrate stimulates both peripheral and central opiate receptors, whereas [D-Ala^2-Met^5]-enkephalinamide stimulates only peripheral opiate receptors. Compounds that inhibit both laryngeal and respiratory effects of fentanyl, e.g., naloxone HCl, can be considered both central and peripheral opiate antagonists. Compounds that inhibit only the peripheral effects of fentanyl, e.g., naltrexone methylbromide, can be considered peripheral opiate receptor antagonists.

Procedure

Laryngeal resistance experiments are carried out in male Sprague Dawley rats weighing 340–360 g and anesthetized with urethane (900 mg/kg, i.p.) The animals are secured in supine position and the left femoral artery and the left and right femoral veins cannulated for recording of arterial blood pressure and administration of drugs. A right-angle polyethylene cannula (ID 1.67 mm, OD 2.42 mm) is inserted into the caudal trachea at

the level of the manubrium for measuring tracheal flow via a small animal pneumotachograph. Care has to be taken to avoid damaging surrounding blood vessels or adjacent bilateral laryngeal nerves. Tracheal air flow is continually sampled (300 ml/min) for the breath-to-breath analysis of end tidal carbon dioxide with an infrared gas analyzer.

Laryngeal resistance in the rat is determined using a modification of the methods described by Stransky et al. (1973), Bartlett et al. (1973), and Willette et al. 1982b). The rostral portion of the trachea is cannulated with a right-angle polyethylene tube (ID 1.67 mm, OD 2.42 mm). This cannula is carefully advanced toward the larynx and secured with a suture (4–0 silk). A constant flow ($V = 30$ ml/min) of compressed air, maintained with a flow meter, is delivered through a cannula. Prelaryngeal pressure is measured with a needle tipped pressure transducer inserted into the lumen of the flow cannula. A 1.75-cm segment of polyethylene tubing (OD 8.4 mm, ID 4.6 mm) is placed into the mouth to retract resistive components in the nasopharyngeal region.

Laryngeal resistance (LR) is calculated by the following equation:

$$LR = (P_{\mathrm{L}}P_{\mathrm{i}} - P_{\mathrm{L}}P_0)/V$$

where $P_{\mathrm{L}}P_{\mathrm{i}}$ is the laryngeal pressure in the cannula directing a constant flow (V) through the larynx. $P_{\mathrm{L}}P_0$ is the pressure in the cannula when it is removed from the trachea.

The agonist fentanyl is administered through the left femoral vein at a dose of 12 μg/kg which is equivalent to 1.5 times the ED_{99} in the conscious rat tail flick assay. The enkephaline analogue [D-Ala^2-Met^5]-enkephalinamide is injected at a dose of 250 μg/kg which acts peripherally and increases laryngeal resistance (Willette et al. 1982a). The opiate receptor antagonists are administered similarly into the right femoral vein.

At the conclusion of the experiment, the pulmonary afferent stimulant phenyldiguanide (25 μg/kg, i.v.) is injected into the right femoral vein to elicit laryngospasm and to determine the viability of the preparation.

Evaluation

All summary values are expressed as the mean plus or minus the standard error of the mean (SEM) Comparisons are made with independent and paired two-tailed *t*-tests.

Modifications of the Method

Inagi et al. (1998) assessed the effect of botulin toxin in the rat larynx by measurement of the optical density of PAS-stained laryngeal muscle after electrical stimulation, spontaneous laryngeal muscle activity, and laryngeal movement.

O'Halloran et al. (1994) studied the effects of upper airway cooling and CO_2 on breathing and on laryngeal and supraglottic resistance in anesthetized rats.

González-Barón et al. (1989) studied the modifications of larynx resistance changing bronchial tone in cats evoked by intravenous administration of 10 μg/kg carbachol as bronchoconstrictor and by fenterol (10 μg/kg) or isoproterenol (0.1 mg/kg) as bronchodilators.

Wang et al. (1999) developed an isolated, luminally perfused laryngeal preparation in anesthetized paralyzed cats in order to compare the effects of solutions with varying levels of pH and pCO_2 on pressure-sensitive laryngeal receptor sensitivity.

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Animal Models of Asthma

Treatment of Asthma

Purpose and Rationale

Several authors described asthma model s in mice. Hammad et al. (2004) found in a mouse model of asthma that activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) in dendritic cells inhibits the development of eosinophilic airway inflammation.

Procedure

Bone Marrow Dendritic Cells

OVA-TCR transgenic mice (DO10.11) on a BALB/c background, bred at the Erasmus University (Rotterdam) (Murphy et al. 1990), are used. Femurs and tibiae of female mice are removed and flushed with RPMI 1640. Vigorous pipetting disintegrates clusters within the marrow suspension. The cells are washed, enumerated, and plated in Petri dishes. Cell culture medium (TCM) is supplemented with gentamicin (60 µg/ml), 2-mercaptoethanol (5×10^{-5} mol/l), and 5 % fetal calf serum (Lutz et al. 1999). At day 0 of the culture, cells are seeded at a concentration of 2×10^6/dish in medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF, 200 IU/ml). At days 6 and 8, half of the medium is collected, centrifuged, and the pellet is resuspended in TCM containing 200 IU/ml of recombinant murine GM-CSF.

At day 9 of the culture, dendritic cells (Banchereau and Steinman 1998) are pulsed overnight with ovalbumin containing the vehicle (dimethylsulfoxide, DMSO) in which the standard PPAR-γ agonist (e.g., rosiglitazone) is suspended or with medium alone as control. Other plates are treated with the test drug or a PPAR-γ antagonist. After antigen pulsing overnight, nonadherent dendritic cells are collected, washed to remove free ovalbumin, and resuspended in phosphate-buffered saline at a concentration of 12.5×10^6 cells/ml.

Eosinophilic Airway Inflammation

For intratracheal injection of dendritic cells (Lambrecht et al. 2000), mice are anesthetized with Avertin and 80 µl of the cell suspension (1×10^6 dendritic cells) instilled through the opening vocal cords. Mice are injected with unpulsed dendritic cells, ovalbulmin-treated dendritic cells, or dendritic cells treated additionally with the PPAR-γ antagonist. From days 10–13, mice are exposed to 30-min ovalbumin aerosols. They are sacrificed 24 h after the last aerosol. Bronchoalveolar lavage is performed with 3×1 ml of Ca^{2+}- and Mg^{2+}-free buffer supplemented with 0.1 mmol/l sodium EDTA. The bronchoalveolar lavage is centrifuged; the cells are resuspended in buffer and enumerated with a hemocytometer. They are differentiated (Vremec and Shortman 1997) by staining for 30 min with anti-I-Ad/I-Ed FITC (macrophages), anti-CCR3 PE (eosinophils), antiCD3-cy-chrome, anti-B220 cytochrome (T and B cells, respectively), and anti-CD11c APC (macrophages) in PBS containing 0.5 bovine serum albumin and 0.01 % sodium azide. Cells are washed and analyzed by flow cytometry (Van Rijt et al. 2002).

Evaluation

The difference between the groups is calculated using the Mann–Whitney *U*-test for unpaired data.

Modifications of the Method

Iwasaki et al. (2001) recommended atopic NC/Nga mice as a model for allergic asthma: after immunization with ovalbumin, severe allergic responses were elicited by a single intranasal challenge.

For testing PPAR agonists, Trifilieff et al. (2003) used a murine model of asthma using lung inflammation induced by ovalbumin or by LPS (Trifilieff et al. 2000). Female BALB/c mice (4 weeks old) were immunized with ovalbumin on days 0 and 14, exposed to an aerosol challenge of ovalbumin or phosphate-buffered saline (PBS) on day 21, and killed on day 23 for measurement of inflammatory cells in the bronchoalveolar lavage. Animals were intranasally treated with compounds 1 h before the aerosol exposure using PBS containing 2 % DMSAO as vehicle.

For LPS-induced lung inflammation mice were challenged intranasally with 0.3 mg/kg of LPS (*Salmonella typhosa*) in 50 ml of sterile PBS or with sterile PBS alone and killed 3 h later for bronchoalveolar lavage. Tumor necrosis factor alpha (TNF-α) and the neutrophil chemokine KC levels were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, UK). Animals were intranasally treated with compounds 1 h before the aerosol exposure using PBS containing 2 % DMSO as vehicle.

Mueller et al. (2003) reported that **peroxisome proliferator-activated receptor γ ligands** attenuate immunological symptoms of allergic asthma. For asthma induction, Balb/c mice were injected with 10 µg of ovalbumin in Alum twice on days 1 and 5 (Zhang et al. 1999). On day 10, they were challenged intranasally with 40 µg ovalbumin in sterile saline, every day for 3 days. On the day after the last intranasal challenge, the mice were sacrificed. The degree of inflammation in the lung was evaluated by histopathology. Gene expression was tested by microarray analysis and serum IgE determined by ELISA.

Regal et al. (2001) described trimellitic anhydride-induced eosinophilia in a mouse model of occupational asthma. Trimellitic anhydride was conjugated to mouse serum albumin. Female BALB/c mice were sensitized on days 1 and 3 intradermally with 0.1 ml of 0.3 % ovalbumin suspended in corn oil. On day 12, animals were additionally sensitized intratracheally with 0.04 ml ovalbumin, trimellitic anhydride conjugated to mouse serum albumin, or mouse serum albumin dissolved in water. For elicitation of the allergic response, mice were challenged intratracheally beginning on day 19 with 0.04 ml of aqueous solutions of ovalbumin, trimellitic anhydride conjugated to mouse serum albumin, or mouse serum albumin under ketamine/xylazine anesthesia. After the last intratracheal instillation, the mice were anesthetized with pentobarbital, EDTA plasma was collected by cardiac puncture, the trachea was cannulated, and the lungs were lavaged with two 0.9-ml aliquots of PBS to obtain bronchial lavage fluid. The lungs were removed for homogenization and analysis of eosinophil peroxidase and myeloperoxidase.

Churg et al. (2001) studied anti-inflammatory effects of alpha-1 antitrypsin and a metalloprotease inhibitor in C57 BL/6 mice or macrophage metalloelastase knockout mice after intratracheal instillation of a single 7-mg dose of crystalline silica (α-quartz, Minusil-5; US Silica Corporation, Clarkstown, W.Va., USA). This dose produced a rapid and persisting acute inflammatory infiltrate. Mice were euthanized 2 or 24 h after dust exposure by halothane overdose and the lungs removed from the chest cavity. A 20-gauge catheter was inserted into the trachea and the lungs lavaged six times with 1 ml of ice-cold saline. For inflammatory cell measurements, the saline lavage was centrifuged at 200 *g* at 4 °C for 10 min. The supernatants were decanted, and the cell pellets were resuspended in 200 µl of saline. Total cell counts were performed using a hemocytometer and differential cell counts performed on a 10-µl drop of the cell suspension heat fixed on a slide and stained with hematoxylin-eosin. Lavage samples were analyzed for desmosine and hydroxyproline.

De Sanctis and Drazen (1997) discussed the genetics of native airway responsiveness in mice.

Several animal species have been used such as **rats**.

Misawa et al. (1987) found strain differences among Wistar, Lewis, and Fischer 344 rats in an allergic asthma model.

Misawa and Sugiyama (1993) described an airway hyperresponsiveness model in rats, inducing allergic asthma with DNP-Ascaris extract.

Uhlig et al. (1998) reported the effects of long-term oral treatment with leflunomide on allergic sensitization, lymphocyte activation, and airway inflammation in rats.

Birrell et al. (2003) investigated the nitric oxide synthase isoform involved in eosinophilic inflammation in a rat model of Sephadex-induced airway inflammation. The rat model of Sephadex-induced airway inflammation was also used by Belvisi et al. (2005) for preclinical studies on ciclesonide, an inhaled corticosteroid for the treatment of asthma.

Many authors used sensitized **Brown Norway rats** in experimental models of asthma (Elwood et al. 1992; Steerenberg et al. 1999; Nonaka et al. 2000; Xu et al. 2000; Blesa et al. 2002; Glaab et al. 2002; Huang et al. 2002; Belvisi et al. 2005; Valstar et al. 2006).

Guinea pigs have been used as animal models for asthma by many authors. Most studies were performed with sensitization either by injection with ovalbumin suspended in an adjuvant (Santives et al. 1976; Banner et al. 1996; Lawrence et al. 1998; Regal et al. 2000; Li et al. 2001; Santing et al. 2001; Mukaiyama et al. 2004; Boskabady and Zarei 2004; Ikezono et al. 2005; Tang et al. 2005) or by repetitive sensitization with inhaled ovalbumin (Sagara et al. 1996, 1997; Smith et al. 1996; Liu et al. 1997; Tohda et al. 1998; Cheng et al. 2001; Zhang et al. 2002) and then challenge by inhaled ovalbumin.

Fujimura et al. (1997) described a guinea pig model of ultrasonically nebulized distilled water–induced bronchoconstriction. Guinea pigs sensitized by intradermal injection of ovalbumin in Freund's adjuvant were treated with aerosols of physiological saline generated by an ultrasonic nebulizer.

Zhou et al. (1998) reported a dose–response relationship between exposure to cockroach allergens and induction of sensitization in an experimental asthma in Hartley guinea pigs.

Larsen and Regal (2002) studied trimellitic anhydride dust-induced airway obstruction and eosinophilia in nonsensitized guinea pigs.

Nishitsuji et al. (2004) described a guinea pig model of cough variant asthma. Bronchial responsiveness to methacholine and cough reflex sensitivity to capsaicin were measured 72 h after ovalbumin inhalation in actively sensitized guinea pigs.

Dogs were used as animal models for asthma by several authors. Many studies preferred the **Basenji Greyhound dog**, which manifests various characteristics of human asthma, including airway hypersensitivity to low concentrations of methacholine (Hirshman et al. 1980; Hirshman and Downes 1981; Chan et al. 1985; Darowski et al. 1989; Emala et al. 1993, 1996).

Redman et al. (2001) studied pulmonary immunity to ragweed in a **Beagle dog** model of allergic asthma.

Some studies were reported using **cats** as an animal model for asthma. Norris et al. (2003) and Norris-Reinero et al. (2004) described an experimental model of allergic asthma in cats sensitized to the house dust mite and Bermuda grass allergen.

Several studies were performed using **monkeys** as animal models for asthma. *Ascaris suum*-sensitive monkeys were used by Mauser et al. (1995) and Zou et al. (2002). Rhesus monkeys (*Macaca mulatta*) were used by Patterson et al. (1975) and Patterson and Harris (1981, 1990, 1992). Turner et al. (1996) used *Macaca fascicularis* to demonstrate in vitro and in vivo effects of leukotriene B_4 antagonism in primates.

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Prevention of Allergic Asthma Reaction

Purpose and Rationale

Type 1 allergy is an abnormal reaction to protein substances that occur naturally. B-lymphocytes produce an antibody against the allergen. An allergic reaction occurs every time the body is exposed to the allergen causing release of histamine. Allergic reactions can be asthma, hay fever, or nettle rash. Sensitization to normally harmless environmental antigens (e.g., pollen, house-dust mite) is the prerequisite for initiating the inflammatory cascade in bronchial asthma. Inflammation of the airway mucosa is orchestrated by Th-2 type T cells, which produce Th-2 cytokines (IL-4, IL-5, IL-9, IL-13, and IL-15), which regulate both IgE production and airway inflammation. At present, no cure of asthma disease is available, but primary and secondary prevention is the therapy of choice (Cieslewicz et al. 1999). Specific immunotherapy is performed by injection of increasing amounts of allergens to induce hyporesponsiveness to the respective allergen. By mucosal application of soluble antigen, mucosal tolerance can be achieved (van Halteren et al. 1997; Astori et al. 2000). Many studies have been performed in spite of the fact that no animal model is available that resembles all features of human bronchial asthma (Herz et al. 1998). In particular, models using recombinant allergens were described (Hoyne et al. 1997, 2000; Herz et al. 2004).

These studies concern specific allergens, such as **ovalbumin** (Renz et al. 1992; Marth et al. 2000; Neuhaus-Steinmetz et al. 2000; Raap et al. 2003; Reader et al. 2003; Wegmann et al. 2005), **mite dust allergen** (Hoyne et al. 1996; Clarke et al. 1999; Yasue et al. 1998a, b, c, 1999; Jarnicki and Thomas 2002), **pollen allergen** (Hirahara et al. 1998; Wiedermann et al. 1999, 2001; Batanero et al. 2002; Repa et al. 2004; Hufnagl et al. 2005; Winkler et al. 2006), **latex protein allergen** (Thakker et al. 1999; Woolhiser et al. 2000; Meade and Woolhiser 2002; Hufnagl et al. 2003), **bee venom allergen** (Von Garnier et al. 2000), and **cat allergen** (Briner et al. 1993; Treter and Luqman 2000).

Wegmann et al. (2005) studied involvement of distal airways in a chronic model of experimental asthma.

Procedure

Animals

Pathogen-free female BALB/c mice (Harlan Winkelmann, Hannover, Germany), weighing

18–22 g and 6–8 weeks of age, were used in all experiments. The animals were maintained under standard housing conditions, fed an ovalbumin (OVA-)-free diet, and supplied with food and water ad libitum.

Sensitization Protocol

Mice were sensitized to OVA by three intraperitoneal injections [10 μg OVA grade VI (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg Al $(OH)_3$ diluted in 200 μl phosphate-buffered saline (PBS)] on days 1, 14, and 21. The mice were challenged with OVA (grade V) aerosol (1 % wt/vol in PBS) via the airways twice a week on 2 consecutive days over a period of 12 weeks (Renz et al. 1992). Sham sensitization and challenges were carried out with sterile $Al(OH)_3$ in PBS. Animals were analyzed after 1 or 12 weeks of OVA aerosol challenge. To investigate persistence of airway inflammation and lung physiological changes mice were analyzed after 6 weeks of OVA aerosol challenge discontinuation following 12 weeks of OVA aerosol challenge.

Differential Cell Counts in Bronchoalveolar Lavage Fluid

At 48 h after the last allergen challenge bronchoalveolar lavage (BAL) was performed and analyzed (Renz et al. 1992).

Measurement of Cytokines in BAL Fluid

IL-4, IL-5, and tumor necrosis factor alpha (TNF-α) were measured in cell-free lavage fluids by cytometric bead array (CBA, BD Biosciences, San Diego, Calif., USA). The detection limit for each of the cytokines was 10 pg/ml. Complete BAL transforming growth factor beta (TGF-β) was measured after acidic activation using chicken antihuman TGF-β in a standard ELISA protocol. The detection limit for TGF-β was 20 pg/ml.

Lung Histology

Lungs were fixed ex situ with 4 % (wt/vol) paraformaldehyde via the trachea, removed, and stored in 4 % paraformaldehyde. Lung tissues were embedded into paraffin, and 3 μm sections were stained with hematoxylin and eosin or periodic acid-Schiff staining. For localization of collagen fibrils, Sirius red staining was performed according to Uhal et al. (1998).

Immunohistochemistry

Indirect immunohistochemistry was used to stain lung sections for smooth muscle cells and myofibroblasts (mouse monoclonal IgG against α-SMA, clone 1A4, Immunotech, Marseilles, France) and nerve cells (polyclonal rabbit antiserum against protein gene product 9.5, Biogenesis, Poole, UK) according to Fehrenbach et al. (2002). To distinguish smooth muscle cells from myofibroblasts a rat monoclonal IgG against fibroblast-specific peptide (clone ER-TR7; Biogenesis) was utilized.

Quantitative Morphology

Paraffin sections stained with Fast Green/Sirius Red and for α-SMA, respectively, were used to quantify changes in airway epithelial cell, collagen, and smooth muscle cell layers of distal airways according to standard stereological methods. A distal airway was defined as the segment of a terminal bronchiolus that, starting at the bronchoalveolar duct transition, extended up to five alveoli along the proximal direction. Using a PC-based Olympus light microscope BX 51 equipped with a CAST-Grid System (Visiopharm, Hoersholm, Denmark), all distal airways of a given section were delineated (at a magnification of $\times$ 426), and the fields of view to be analyzed (at a final magnification of $\times$ 1.700) were automatically defined according to systematic uniform random sampling. The arithmetic mean thicknesses were determined as the volume of the respective component, determined by counting all points hitting airway epithelium, Sirius Red- and α-SMA-positive components, respectively. Results were referred to the reference surface determined by counting all intersections with the airway epithelial basal membrane (Reader et al. 2003). The arithmetic mean thicknesses were calculated.

Electron Microscopy and Definition of Proximal and Distal Airways

For analysis of airway wall ultrastructure, lungs of mice chronically challenged with OVA and

control mice challenged with PBS ($n = 3$ per group) were fixed by instillation of 4 % (wt/vol) paraformaldehyde via the trachea. Beginning at the lobar bronchus, the airways were microdissected along the axial pathway according to Plopper et al. (2001). For histopathological and ultrastructural analysis, proximal airways were defined as the lobar bronchi and the axial pathway down to the fourth intrapulmonary branch point. Mid-level airways were defined as the axial pathways between the intrapulmonary branch points 6–12. Terminal bronchioles with direct connection to the alveolar ducts were defined as distal airways (Postlethwait et al. 2000).

Noninvasive Measurement of Mid-Expiratory Airflow at Baseline and of Bronchial Responsiveness to Methacholine

The mid-expiratory airflow (EF_{50}) was measured 24 h after the last OVA aerosol challenge using head-out body plethysmography (Glaab et al. 2001).

Evaluation

Results are presented as mean values ± SD unless stated otherwise. One-way ANOVA test or Student's unpaired *t*-test was used to determine the significance of differences between animal groups.

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Bleomycin-Induced Pulmonary Fibrosis

Purpose and Rationale

Pulmonary fibrosis has been induced by bleomycin in various species: golden Syrian **hamsters** (Giri et al. 1986; Chen et al. 1997; Gurujeyalakshmi et al. 1999; Iyer et al. 1999, 2000), **mice** (Taooka et al. 1997; Tamagawa et al. 2000; Keerthisingam et al. 2001; Terasaki et al. 2000; Terasaki 2001; Atzori et al. 2004), and **rats** (Howell et al. 2001; Simler et al. 2002; Wang et al. 2002; Morcillo and Bulbena 2003).

Iyer et al. (1999) determined the effects of pirfenidone on procollagen gene expression at the transcription level in a bleomycin **hamster** model of lung fibrosis.

Procedure

Treatment of Animals

Male golden Syrian hamsters weighing 90–110 g were housed in groups of four in facilities with filtered air and constant temperature and humidity. A 12-h/12-h light/dark cycle was maintained, and the animals had access to water and either pulverized Rodent Laboratory Chow 5001 or the same pulverized chow containing pirfenidone (0.5 % w/w). The hamsters were fed these diets 2 days before intratracheal instillation and throughout the study period. Hamsters were instilled intratracheally with saline or bleomycin (7.5 units/kg per 5 ml). The animals were sacrificed at 3, 7, 10, 14, and 21 days after the bleomycin or saline instillation by decapitation, and their lungs were removed and freeze clamped, dropped in liquid nitrogen, and stored at −80 °C. The major portion of the sample was used for direct total RNA isolation, and the remainder was used for other biochemical studies.

Tissue Processing for Biochemical Study

The frozen lungs were thawed and homogenized in 0.1 M KCl/0.02 M Tris · HCl buffer, pH 7.6, with a Polytron homogenizer. After recording the total homogenate volume (5–6 ml), it was mixed,

divided into aliquots, and stored at −80 °C, except for the aliquots for lipid peroxidation and hydroxyproline assays, which were processed and assayed the same day on which the lungs were homogenized.

Determination of Lipid Peroxidation

The lung malondialdehyde equivalent (MDAE) level, an index of lipid peroxidation, was determined in the whole homogenate according to the method of Ohkawa et al. (1979).

Determination of Prolyl Hydroxylase Activity

The method for prolyl hydroxylase assay is based on the release of tritiated water from 3,4-[^{3}H] proline-labeled unhydroxylated procollagen substrate prepared in vitro using 10-day-old embryonic chick tibiae (Giri et al. 1983). During the reaction, tritium is released in stoichiometric proportion to prolyl hydroxylation as tritiated water, which is collected and counted as a measure of the prolyl hydroxylase activity (Hutton et al. 1966). The activity was expressed as the total dpm released/lung per 30 min.

Determination of Prolyl Hydroxylase Activity In Vitro

Control hamsters not subjected to any treatment were first anesthetized with sodium pentobarbital (80–100 mg/kg). Their lungs were perfused with ice-cold isotonic saline; then, all lung lobes were dissected out and rinsed in saline. They were immediately homogenized in buffer containing 0.1 M Tris and 0.05 % Triton X. The homogenate was spun down at 6,000 g for 20 min at 4 °C. The supernatant was gently aspirated and used to determine prolyl hydroxylase activity and its protein content. The procedure to measure the prolyl hydroxylase activity was essentially the same as described above. Briefly, the reaction mixture in a total volume of 2.2 ml consisted of 200 µl of α-ketoglutarate (0.001 M), 200 µl of ferrous ammonium sulfate (0.005 M), 250 µl of supernatant as enzyme source, 200 µl of pirfenidone to produce the desired final concentration, 200 µl of Tris · HCl (1 M), and 20 µl of ^{3}H-unhydroxylated PC substrate (400,000 cpm). The reaction mixture was first preincubated with pirfenidone at different concentrations for 30 min at 37 °C in a shaking water bath. The reaction was started by adding 200 µl of ascorbic acid (0.005 M); 30 min later, the reaction was terminated by adding 200 µl of 50 % trichloroacetic acid. The tritiated water released was collected by vacuum distillation. Then 1 ml of the tritiated water was mixed with 10 ml of Ready Safe liquid scintillation cocktail (Beckman), and the radioactivity was determined at 45 % counting efficiency in a scintillation counter. The protein content of the supernatant was determined according to the method of Lowry et al. (1951). The prolyl hydroxylase activity was expressed as total dpm associated with tritiated water released in the reaction mixture/mg protein per 30 min.

Determination of Hydroxyproline

For assay of lung hydroxyproline as a measure of collagen content, 1 ml of whole homogenate was precipitated with 0.25 ml of ice-cold 50 % (w/v) trichloroacetic acid and centrifuged, and the precipitate was hydrolyzed in 2 ml of 6 N HCl for 18 h at 110 °C. The hydroxyproline content was measured according to the method of Woessner (1961).

Evaluation

All data are expressed as mean ± SEM. Bleomycin treatment increases the amount of proteins of extrapulmonary origin that can result in the artificial lowering of all values (Karlinsky and Goldstein 1980; Goldstein and Fine 1986). Thus, the in vivo data are expressed on a per-lung basis. The data were compared within the groups at the corresponding times using the two-way ANOVA where four groups were involved and the t test between the two groups. A value of $p \leq 0.05$ was considered to be the minimum level of statistical significance.

Modifications of the Method

Howell et al. (2001) induced pulmonary fibrosis in **rats**. Male Lewis rats, aged 6 weeks and weighing 140–170 g, were anesthetized by intramuscular injection of 0.75–1.0 ml/kg Hypnorm (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml). Bleomycin sulfate was administered

by a single intratracheal injection (l.5 mg/kg body weight in 0.3 ml of sterile saline). Control animals received 0.3 ml of saline alone. In initial experiments, groups of six rats were killed by pentobarbitone overdose after 6 days to allow assessment of thrombin levels in bronchoalveolar lavage fluid. Separate groups of two rats were sacrificed 1, 3, 6, and 14 days after bleomycin instillation for immunohistochemical assessment of thrombin and protease-activated receptor1. Lungs were fixed by intratracheal instillation of 4 % paraformaldehyde, the trachea ligated, and the inflated lungs and heart removed *en bloc*. Tissues were fixed and transferred to 15 % sucrose in phosphate-buffered saline, before alcohol dehydration and embedding in paraffin wax. An additional series of animals was killed 6 days after bleomycin or saline instillation for measurement of blood coagulation parameters, total and differential cell counts in bronchoalveolar lavage fluid, and for Northern blot analysis of lung tissue connective tissue growth factor and α_1(I) procollagen mRNA levels. For measurement of coagulation parameters, blood was collected from the inferior vena cava of animals after laparotomy and was immediately mixed 10:1 with a solution of 3.8 % trisodium citrate (w/v). For measurement of total lung collagen and connective tissue growth factor and procollagen mRNA levels, the vasculature was perfused with 5 ml of sterile saline containing 100 U/ml heparin. The lungs were removed, weighed, and immediately snap frozen in liquid N_2 after removing the trachea and major airways.

Adachi et al. (2003) and Azoulay et al. (2003) studied the effects of granulocyte colony-stimulating factor (G-CSF) on lung injury induced by various doses of bleomycin in rats.

Sogu et al. (2004) found that endosteine, an antioxidant, prevents bleomycin-induced pulmonary fibrosis in rats.

Chen et al. (2006) reported that short courses of low-dose dexamethasone delay bleomycin-induced lung fibrosis in rats.

Chaudhary et al. (2006) described the time course of inflammation and fibrosis in the rat bleomycin model and studied the effect of timing of anti-inflammatory and antifibrotic treatments on efficacy.

Barrio et al. (2006) studied in vitro tracheal hyperresponsiveness to muscarinic receptor stimulation by carbachol in the rat model of bleomycin-induced pulmonary fibrosis.

Terasaki et al. (2000) and Terasaki (2001) studied the effect of epimorphin in bleomycin-induced pulmonary fibrosis in **mice**. Pulmonary fibrosis was induced in 8-week-old male ICR mice by a single intratracheal instillation of bleomycin. On selected days after injection (days 0, 3, 7, 14, 21, 28, 35, 42, and 56), the lungs were harvested and investigated. Immunohistochemical analysis was performed for epimorphin using light microscopy and confocal microscopy and also the analysis by immunoelectron microscopy and in situ hybridization.

Avivi-Green et al. (2006) reported that discoidin domain receptor 1-deficient mice are resistant to bleomycin-induced lung fibrosis. Matsuyama et al. (2006) found that suppression of discoidin domain receptor 1 by RNA interference attenuates lung inflammation induced by intratracheal instillation of bleomycin in mice.

Inayama et al. (2006) reported that a Iκ B kinase-β inhibitor ameliorates bleomycin-induced pulmonary fibrosis in mice.

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Influence of Cytokines on Lung Fibrosis

Purpose and Rationale

Several cytokines (Murphy et al. 2000) are involved in development of pulmonary fibrosis, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNFα), and transforming growth factor beta (TGFβ) (Kelly et al. 2003; Agostini and Gurrieri 2006).

GM-CSF is involved in fibrotic reactions of the lung (Xing et al. 1996; Adachi et al. 2002, 2003).

TGFβ is a central modulator of pulmonary and airway inflammation and fibrosis (Sime et al. 1997; Kolb et al. 2002; Gauldie et al. 2003; Xu et al. 2003; Yao et al. 2004; Hardie et al. 2006; Lee et al. 2006; Sheppard 2006).

Smad proteins are involved in the TGFβ-mediated pulmonary fibrosis (Zhao et al. 2000; Bonniaud et al. 2004, 2005; Kobayashi et al. 2006).

TNFα induces TGFβ (Sime et al. 1998; Warshamana et al. 2001; Sullivan et al. 2005).

Xing et al. (1996) reported that the **transfer of** GM-CSF **gene** to rat lung induces eosinophilia, monocytosis, and fibrotic reactions.

Procedure

Construction of Recombinant Adenovirus Vectors

An 800-bp fragment of murine GM-CSF cDNA was isolated from pCDSRα by digestion with *Bam*HI and *Dra*I. The shuttle plasmid pACCMV containing 0–17 mu human type 5 adenovirus genome with a CMV promoter (760 bp), multicloning sites, and SV40 splicing junction/polyA signal (430 bp) inserted in the E1 region of viral genome was first digested with *Sal*I, and the ends were repaired using T4 kinase and dNTPs (New England Biolabs, Beverly, Mass., USA), followed by a secondary digestion with *Bam*HI to generate the 3′ complimentary ends. The GM-CSF fragment was then subcloned into the *Bam*HI/*Sal*I site in PACCMV using T4 ligase (New England Biolabs) to generate the recombinant plasmid PACCMVmGM-CSF. The presence of the GMCSF insert was confirmed by restriction digestions. The PACCMVmGM-CSF was cotransfected, following a standard procedure described previously (Graham and Prevec 1991), into 293 cells along with a plasmid pAdBHG10 which contained the most rightward sequences (3.7–100 mu) of human type 5 adenovirus genome with a partial deletion in the E3 region

(Bett et al. 1994). The recombinant replication-deficient adenovirus Ad5E1PACCMVmGM-CSF (Ad5E1GM-CSF) was rescued by homologous recombination. The presence of GM-CSF cDNA in the viral genome was verified by analyzing viral genome fragments upon *Hind*III digestion and by Southern hybridization (Graham and Prevec 1991). The control virus Ad5dl70–3 was constructed and characterized as previously described (Bett et al. 1994), and similar to Ad5E1GM-CSF, this virus had the E1 region crippled, hence incapable of replication.

High titers of the above viruses were generated (Graham and Prevec 1991). Briefly, viruses purified by two rounds of CsCl gradient centrifugation were subjected to chromatography using PD-10 Sephadex columns (Pharmacia Biotech, Baie d'UrFe, Quebec, Canada) to remove CsCl. The virus fractions were collected in PBS containing 10 % glycerol, measured for conductance to ensure complete removal of CsCl, pooled, titered, aliquoted, and stored at −70 °C until use.

Characterization of Recombinant Adenovirus Vector Expressing GM-CSF In Vitro

GM-CSF transgene mRNA was examined by Northern hybridization analysis (Xing et al. 1993) using total RNA from 293 cells infected with 10 plaque-forming units (pfu)/cell of Ad5E1GM-CSF for 24 and 48 h. The supernatants from these cells and from infected rat alveolar macrophages were assayed for GM-CSF using an ELISA kit (Endogene, Cambridge, Mass., USA). This ELISA was specific for mouse GM-CSF without cross-reactivity with rat GM-CSF, with a sensitivity of 4 pg/ml.

Delivery of Recombinant Adenovirus Vectors to the Lung

Following a standard procedure (Xing et al. 1994), 300 µl of Ad5E1GM-CSF or control virus Ad5dl70–3 diluted in PBS to a concentration of 1×10^9 pfu was instilled intratracheally to the lung of Sprague Dawley male rats weighing 220–280 g (Charles River Laboratories, Ottawa, Canada). At the end of 1, 2, 4, 7, 12, 18, and 24 days after gene transfer, rats were anesthetized, blood samples were taken from the abdominal aorta, and serum preparation and bronchoalveolar lavage (BAL) were performed.

Transgene and Transgene Protein Expression in the Lung

The left lung of rats obtained at each time point was snap frozen in liquid nitrogen. Total lung RNA extraction and Northern hybridization were performed. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine transgene mRNA expression with total lung RNA using PCR reagents from Promega (Madison, Wis., USA) following the standard protocol. The specific primers for PCR were chosen to ensure the amplification of the transgene-specific GM-CSF mRNA but not the endogenous rat GM-CSF mRNA (Miyatake et al. 1985; Smith et al. 1994). The sense and antisense primer sequences were 5′-GTCTCTAA CGAGTTCTCCTTCAAG-3′ and 5′-TTCAG AGGGCTATACTGCCTTCCA-3′, respectively. The primers for rat GAPDH were designed as described by Rosenfeld et al. (1992). BAL samples were collected at various times and assayed for transgene protein GM-CSF by ELISA as described above or for TNFα by ELISA specific for both murine and rat TNFα (Genzyme, Cambridge, Mass., USA). Serum samples collected from the same animals were also assayed for circulating levels of GM-CSF by ELISA.

Cytological Examination of BAL and Blood Samples

Total cell numbers in BAL were determined using a hematocytometer. Differential cell types were determined on cytospins stained with Diff-Quik (Baxter, McGaw Park, Ill., USA) by randomly counting 300–400 cells/cytospin. To analyze the total peripheral blood leukocyte counts and differentials, blood samples were collected into heparin-coated tubes. Total leukocyte numbers were counted on a hematocytometer after lysing red blood cells with a lysis buffer containing 94 % H_2O, 3 % acetic acid, and 3 % Diff-Quik purple stain. Differential leukocytes were determined on blood smears stained with Diff-Quik by counting 500–700 cells/blood smear.

Histopathologic Examination of Lung and other Tissues

The right lung and in some instances the whole lung of each animal were fixed by perfusion with 10 % formalin (Fisher Scientific, Fairlawn, N.J., USA). Tissues from heart, liver, spleen, and kidney were also fixed in 10 % formalin. Multiple sections from different lobes of the lung or from other organs were stained with hematoxylin/eosin for routine histopathology, with Congo Red for identification of tissue eosinophils, or with Elastic van Gieson for collagen and elastin.

Modifications of the Method

Underwood et al. (2000) demonstrated in guinea pigs and rats that a p38 MAPK inhibitor reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lungs.

Kolb et al. (2001) reported that the proteoglycans decorin and biglycan differentially modulate TGF-β-mediated fibrotic responses in the lungs of mice.

Uhal et al. (2003) showed that in rats amiodarone induced lung fibrosis and alveolitis, which could be partially inhibited by angiotensin system antagonists.

Jiang et al. (2004) described regulation of pulmonary fibrosis by the chemokine receptor CXCR3, which is the receptor for the interferon-γ-inducible C-X-C chemokines MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11.

Kim et al. (2006) tested the alveolar epithelial-to-mesenchymal cell transition, which develops in vivo during pulmonary fibrosis, and found regulation by the extracellular matrix.

Shi-Wen et al. (2006) found that constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis and gave evidence of an autocrine endothelin loop operating through the endothelin A and B receptors.

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Emphysema Models

Purpose and Rationale

Several animal models of genetically determined emphysema are known, such as the **tight-skin mouse** (Rossi et al. 1984; Martorana et al. 1989; Gayraud et al. 2000), beige mutants of the C57BL6J strain (O'Donnell et al. 1999; Lucattelli et al. 2003), the **blotchy mouse** (Ranga and Kleinerman 1981; McCartney et al. 1988), and **homozygous mutant *klotho* (*KL*$^{--}$) mice** (Suga et al. 2000).

Hoyle et al. (1999) described emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. LeCras et al. (2004) reported that vascular endothelial growth factor causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. Tsa et al. (2004) reported that overexpression of placenta growth factor contributes to the pathogenesis of pulmonary emphysema in mice.

Blanco et al. (1989), Whitney et al. (1999), Massaro et al. (1995), Massaro and Massaro (2000, 2003), and Dirami et al. (2004) reported that in **rats** septation of gas exchange saccules occurs during the first 2 postnatal weeks. Treatment with dexamethasone irreversibly impairs septation. Treatment with all-*trans*-retinoid acid prevents the dexamethasone-induced inhibition of septation.

Hind and Maden (2004) and Maden and Hind (2004) found that retinoic acid induces alveolar regeneration in the adult mouse lung after damage by disulphiram treatment. These data could not be confirmed in an elastase-induced emphysema model by Fujita et al. (2004).

Several authors used elastase instillation to produce emphysema-like lesions in **rats** (Tepper et al. 2000; March et al. 2004), **mice** (Inoue et al. 2003; Murakami et al. 2005), **rabbits** (Qi et al. 2004), and **dogs** (Morino et al. 2005).

Kuraki et al. (2002) described inhibition of human neutrophil elastase-induced emphysema in rats by an oral neutrophil elastase inhibitor.

Procedure

Rat Emphysema Model

Male Wistar rats weighing 228 ± 15 g were divided into controls (saline treated), low-dose group (treated with 200 U human neutrophil elastase), and a high-dose group (treated with 400 U human neutrophil elastase). Human neutrophil

elastase was sprayed above the carina of the trachea using a microsprayer without tracheotomy. Eight weeks after human neutrophil elastase application, rats were sacrificed and lungs dissected out to evaluate the morphological changes.

Effects of Neutrophil Elastase Inhibitor

Rats were divided into four groups: saline control, human neutrophil elastase + CMC, human neutrophil elastase + low dose of inhibitor, and human neutrophil elastase + high dose of inhibitor given orally. Six hours after human neutrophil elastase, lung hemorrhage and neutrophil accumulation in the lung were determined. Eight weeks after the application, the functional and morphological changes were determined.

Lung Hemorrhage and Neutrophil Accumulation in the Lung

After tracheostomy, bronchoalveolar lavage was performed for determination of neutrophil counts and hemoglobin in lavage fluid. In addition, neutrophil accumulation was estimated using the myeloperoxidase activity.

Lung Volume and Pulmonary Mechanics

Eight weeks after human neutrophil elastase application, rats were anesthetized and tracheotomy performed. Using a whole-body plethysmograph for small animals, the functional residual capacity (FRC), total lung capacity, and static lung compliance were determined.

Evaluation

Data were presented as mean ± SD. Differences between groups were evaluated for statistical significance using one-way analysis of variance.

Modifications of the Method

Boström et al. (1996) showed that platelet-derived growth factor A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis.

Kirschvink et al. (2005) induced production of pulmonary MMP-2 and MMP-9 and emphysema by repeated cadmium nebulizations in rats.

Corteling et al. (2002) studied the migration and activation of neutrophils into the airway in pathological conditions such as pulmonary emphysema in BALB/c and C57BL/6 **mice** and in **golden hamsters**. The animals were sequentially treated intranasally with 0.3 mg/kg lipopolysaccharide and with 0.5 mg/kg *N*-formyl-Met-Leu-Phe.

Selman et al. (2003) induced emphysema in **guinea pigs** by exposure to the whole smoke of 20 cigarettes per day, 5 days per week, for 1 month, 2 months, and 4 months through a whole-body exposure chamber. Half of the animals received a matrix metalloproteinase inhibitor. After death, the lungs were lavaged with saline solution, and matrix metalloproteinases in the lavage fluid were determined by zymography and immunoblot. Lungs were fixed for histology, immunochemistry, and morphometry.

Lucattelli et al. (2003) described collagen phagocytosis by lung alveolar macrophages in animal models of emphysema.

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Models of Chronic Obstructive Pulmonary Disease (COPD)

Purpose and Rationale

Chronic obstructive pulmonary disease (COPD) is a severe respiratory condition that is increasing in prevalence worldwide. The disease is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with abnormal inflammatory response of the lungs to noxious particles and gases. Three conditions comprise COPD, namely, mucus hypersecretion, emphysema, and bronchiolitis. Cigarette smoking is the major risk factor for development of COPD and accounts for the majority of cases (Donnelly and Rogers 2003). The relevance of the present animal models for COPD has been questioned (Canning 2003). Several authors used chronic cigarette-smoke exposure in mice (Hautamaki et al. 1997; Cavarra et al. 2001a, b; Wright and Churg 2002; Bartalesi et al. 2005; Martorana et al. 2005), in rats (Escolar et al. 1995; Lee et al. 2005), in guinea pigs (Wright 2001; Meshi et al. 2002), or in dogs (Frasca et al. 1983).

Lee et al. (2005) reported inhibition of cigarette-smoking-induced emphysema and pulmonary hypertension in rat lungs by a HMG-CoA reductase inhibitor.

Martorana et al. (2005) found that a selective phosphodiesterase-4 (PDE4) inhibitor fully prevents emphysema in mice chronically exposed to cigarette smoke.

Procedure

Six-week-old C57Bl/6 J male mice were, in acute studies, exposed either to room air or the smoke of five cigarettes (Virginia filter cigarettes: 12 mg of tar and 0.9 mg of nicotine) for 20 min. In chronic studies, the mice were exposed to either room air or to the smoke of three cigarettes/day for 5 days/week for 7 months. Antioxidant capacity was assessed at the end of exposure in bronchoalveolar lavage fluid. Cytokines and chemokines were determined.

In acute studies, mice were divided in 3 groups of 40 animals each. These groups were then divided into 4 subgroups of 10 mice: no treatment/air exposed; no treatment/smoke exposed; low dose of test compound/smoke exposed; high dose of test compound/smoke exposed.

In chronic studies, five groups of animals were used: no treatment/air exposed; drug treatment/air exposed; no treatment/smoke exposed; low dose of test compound/smoke exposed; high dose of test compound/smoke exposed. After 7 months,

animals were sacrificed and the lungs fixed intratracheally with 5 % formalin at a pressure of 20 cmH_2O. Lung volume was measured by water displacement. Assessment of emphysema included mean linear intercept and internal surface area. The volume density of macrophages, marked immunohistochemically with antimouse Mac-3 monoclonal antibodies, was determined by point counting.

Evaluation

The significance of the differences was calculated using one-way analysis of variance.

Modifications of the Method

Kumar et al. (2003) compared a selective PDE4 inhibitor with pentoxifylline (a nonselective phosphodiesterase inhibitor) and dexamethasone in ameliorating the lesions of chronic asthma in BALB/c mice sensitized to ovalbumin and chronically challenged with aerosolized antigen for 6 weeks.

Kodavanti et al. (2000) reported that the combination of elastase and sulfur dioxide exposure causes COPD-like lesions in the rat.

The potential of tachykinin receptor antagonists in airway diseases was discussed by Joos and Pauwels (2001).

Sturton and Fitzgerald (2002) reviewed PDE4 inhibitors for the treatment of COPD.

Billah et al. (2002) described the pharmacology of an orally active PDE4 inhibitor.

Pitfalls and opportunities for modeling allergic asthma in mice were discussed by Kumar and Foster (2002).

Inoue et al. (2003) described an impaired pulmonary inflammation response as a prominent feature of streptococcal pneumonia in mice with experimental emphysema.

Dual dopamine D_2 receptor and β_2-adrenoreceptor agonists were proposed for the treatment of COPD (Dougall et al. 2003; Ind et al. 2003).

Lessons from transgenic mice for the relationship between asthma and COPD were discussed by Elias (2004).

Lappalainen et al. (2005) found that interleukin-1β causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung.

Romano (2005) discussed selectin antagonists for their therapeutic potential against asthma and COPD.

Jones et al. (2002) described a model for the continuous monitoring of polymorphonuclear leukocyte trapping in the pulmonary vasculature of the **rabbit**.

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Antitussive Activity

Kristy D. Bruse

Contents

Antitussive Activity After Irritant Inhalation in Guinea Pigs

Purpose and Rationale

Cough is thought to be caused by a reflex. The sensitive receptors are located in the bronchial tree, particularly in the bifurcation of the trachea. These receptors can be stimulated mechanically or chemically, e.g., by inhalation of various irritants. Nerve impulses then activate the cough center in the brain. Several animal species and several irritants have been used, most frequently the citric acid–induced cough in guinea pigs (Charlier et al. 1961; Karlsson et al. 1989; Braga et al. 1993). The pharmacology of cough was reviewed by Reynolds et al. (2004).

Procedure

Guinea pigs of either sex weighing 300–400 g are used. The animal is placed in a cylindrical glass vessel, with two tubes at either end. One serves as the entrance of the aerosol, the other for its efflux. The latter tube has a sidearm connecting to a tambour, from which changes in pressure can be registered. A pinch clamp with a variable screw is placed on the efflux tube beyond the sidearm, permitting the regulation of the sensitivity of the system, so that the normal respiration is not registered, while the displacement of air in the

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enclosure caused by coughing of the animal is registered. The guinea pig is exposed to the aerosol of 7.5 % citric acid in water for 10 min. Each animal is tested first to obtain the control response. The number of tussive responses is registered. One hour later, the test substance is applied either s.c. or orally, and 30 min later the guinea pig is subjected to the aerosol again. The number of coughs during 10 min is recorded.

Evaluation

The number of coughs after treatment is expressed as percentage of the control period. Using various doses, ED_{50} values can be calculated.

Critical Assessment of the Method

The citric acid–induced coughing in guinea pigs has been proven to be an effective method to test antitussive agents.

Modifications of the Method

The citric cough model in guinea pigs was used by Adcock et al. (1988) to study the effects of codeine, morphine, and an opioid pentapeptide, by Hay et al. (2002) to study a potent and selective neurokinin-3 receptor antagonist, and by Brown et al. (2004) to study antitussive activity of sigma-1 receptor agonists.

Cough elicited by capsaicin inhalation in guinea pigs was used by Bolser et al. (1993, 1997) to study antitussive effects of $GABA_B$ agonists or NK1 and NK2 tachykinin receptor antagonists, by McLeod et al. (1998) to study the antitussive action of antihistamines, and by Trevisani et al. (2004) to investigate the activity of iodo-resiniferatoxin, an ultrapotent antagonist of the transient receptor potential vanilloid-1.

Forsberg et al. (1988) studied cough and bronchoconstriction mediated by aerosols of capsaicin or citric acid or nicotine or histamine in guinea pigs.

Püschmann and Engelhorn (1978) studied the inhibition of the coughing reflex induced by inhalation of a citric acid spray in ***rats***.

Other irritants have been used to induce cough, e.g., ammonia in dogs, guinea pigs, and cats (Rosiere et al. 1956; Källqvist and Melander 1957; Chen et al. 1960; Sallé and Brunaud 1960; Ellis et al. 1963), or nebulized sulfuric acid or sulfur dioxide in guinea pigs, rats, cats, or dogs (Eichler and Smiatek 1940; May and Widdicombe 1954; Winter and Flakater 1952, 1954; Friebel et al. 1955; Reichle and Friebel 1955; Wiedemeijer et al. 1960; Charmat et al. 1966; Karttunen et al. 1982). Capsaicin aerosol was used by Forsberg and Karlsson (1986), Gallico et al. (1997).

Winter and Flakater (1955) exposed sensitized guinea pigs to aerosol of a specific antigen.

Kamei et al. (1989) induced cough in rats by a nebulized solution of capsaicin. The cough reflex was measured as airflow into or out of the chamber of a body plethysmograph by a pneumotachometer head.

Carotis sinus excitation in dogs induced by injection of lobeline resulting in coughing could be suppressed by codeine (Gross 1957).

Sanzari et al. (1968) induced cough in cats anesthetized with α-chloralose by intravenous injection of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a ganglionic stimulant which is more potent than lobeline and possesses only marginal ganglionic blocking properties. The number of coughs was found to be a linear function of the dose of the irritant. Coughs were recorded as spikes superimposed on the respiratory pattern. The method is suitable for quantitative evaluation of antitussive activity.

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Cough Induced by Mechanical Stimulation

Purpose and Rationale

Cough can be induced by mechanical stimulation of the trachea in anesthetized guinea pigs (Takagi et al. 1960; Gallico et al. 1994).

Procedure

Male guinea pigs weighing 350–400 g are maintained in conditioned quarters (temperature 21±2 °C, relative humidity 55±10 %, 12 h on–12 h off light cycle) with food and water ad libitum for at least 1 week before use.

After overnight fasting with water ad libitum, the guinea pigs are lightly anesthetized with 25 % urethane (4 ml/kg i.p.), which induces surgical levels of analgesia without depressant effects on respiratory function. Analgesia is monitored throughout the experiment as the disappearance of head shaking in response to ear pinch. The animals are maintained at a constant body temperature of 37 °C by means of a heated plate. A thin steel wire is gently inserted into the trachea through a small incision near the cricoid cartilage. Coughs are evoked by pushing the steel wire to reach the bifurcation of the trachea 35 and 5 min before oral drug administration and 30, 60, and 120 min after treatment. One violent cough occurs upon each stimulation. Only those animals that respond to both mechanical stimulations before dosing are selected and then randomly assigned to receive the test drug at various doses or the standard (codeine 15, 30, and 60 mg/kg). Ten animals per dose are used.

Evaluation

Evaluation of the statistical significance of the results is performed with Student's *t*-test for paired data. ED_{50} values are determined by logit transformation.

Modifications of the Method

Several other ways of mechanical stimulation have been used, e.g., by a nylon-bristled stimulator thrust into the trachea in **dogs** (Kasé 1952, 1954), a silver thread in **decerebrated guinea pigs** (Lemeignan et al. 1966), vibration of an iron slung in the trachea of a dog induced by an electromagnet (Tedeschi et al. 1959), electrical stimulation of the trachea via a bronchoscope (Gross et al. 1958), or through implanted copper electrodes (Stefko and Benson 1953; Benson et al. 1953; Granier-Doyeux et al. 1959; Stefko et al. 1961).

Hara and Yanaura (1959), Yanaura et al. (1974, 1982) induced cough in unrestrained animals after implantation of electrodes in the trachea.

Combined mechanical and chemical stimulation has been applied by Kroepfli (1950).

Bolser et al. (1999, 2001) and McLeod et al. (2002) studied the influence of antitussive drugs on the cough motor pattern in anesthetized cats. Coughing was produced by mechanical stimulation of the intrathoracic trachea with a thin flexible polyethylene cannula for 10 s per stimulus trial. During each trial, the cannula was repetitively moved in the trachea at a frequency of ~2 Hz. EMGs from the diaphragm and rectus abdominis muscles were recorded with the use of bipolar tungsten wire electrodes. The diaphragm electrodes were placed through a small midline abdominal incision, which was subsequently closed. Cough is characterized by coordinated bursts of activity in inspiratory and expiratory muscles. Cough was defined as a large burst of EMG activity in the diaphragm that is immediately followed by a burst of EMG activity in the rectus abdominis muscle. This definition differentiates augmented breaths, the aspiration reflex, or the expiration reflex from cough. The antitussive activity of selected drugs was evaluated from cumulative dose responses after intravertebral artery administration of each compound. The protocol consisted of the application of five consecutive mechanical stimulus trials after vehicle administration. Stimulus trials were applied at 1-min intervals after each dose of compound for a total of five stimulus trials between doses. Approximately 7 min elapsed between each dose of compound.

Kasé et al. (1976) studied the antitussive activity of D-3-methyl-*N*-methylmorphinan in conscious mongrel **dogs**. Coughing was induced by mechanical stimulation with a stimulator consisting of five hog bristles on the mucosa of tracheal bifurcation through a chronically built tracheal fistula and in lightly anesthetized cats with a stimulator consisting of five whiskers of a rabbit.

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Cough Induced by Stimulation of the Nervus Laryngicus Superior

Purpose and Rationale

The probable pathways in the cough reflex arc are receptors in the area of the trachea and the large bronchi, afferent nerves mainly in the branches of the vagus nerve, a "cough center" located in the medulla oblongata, and efferent nerves closing the glottis and reinforcing the expiratory thrust. Stimulation of the nervus laryngicus superior induces coughing. Antitussive agents with predominantly central action suppress the coughing reflex.

Procedure

Cats of either sex weighing 2–3 kg are anesthetized with 40 mg/kg i.p. pentobarbital, placed on a heated operating table, and their extremities secured. Since deep anesthesia suppresses coughing the dose of pentobarbital has to be adjusted. The fur is shaved ventrally at the neck. Small incisions are made at both sides of the larynx. The superior laryngeal nerves (forming a loop) are prepared carefully. After a median skin incision, the trachea is exposed and cannulated. The cannula is connected with a Fleisch tube (size 00). One femoral artery is cannulated for registration of blood pressure via a Statham pressure transducer. One femoral vein is cannulated for intravenous application of test substances. Small hook electrodes are attached to each laryngeal nerve. At the end of an inspiration square wave impulses with a frequency of 50 Hz, an impulse width of 0.5 ms, an amplitude of 0.2–1.0 V, and a duration of 1–10 s are applied every 5 min. The intensity of the forced expiration is measured by the Fleisch pneumotachograph and recorded simultaneously with blood pressure on a polygraph. Prior to the intravenous application of the test compound, the response to three stimuli is recorded serving as control. After injection of the test compound or the standard, the stimuli are repeated every 5 min. Suppression or diminution of the forced expiration is recorded

over 1 h. Then, the next dose or the standard (codeine phosphate 1–2 mg/kg i.v.) is applied.

Evaluation

Total or partial suppression of the forced expiration are recorded over time and expressed as percentage of control. Intensity and duration of the effect are compared with the standard.

Critical Assessment of the Method

The method described by Domenjoz (1952) is very useful to detect centrally active antitussive agents like codeine but by definition cannot determine compounds which act on cough receptors in the bronchial area. Moreover, even light anesthesia influences the cough reflex.

Modifications of the Method

Several other assays have been described which elicit the cough reflex by central or nerve stimulation. Toner and Macko (1952) also stimulated the superior laryngeal nerve in anesthetized cats to induce a definite cough as indicated by rapid contractions of the abdominal musculature.

Mattalana and Borison (1955)and Chakravarty et al. (1956) used decerebrated cats to study the central effects of antitussive drugs on cough and respiration. Cough responses were obtained by electrical stimulation of the dorsolateral region of the medulla with bipolar needle electrodes oriented by means of a stereotactic instrument.

Lindner and Stein (1959) evaluated derivatives of diphenyl-piperidono-propan, a series of antitussive drugs using a modification of the method originally described by Domenjoz (1952).

Schröder (1951) and Bobb and Ellis (1951) elicited cough in **conscious dogs** by stimulation of the vagus nerve in a surgically prepared skin loop. In anesthetized cats, coughs were elicited by electrical stimulation of the dorsolateral region in the upper medulla (Kasé et al. 1970).

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Effects on Tracheal Cells and Bronchial Mucus Secretion

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Contents

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In Vitro Studies of Mucus Secretion

Purpose and Rationale

Mucus secretion has been studied in isolated tracheas from ferrets and dogs (Borson et al. 1980; Kyle et al. 1987).

Procedure

Ferrets of either sex weighing 0.6–1.5 kg are anesthetized with sodium barbital intraperitoneally. The trachea is exposed and cannulated with a special Perspex cannula about 5 mm below the larynx. The animal is then sacrificed with an overdose of the anesthetic and the chest opened along the midline. The trachea is exposed to the carina, cleared of adjacent tissue, removed from the animal, and cannulated just above the carina. The trachea, with its laryngeal end down, is then mounted in a water-jacketed organ bath and bathed on its submucosal site with Krebs-Henseleit solution plus 0.1 % glucose at 37 °C and bubbled with 95 % O_2 and 5 % CO_2. The lumen of the trachea remains air filled. A plastic catheter is inserted into the lower cannula to form an airtight seal into which secretions can periodically be withdrawn and collected. Volumes of secretions are estimated by the weight difference of catheter lengths with and without secretions.

Simultaneous measurements of both mucus secretion and changes in tissue volume in vitro are achieved by mounting portions of ferret trachea cut longitudinally along the posterior wall, flattened out, and pinned to a Perspex chamber. Krebs-Henseleit solution at 37 °C and gassed with 95 % O_2 and 5 % CO_2 is circulated on the submucosal side of the tissue, while the luminal side is exposed to the atmosphere. The surface area of the exposed tissue is about 50 mm^2. Mucus secretion is promoted by electrical field stimulation at 50–100 V, 20 Hz, 1–2 ms duration, applied through the pins holding the tissue. Before the start of each experiment, surface fluid is gently wiped off from the luminal surface with a tissue pledglet. The epithelium is coated with a layer of powdered tantalum dust; as mucus secretion from submucosal glands occurs through gland ducts, the layer of tantalum effectively traps the secreted mucus above the duct and under the tantalum layer. Nearly hemispherical hillocks are formed. The surface is photographed at intervals through a dissecting microscope and hillock diameters measured. Assuming the hillocks to be hemispheres, the secretion volume per unit area is calculated. Drugs are added to the submucosal bath.

Evaluation

Secretory response after electrical stimulation in the presence or absence of drugs is recorded after 45, 90, and 135 min.

Modifications of the Method

Quinton (1979) used isolated tracheae from cats. A segment of the trachea was mounted in a chamber such that the serosal side was constantly bathed in Ringer solution, whereas the epithelial surface was coated with water-saturated paraffin oil. Secretion was stimulated by adding appropriate drug concentrations to the bath. Under a dissecting microscope, small droplets of secretory fluid were observed to form on the tracheal epithelial surface shortly after stimulation. Timed collection of droplets secreted from three to four glands were taken up between oil blocks in constant-bore capillaries (78 μm inner diameter), and droplet volumes were measured for rate determinations usually over a period of 5 min.

Critical Assessment of the Methods

Both modifications of the in vitro methods need at least as many animals and are as time consuming as the in vivo methods.

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Acute Studies of Mucus Secretion

Purpose and Rationale

Many diseases of the respiratory tract cause both qualitative and quantitative changes in the mucus that covers and protects the airway epithelium. To study the influence of drugs, methods of collecting bronchial mucus are necessary (Braga 1988). Perry and Boyd (1941) described a method for collecting bronchial mucus from the rabbit.

Procedure

Rabbits weighing 2.5–3.5 kg are anesthetized by intraperitoneal injection of 1.1–1.4 g/kg urethane. The trachea is exposed by blunt dissection and half opened, 2 cm below the cricoid cartilage. One arm of a T cannula with a large enough diameter to slightly distend the trachea is inserted into the trachea. The perpendicular arm is connected to an air outlet of a humidifier (temperature 35–38 °C, relative humidity 80 %). The other arm is connected to a collection tube. The rabbit is restrained in the supine position on a 60° inclined board with its head downward. Respiratory tract fluids are collected in centrifuge tubes at 1 h intervals. Mucus secretion can be stimulated by vagal stimulation or by ammonium chloride given by stomach tube or by pilocarpine given i.p.

Evaluation

Time response curves after stimulants of mucus secretion are compared with data from untreated animals.

Modifications of the Method

A method for collecting mucus from cats, using a segment of cervical trachea about 5 cm long isolated in situ, with nerve and blood supplies intact and a glass cannula inserted to each end, has been described by Gallagher et al. (1975).

A method to collect mucus from the upper tract trachea and the nasopharynx in dogs in acute experiments has been proposed by Proctor et al. (1973).

Engler and Szelenyi (1984) described a new method for screening mucosecretolytic compounds using tracheal phenol red secretion in **mice**. Phenol red at a dose of 500 mg/kg was injected intraperitoneally in male mice. Thirty minutes later, the animals were sacrificed by carbon dioxide. The whole trachea was dissected free from surrounding tissue and excised. Each trachea was washed for 30 min in 1 ml physiological saline. Afterward, 0.1 ml 1 M NaOH was added to the washing to stabilize the pH of the lavage fluid. The concentration of phenol red was measured photometrically. Agonists were

administered subcutaneously 15 min or intragastrally 30 min before phenol red was injected. Antagonists were given 5 min prior to the administration of agonists.

Other dyes such as Evans blue or sodium fluorescein also are reported to be eliminated in the respiratory tract fluid of mice (Graziani and Cazzulani 1981).

Dye methods reported for mice can also be used for rats (Quevauviller and Vu-Ngoc-Huyen 1966). Alcian blue was used to stain the normal bronchial tree. After chronic treatment with sulfur dioxide, there were changes in bronchial coloration. Administration of drugs protected against the effects of sulfur dioxide.

Secretion from tracheal submucosal glands can be studied in **dogs** (Davis et al. 1982; Johnson and McNee 1983, 1985). In anesthetized dogs the epithelial surface of the upper trachea is exposed and coated with powdered tantalum. Secretions from the submucosal gland ducts form elevations (hillocks) in the tantalum layer. The number of hillocks that appear in a 1.2 cm^2 field is counted.

A micropipette method for obtaining secretions from single submucosal gland ducts in vivo in **cat** tracheas has been described (Ueki et al. 1979, 1980; Leikauf et al. 1984). In anesthetized cats an endotracheal tube was inserted into the lower trachea and connected to a constant-volume respirator. The remainder of the trachea above the endotracheal tube was then dissected open by a midline incision. Paraffin oil equilibrated with HEPES buffer was then placed on the exposed mucosa to prevent drying and to aid visualization of the gland duct openings. The secretions from the gland duct openings were collected with constant-bore (99 μm ID) glass micropipettes. The volume and the viscosity of the secreted mucus were determined.

Critical Assessment of the Methods

For the methods of mucus collection in rabbits, cats, or dogs a rather high number of animals is necessary to achieve data suitable for statistical analysis. For screening procedures the methods using dye elimination into the trachea of mice or rats seem to be preferable.

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Studies of Mucus Secretion With Chronic Cannulation

Purpose and Rationale

Several techniques have been developed for chronic collection of mucus (Wardell et al. 1970; Yankell et al. 1970; Scuri et al. 1980).

Procedure

Beagle dogs weighing 9–11 kg are anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium. The cervical trachea is exposed by a midline skin incision and blunt dissection of the muscles. A segment approximately 10 rings in length, with an intact blood and nerve supply, is transected. The cephalic and caudal parts of the trachea are anastomosed end to end with interrupted gut sutures to reestablish a patent airway. The isolated segment is loosened slightly from the surrounding tissue and turned 180° to reverse cilia movement. A funnel-shaped silicone cannula is attached to the outer surface of the proximal end of the tracheal segment with surgical mesh and sutured in place. With cannulation completed, the tracheal segment is placed in a pocket below the sternohyoid muscle and the cannula brought to the surface and exteriorized through a stab wound. Alternatively, the isolated segment is closed at its caudal end with interrupted gut sutures. The mucosal surface of the cervical end of the isolated tracheal segment is sutured with interrupted silk sutures to the overlying subcutaneous tissue through a small incision in the cervical skin. Muscles and skin are sutured normally. In 2 or 3 weeks the skin heals over the small stoma resulting in a subcutaneous pouch of functioning tracheal tissue. Mucus samples can be collected for months. In this modification, a balloon can be placed into the pouch. Pressure changes in the balloon due to contraction of the smooth tracheal muscles after physostigmine injection or vagal stimulation or relaxation after atropine injection are recorded demonstrating parasympathetic innervation.

Evaluation

Parasympathomimetic stimulation (0.5 mg/kg pilocarpine s.c.) increases the flow rate of tracheal fluids. Pressure changes in the balloon after injection of parasympathomimetic or sympathomimetic drugs are compared with baseline values.

Modifications of the Method

Scuri et al. (1980) inserted a T-shaped cannula into the trachea of anesthetized rabbits. The wound was sutured, the third arm was connected with a collecting tube, and after 3 days of antibiotic administration, the mucus was collected at different times to establish basal production. For the experiments, mucus was collected during a 4 h control period, then drugs were given intravenously, orally, or as aerosol inhalation. Mucus was further collected during the periods of 0–4 and 4–24 h and analyzed for sialic acid, fucose, and protein content.

A tracheal pouch method in ferrets has been described by Barber and Small (1974).

Several authors published methods to determine **viscoelastic properties and rheological behavior of tracheal and bronchial mucus**: Philippoff et al. (1970), Lopez-Vidriero et al. (1977), Martin et al. (1980) Kim et al. (1982), Braga (1988), King (1988), Majima et al. (1990).

Critical Assessment of the Methods

The methods using pouches in dogs may be useful for physiological studies, but for pharmacological purposes the rabbit method of Scuri et al. (1980) seems preferable.

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Bronchoalveolar Lavage

Purpose and Rationale

Isolation of bronchial cells from bronchoalveolar lavage was described by Myrvik et al. (1961), Bassett et al. (1988), Fryer et al. (1994, 1997), and Wang et al. (1996).

Procedure

After determination of mechanical respiratory parameters in anaesthetized guinea pigs, bronchoalveolar lavage is performed via the tracheal cannula. The lungs are lavaged with 5 aliquots of 10 ml phosphate-buffered saline containing 3 mM EDTA and 100 μM isoproterenol (pH 7.2–7.4). The recovered lavage fluid (40–45 ml) is centrifuged, the cells are resuspended in 20 ml of phosphate-buffered saline, and total cells are counted using a hemacytometer. The remaining aliquot is centrifuged again and cells stained to determine cell differentials.

Evaluation

The differences in cells recovered from bronchoalveolar lavage between treatment groups are tested by use of a one-factor analysis of variance. $P < 0.05$ is considered significant.

Modifications of the Method

Gossart et al. (1996) determined TNF-α activity in the supernatant of bronchoalveolar lavage by the cytotoxicity against TNF-α-sensitive L929 murine fibroblasts.

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Ciliary Activity

Purpose and Rationale

Ciliary activity is a natural defense mechanism of the mucosa in the respiratory tract against harmful extraneous agents resulting in a continuous transportation of secreted mucus. Many attempts have been made to visualize and to quantify this phenomenon. Several authors used the light beam reflex method which can be used for in vivo as well as for in vitro experiments (Dalhamn 1956, 1964; Dalhamn and Rylander 1962; Hakansson and Toremalm 1963; Mercke et al. 1974; Baldetorp et al. 1976; Lopez-Vidriero et al. 1985).

Procedure

Rats are anesthetized by intraperitoneal injection of tribromo-ethanol (Avertin). The trachea is exposed and its soft parts incised by electrocoagulation so that bleeding is avoided. The cartilaginous rings are opened sufficiently to permit microscopy. The rat is immediately placed in a moist chamber. The trachea opening is linked to a microscope (Leitz Ultropak) by means of a rubber bellows which is fitted around the lens of the microscope and is made to embrace the trachea by means of a piece of rubber tubing that is slit along its length and secured to the bellows. The beam of an illuminating lamp is concentrated to a surface of about 1 mm^2. By placing a heat-reflecting filter in the path of the beam the rise in temperature can be reduced. For registration of the reflected light high-speed cameras with a speed of 220 exposures are used. Alternatively, the reflected light from the microscope is directed to a TV camera and amplified to be displayed on a TV screen. The frequency of ciliar beats is recorded over 1 h.

Evaluation

The beat frequency of treated animals is compared with that of controls.

Modifications of the Method

Mercke et al. (1974) described a stroboscopic method for standardized studies of mucociliary activity in rabbit tracheal mucosa.

Lierle and Moore (1935) inserted windows into anesthetized rabbits for observation of the ciliary activity in the maxillary sinus of living animals.

With modern equipment, a similar technique has been used by Hybbinette and Mercke (1982a, b, c), Lindberg and Mercke (1986), Lindberg et al. (1986), andMercke et al. (1987) to study the role of several pharmacological agents in the mucociliary defense of the rabbit maxillary sinus.

Corssen and Allen (1958) compared the toxic effects of various local anesthetic drugs on human ciliated epithelium in vitro by observation of rotating globes of human tracheal epithelium in tissue culture.

Cheung (1976) performed high-speed cinemicrographic studies on rabbit tracheal (ciliated) epithelia.

Iravani (1967, 1971; Iravani and Melville 1975) studied the ciliary activity in the intrapulmonary airways of rats by incident light microscopy.

Lee and Verdugo (1976)and Verdugo et al. (1980) recommended laser light-scattering spectroscopy for the study of ciliary activity.

Manawadu et al. (1978) studied the effects of local anesthetics on ciliary activity using ferret tracheal rings in vitro. The ferret possesses a long neck, and 100 or more tracheal rings can be obtained from a single ferret. The tracheal rings were maintained in sterile tubes. The ciliary activity was graded by determining the percentage of cilia beating on each ring, using transmitted light and the 10 × objective of an inverted microscope.

Rutland and Cole (1980) and Hesse et al. (1981) used a noninvasive method for obtaining nasal ciliated epithelium which is suitable for measurement of ciliary beat frequency.

Van de Donk et al. (1980) used isolated chicken embryo tracheas to measure the effects of preservatives on ciliary beat frequency. Maurer et al. (1982) studied the role of ciliary motility in acute allergic mucociliary dysfunction in cultivated ciliated cells from sheep.

Lopez-Vidriero et al. (1985) studied the effect of isoprenaline on the ciliary activity of an in vitro preparation of rat trachea.

Braga et al. (1986) described a simple and precise method for counting ciliary beats directly from the TV monitor screen using specimens of human ciliated epithelium obtained by brushing the nasal mucosa.

Curtis and Carson (1992) used pieces of human nasal epithelium for computer-assisted video measurement of ciliary beat frequency in vitro. Ciliary beat frequency was viewed with a microscope equipped with a phase-contrast objective. The microscopic image was recorded by a camera and data stored by a videorecorder. For measuring ciliary beating, tapes were displayed with amplification on a monitor. A photoelectric transducer was positioned over the video image of the cilia. Movement of the cilia interrupting the light path caused changes in light intensity recorded by the photocell transducer.

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Studies of Mucociliary Transport

Purpose and Rationale

Mucus flow has been studied in in vitro and in vivo experiments (Iravani 1971; Ahmed et al. 1979). The rate of mucus flow can be

estimated by measuring the time needed for certain particles to travel a known distance in the trachea. Numerous substances have been used such as charcoal particles (Dalhamn 1956), Teflon disks (Ahmed et al. 1979), and other pulverized materials (Deitmer 1989). The effect of local radioactivity on tracheal mucous velocity of sheep has been studied in vitro and in vivo by Ahmed et al. (1979).

Procedure

For in vitro experiments, sheep are sacrificed during anesthesia; the trachea is exposed, clamped, and resected just below the cricoid cartilage. The chest is opened and the trachea resected at the level of the carina. Then the trachea is slit open along the posterior membranous wall, pinned with gentle stretching on a board, and slanted upward at an angle of 25°. A metric ruler is placed along the board as a measuring reference. The board is then placed in a Plexiglas chamber with a constant temperature of 37 °C and 100 % humidity. Teflon disks are spread on the mucous layer. Tracheal mucus velocity is estimated by filming the movements of the radiopaque Teflon disks visualized on a television monitor connected to a camera. Disk motion is recorded for 1–2 min on a videotape, and the distance measured during the elapsed time is obtained from the videomonitor.

For in vivo measurements of tracheal mucus velocity, the roentgenographic method of Friedman et al. (1977)and Sackner et al. (1977) is used. The sheep are restrained, and their heads are immobilized with a sling. The nasal mucosa is sprayed with a 2 % lidocaine solution for topical anesthesia. A bronchofiberscope is inserted transnasally, and its tip is placed just below the vocal cords. Radiopaque Teflon disks 1.0 mm in diameter, 0.8 mm thick, and weighing 1.76 mg are blown through the inner channel of the bronchofiberscope onto the tracheal mucosa in a circumferential distribution. The cervical trachea containing the disks is visualized in the lateral projection with a television monitor. Disk motion is recorded on a videotape while the time is displayed on a digital clock. The disk image is marked to obtain the distance traveled. This distance is measured with a ruler, and the linear velocity of the disk is computed by dividing the distance by the elapsed time. This procedure is repeated for all disks in the field of view. To compute a mean tracheal mucus velocity, data from 10 to 15 disks are obtained in each filmed run.

Evaluation

Disk velocities measured in vitro or in vitro are compared before and after treatment.

Modifications of the Method

Mucociliary transport has been studied on the hard palate of decapitated frogs measuring the transport velocity of small particles, e.g., pieces of cork or charcoal (Kochmann 1930; Sadé et al. 1970).

Suzuki (1966) measured the movement of a standard object (1 mm^2 aluminum foil) on the ciliated surface of the palate of frogs.

Mucociliary clearance by the in vitro frog method was used by Leitch et al. (1985) to study the effects of ethanol.

Movement of poppy seeds in rabbit tracheal preparations was studied by Kensler and Battista (1966).

In chicken nasal mucosa the interaction between mucociliary transport and the ciliary beat was studied by Ukai et al. (1985).

Mucus transport in the respiratory tract of anesthetized cats was measured with uniform particles of lycopodium spores triturated with lamp black (Carson et al. 1966).

Mucociliary clearance velocities were determined by a radioisotopic method in dogs (Giordano et al. 1977, 1978).

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Culture of Tracheal Epithelial Cells

Purpose and Rationale

Marked morphological changes of the airway epithelium, up to severe damage, are frequently observed in inflammatory airway diseases and appear to play an important role in the pathogenesis of the broncho-obstructive symptoms (Webber and Corfield 1993; Hay et al. 1994). Freitag et al. (1996) studied the effects of lipopolysaccharides (LPS) and TNF-α on cultured rat tracheal epithelial cells and determined NO synthase activity.

Procedure

Trachea of newborn rats, cut into small pieces, are explanted with the epithelial surface downward onto 60 mm culture dishes (Lechner and LaVeck 1985) and cultured in low-calcium (60–80 µmol/l) RPMI-1640 medium at 37 °C and 5 % CO_2. The medium containing 16 % fetal calf serum is supplemented with epidermal growth factor and other growth-promoting factors, such as 80 ng/ml cholera toxin, 2.6 ng/ml estradiol, 180 ng/ml hydrocortisone, 2.5 µg/ml insulin, 12.5 µg/ml transferrin, 100 U/ml

penicillin, 100 µg/ml streptomycin, and 3.5 µg/ml amphotericin B to allow a selective outgrowth of epithelial cells (Emura et al. 1990). Confluent epithelial layers are obtained after about 6–10 weeks at which time all cells show a positive staining with a pancytokeratin antibody. Confluent cells of following passages are treated for four subsequent days with LPS (10 µg/ml) or TNF-α (500 U/ml).

The **morphology** of the cells is evaluated by daily inspection using a phase-contrast microscope with photographic documentation. Cell density in the culture is determined in each culture dish by counting daily the number of cells in four marked areas (each 2000 μm^2). For immunocytochemical staining, the cells are fixed by incubation in 100 % methanol at −20 °C for 20 min. The cells are washed with Ca/Mg-free phosphate-buffered saline and several areas marked off by nail varnish. A solution of the anticytokeratin (pan) antibody (monoclonal mouse IgG, Boehringer Mannheim) is added, incubated for 2 h at room temperature, and after washing incubated for 30 min with a secondary antibody (FITC-coupled, polyclonal rabbit antimouse IgG, Sigma). After washing, fluorescence microscopy is performed using a microscope with a FITC-specific filter combination. Unspecific fluorescence is excluded by performing the staining procedure in a different area of the same culture, but without the addition of the primary antibody.

For **determination of NO synthase activity**, confluent cultures are washed with oxygenated and prewarmed (37 °C) Krebs-HEPES medium. Then the cells are incubated for 1 h in the same medium containing 37 kBq ^{3}H-L-arginine (100 nmol/l). After collection of the supernatants the cells are extracted in 1 ml of 0.4 mol/l $HClO_4$ for 2 h at 0–4 °C.

By **HPLC analysis** ^{3}H-compounds (^{3}H-L-citrulline, ^{3}H-L-ornithine, and ^{3}H-L-arginine) in incubation media and cell extracts are separated on a reverse-phase column (length 250 mm, inner diameter 4.6 mm, prepacked with Shadon ODS-Hypersil, 5 mm) using as mobile phase 0.1 mmol/l sodium phosphate buffer (adjusted to pH 1.8) which contains octane sulphonic acid sodium salt (400 mg/l), Na_2EDTA (0.3 mmol/l), and methanol (6.25 % v/v) with a flow rate of 1 ml/min (Hey et al. 1995). The eluate is collected in 1 min fractions into counting vials. After addition of a commercial scintillation cocktail the radioactivity is determined by liquid scintillation spectrophotometry. External standardization is used to correct for counting efficiency. The retention time is determined by the use of ^{14}C-labeled (L-citrulline or L-ornithin ^{3}H-labeled (L-arginine) standards. Protein content in cells is determined by a commercially available assay.

Evaluation

The amounts of ^{3}H-L-citrulline in supernatants or cell extracts are expressed as DPM/µg protein. Changes in cell density are expressed as % of the density observed in each culture dish at the start of the experiment. Mean values are given ± SEM. The significance of difference is evaluated by Student's *t*-test.

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Alveolar Macrophages

Purpose and Rationale

Alveolar macrophages have been used for various purposes. A rat pulmonary alveolar macrophage cell line (NR8383) was initiated in culture by Helmke et al. (1987) in the presence of a gerbil lung conditioned medium and has been propagated continuously in culture. Sun et al. (1999) tested the inhibition of Ca^{2+} influx by pentoxifylline in NR8383 alveolar macrophages.

Procedure

Cell Culture

The NR8383 alveolar macrophage cell line is grown in plastic tissue culture flasks in Ham's F12 medium containing 15 % fetal bovine serum, 100 g/ml penicillin, and 100 U/ml streptomycin sulfate. The alveolar macrophages are cultured at 37 °C in an atmosphere of 5 % CO_2 in air. The medium is routinely changed twice weekly. The viability of alveolar macrophages is routinely measured by trypan blue exclusion before and 1.5 h after fura-2 loading, when all $[Ca^{2+}]_i$ determinations are completed.

Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ is determined using the Ca^{2+}-sensitive fluorescent indicator fura-2 (Zhang et al. 1997; Mörk et al. 1998). NR8383 alveolar macrophages are loaded with fura-2 by incubation in PSS containing 2 mM (final concentration) fura-2/AM and 0.01 % BSA for 20 min at 37 °C. The cells are then rinsed twice with PSS containing 0.01 % BSA and resuspended in the same medium (1.75×10^6 cells/ml). For $[Ca^{2+}]_i$ determination, a 2-ml aliquot of fura-2-loaded cells is centrifuged at 50 *g* for 2 min, resuspended in PSS containing 0.01 % BSA, and placed in a 4-ml cuvette. $[Ca^{2+}]_i$ is measured at 37 °C using a PTI Deltascan fluorometer (PTI, South Brunswick, N.J., USA). The excitation wavelengths used are 340 and 380 nm and the emission wavelength 505 nm. Calibration of $[Ca^{2+}]_i$ is performed for each measurement trace. Then, 1 mM $CaCl_2$ (for Ca^{2+}-free medium) and 50 mM ionomycin are added to obtain the limiting ratio for the Ca^{2+}-saturated form (R_{max}) of fura-2. Then, 0.0005 % digitonin and 10 mM EGTA are added to obtain the limiting ratio for the free form of fura-2 (R_{min}). Fluorescence ratios of the 340- and 380-nm excitation and 505-nm emission are converted to $[Ca^{2+}]_i$ according to Grynkiewicz et al. (1985) using 224 nM as the K_d of fura-2 for Ca^{2+}.

Measurement of Store Depletion–Activated Ca^{2+} Influx

The Ca^{2+} influx activated by depletion of the inositol 1,4,5-trisphosphate- (IP_3-)-sensitive intracellular store is measured according to Zhang et al. (1997) and Zhu and Birnbaumer (1998)). Cells are exposed to either ATP or thapsigargin (TG) in Ca^{2+}-free medium for 5 min, and 1 mM Ca^{2+} is then added to initiate Ca^{2+} influx. The initial linear portion of $[Ca^{2+}]_i$ changes after addition of Ca^{2+} is used to calculate Ca^{2+} influx rate (nM/min).

Evaluation

Results are presented as the mean ± SEM of separate determinations using different cell preparations. Comparisons are made using the unpaired Student's *t*-test or the analysis of variance. P values <0.05 are considered significant.

Modifications of the Method

Sirois et al. (2000) studied the influence of histamine in the cytokine network in the lung through

H_2 and H_3 receptors. Alveolar macrophages from humans, Sprague Dawley rats, and the alveolar macrophage cell line NR8383 were treated with different concentrations of histamine prior to their stimulation with suboptimal concentrations of lipopolysaccharide (LPS). Release of tumor necrosis factor (TNF) and interleukin-10 (IL-10) was measured.

Using the rat alveolar macrophage cell line NR8383, Gazin et al. (2004) found that uranium induces TNF-α secretion and activates the p38 - mitogen-activated protein kinase (p38 MAPK).

Yang et al. (2004) studied in mice the synergy between a signal transducer and activator of transcription 3 and retinoic acid receptor-α in the regulation of the surfactant protein B gene in the lung.

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Binding Tests in Respiratory System

Kristy D. Bruse

Contents

Histamine (H_1) Receptor Binding

Purpose and Rationale

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H_1 receptor couples mainly to $G_{q/11}$, thereby stimulating phospholipase C, whereas the H_2 receptor interacts with G_s to activate adenylyl cyclase. The histamine H_3 and H_4 receptors couple to G_i proteins to inhibit adenylyl cyclase and to stimulate MAPK (Hough 2001).

Histamine is considered to play a major role in asthmatic attacks (Bryce et al. 2006). H_1 antagonists have been used since decades as therapeutic agents. This assay is used to determine the affinity of test compounds to the histamine H_1 receptor by measuring their inhibitory activities on the binding of the H_1 antagonist ^{3}H-pyrilamine to a plasma membrane preparation from guinea pig brain.

Procedure

Brains from guinea pigs are homogenized in ice-cold Tris buffer (pH 7.5) in a Potter homogenizer (1 g brain in 30 ml buffer). The homogenate is centrifuged at 4 °C for 10 min at 50,000 g. The supernatant is discarded, the pellet resuspended in buffer, centrifuged as before, and the final pellets

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resupended in Tris buffer (1 g fresh weight/5 ml). Aliquots of 1 ml are frozen at −70 °C.

In the competition experiment, 50 µl ^{3}H-pyrilamine (one constant concentration of 2×10^{-9} M), 50 µl test compound (>10 concentrations, 10^{-5}–10^{-10} M) and 100 µl membrane suspension from guinea pig whole brain (approx. 10 mg wet weight/ml) per sample are incubated in a shaking bath at 25 °C for 30 min. Incubation buffer: 50 mM Tris–HCl buffer, pH 7.5.

Saturation experiments are performed with 11 concentrations of ^{3}H-pyrilamine ($0.1–50 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer; nonspecific binding is determined in the presence of mepyramine or doxepin (10^{-5} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free radioactivity. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

Evaluation of Results

The following parameters are calculated:

- Total binding of ^{3}H-pyrilamine
- Nonspecific binding: binding of ^{3}H-pyrilamine in the presence of mepyramine or doxepin
- Specific binding = total binding–nonspecific binding
- % inhibition of ^{3}H-pyrilamine binding: 100–specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-pyrilamine versus nonlabeled drug by a computer-supported analysis of the binding data.

Modifications of the Method

De Backer et al. (1993) reported genomic cloning, heterologous expression in COS-7 cells, and pharmacological characterization of a human H_1-receptor.

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Muscarinic Receptor Binding

Purpose and Rationale

Muscarinic receptors in the airways are important in both the normal physiology and the pathophysiology of pulmonary function. Acetylcholine

released from parasympathetic nerve terminals causes contraction of airway smooth muscle. Animals with asthma or other chronic inflammation of the airways exhibit hypersensitivity of the airways to muscarinic agonists, and muscarinic antagonists are used therapeutically in patients with asthma and chronic obstructive pulmonary disease (Nathanson 2000). Muscarinic receptors are present in neurons in the central and peripheral nervous system, cardiac and smooth muscles, and a variety of exocrine glands. Mammals possess genes encoding five different subtypes of mAChR, termed M_1–M_5, which can be divided into two broad functional categories: the M_1, M_3, and M_5 receptors preferentially couple to the Gq family of G-proteins whereas the M_2 and M_4 receptors preferentially couple to the Gi family of G-proteins.

For involvement of acetylcholine receptors in the gastrointestinal tract see chapter "▶ Pharmacological Effects on Gastric Function".

Several papers deal with the distribution of muscarinic receptors in the lung, their role in pulmonary disease, and the use of muscarinic antagonists for the treatment of obstructive airway disease (Mak and Barnes 1990; Barnes 1993, 2001, 2004; Disse 2001; Disse et al. 1993, 1999; Haddad et al. 1994; Barnes et al. 1995, 1997; Patel et al. 1995; Peták et al. 1996; Alabaster 1997; Matsumoto 1997; Chelala et al. 1998; Hislop et al. 1998; Okazawa et al. 1998; Wale et al. 1999; Rees 2002; Sarria et al. 2002; Tohda et al. 2002; Costello et al. 2006).

Hirose et al. (2001) described the pharmacological properties of a muscarinic antagonist with M_2-sparing antagonistic activity.

Procedure

Binding Affinity for Human and Rat Muscarinic Receptor Subtypes

In competition studies, specific binding of [^{3}H]*N*-methylscopolamine (NMS; New England Nuclear, Boston, Mass., USA) was determined using membranes from Chinese hamster ovary (CHO) cells expressing cloned human m1, m2, m3, m4, or m5 receptors (Receptor Biology, Baltimore, Md., USA), rat m1 or m3 receptors (American Type Culture Collection, Manassas, Va., USA), and rat heart tissue. These CHO cells expressing cloned rat m1 or m3 receptors and rat heart tissue were homogenized in 3 vols of 50 mM Tris–HCl (pH 7.4) and 1 mM EDTA containing 20 % sucrose with a Polytron PT-10. The homogenates were centrifuged at 10,000 g for 30 min at 4 °C. The supernatants were centrifuged at 100,000 g for 60 min at 4 °C. The pellets were suspended in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ and centrifuged at 100,000 g for 60 min at 4 °C. The pellets were resuspended in the abovementioned buffer (25 mg/ml for CHO cells expressing cloned rat m1 or m3 and 50 mg/ml for rat heart tissue) and stored at −80 °C as membrane preparations. In the binding assay, the membrane preparations were incubated with 0.19 to 0.2 nM [^{3}H]NMS in 50 mM Tris–HCl, 10 mM $MgCl_2$, and 1 mM EDTA (pH 7.4) for 2 h at room temperature. Final protein concentrations were 22 µg/ml (human m1), 70 µg/ml (human m2), 54 µg/ml (human m3), 20 µg/ml (human m4), 116 µg/ml (human m5), 481 µg/ml (rat m1 and m3), and 2500 µg/ml (rat heart). Assays were performed in a total volume of 500 µl. Nonspecific binding was measured in the presence of 1 µM NMS and was less than 2 % of total binding. Free and membrane-bound [^{3}H]NMS were separated by filtration over glass filters (UniFilter-GF/C; Packard Instruments, Meriden, Conn., USA) using a cell harvester (Filtermate 196; Packard Instruments). Radioactivity was counted by a liquid scintillation counter (TopCount; Packard Instruments).

Evaluation

For saturation studies, membranes from CHO cells expressing human m3 were incubated with an increased concentration of [^{3}H]NMS (0.1–3.2 nM) in the presence or absence of 10 nM compound A, and specific binding of [^{3}H]NMS was determined after incubation for 2 h.

Competition binding data were analyzed by a nonlinear regression fitting program using

GraphPad Prism Software (San Diego, Calif., USA). Saturation binding data were transformed to make a Scatchard plot and analyzed by a linear regression fitting program using GraphPad Prism Software.

The K_i values were calculated from the IC_{50} values by using the following equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where K_d is the dissociation constant of [^{3}H]NMS in each receptor subtype and $[L]$ is the concentration of [^{3}H]NMS (Cheng and Prusoff 1973). K_d values of [^{3}H]NMS in each receptor subtype were determined by Scatchard plot analysis. The K_d and B_{max} values below were used in this study. Data of human cloned receptors were extracted from Receptor Biology's Product Information Sheets (Receptor Biology).

Human m1 receptor	K_d = 51 pM	B_{max} = 1.28 pmol/mg of protein
Human m2 receptor	K_d = 290 pM	B_{max} = 1 pmol/mg of protein
Human m3 receptor	K_d = 86 pM	B_{max} = 0.65 pmol/mg of protein
Human m4 receptor	K_d = 56 pM	B_{max} = 1.44 pmol/mg of protein
Human m5 receptor	K_d = 200 pM	B_{max} = 0.59 pmol/mg of protein
Rat m1 receptor	K_d = 62 pM	B_{max} = 0.039 pmol/mg of wet weight
Rat m2 receptor	K_d = 210 pM	B_{max} = 0.0090 pmol/mg of wet weight
Rat m3 receptor	K_d = 72pM	B_{max} = 0.019 pmol/mg of wet weight

In saturation studies, the K_i value was calculated using the following equation:

$$K_i = K_d/\left(K'_d - K_d\right) \times [C]$$

where K'_d or K_d is the dissociation constant of [^{3}H]NMS in human m3 receptors in the presence or absence of an inhibitor, respectively, and $[C]$ is the concentration of the test drug (Nishikibe et al. 1999).

Modifications of the Method

Struckmann et al. (2003) investigated the role of muscarinic receptor subtypes in the constriction of peripheral airways by studies on receptor-deficient mice.

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Part V

Psychotropic and Neurotropic Activity

Effects on Behavior and Muscle Coordination

Mary Jeanne Kallman

Contents

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Spontaneous Behavior

General Considerations

The effects of drugs on the central and peripheral nervous systems can be easily recognized in normal animals. This does not necessarily mean that these effects can be used in therapy. Observing the global effects of drugs during LD_{50} determinations, pharmacologists can detect psychotropic activity. Only if these effects occur also in doses considerably below the LD_{50} are further evaluations justified. This basic experience resulted in the development of a variety of observational tests and activity measurements.

Observational Assessment

Purpose and Rationale

A systematic, quantitative procedure assessing the behavioral state of mice for the evaluation of drugs has been described by Irwin (1964, 1968). The method is applied in the beginning of pharmacological screening to detect psychotropic activities. It allows to identify and differentiate the profile pattern of various classes of pharmacological agents. Furthermore, observational assessment allows into the safety and potential toxicity profile of a new drug.

Procedure

Mice of either sex (NMRI strain) with a weight between 18 and 22 g are kept under standard

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laboratory conditions 5 days before the experiment. Animals tested for oral administration have water ad libitum but are deprived of food 16 h before the test. Animals for intravenous or intraperitoneal administration have access to food and water ad libitum until the test. For unknown substances the following doses are given:

- i.v. administration: 100, 200, 400 mg/kg
- i.p. administration: 300, 600, 1200 mg/kg
- Oral administration: 500, 1000, 2000 mg/kg

Three animals are used for each dose. One group of three animals receiving the vehicle only serves as control group.

Immediately after drug administration, the animals are closely observed for 2 h (following i.v. or i.p. administration) and for 5 h (following oral administration) by a "blind" observer. The following parameters are checked and compared to the vehicle control group Table 1.

Depending on the route of administration, the observations are performed at different time intervals: after intravenous and after intraperitoneal administration during the first 30 min, and after 1 and 2 h; after oral administration during the first 60 min, and after 2 and 6 h. The number of deaths is counted during the first 24 h and after 7 days in order to evaluate acute and late toxicity. Arbitrary scores are chosen for each symptom. If positive effects are seen with the lowest dose, the experiment is repeated with lower doses being decreased by a factor of 3.

Table 1 Effects of tests

Effects on CNS	Effects after manipulations I
Spontaneous motor activity	Auditory stimulus response
Restlessness	Escape after touch
Grooming behavior	Righting reflex
Squatting	Paresis of hind limbs
Staggering	Paresis of forepaws
Ataxic gait	Catalepsy in induced positions
Lying flat on the belly	Effects on reflexes
Lying flat on the side	
Lying flat on the back	Pinna reflex
Sleeping	Corneal reflex
Narcosis	Pain following stimulation
Bizarre behavior	
Timidity	Effects on autonomic nervous system
Straubs's phenomenon	
Writing	Pupil diameter
Tremors	(Constriction or dilatation)
Twitches	Eyelids
Opisthotonus	(Closure or exophthalmus)
Clonic convulsions	Secretion of sweat
Tonic convulsions	Salivations
Rolling and jumping convulsions	Lacrimation Cyanosis Piloerection Defecation Urination

Critical Assessment of the Test

The test has been used by almost every laboratory in the world involved in screening of potential new drugs. Almost every laboratory has introduced its own modifications. In particular, the scores and the calculations differ from one laboratory to the other. Additional tests, such as grip strength or rotarod, described below, have been included into the primary screen. Graph-bar profiles have been established for known drugs in order to rate new substances accordingly. The test is definitively a useful tool for primary screening resulting in hints on psychotropic activity but also for actions on other systems. Nevertheless, this test cannot substitute for more sophisticated tests for the evaluation of psychotropic activity.

Modifications of the Method

Based on the guidelines of the United States Environmental Protection Agency (USEPA) (1991), Mattsson et al. (1996) described a performance standard for clinical and functional observational battery (FOB) for examinations of **rats.** The performance standard was an idealized composite of FOB data from experienced laboratory personnel; each person tested on a separate set of four groups of rats. The rats were examined in random order, and treatments were either (a) saline, (b) chlorpromazine, (c) atropine, followed by physostigmine, or (d) amphetamine.

Testing of neurotoxicity with new pharmaceuticals was reported by Haggerty (1991). He proposed a primary screen for rodents, consisting of a functional observation battery and an automated test for motor activity. In addition, a functional observation battery for dogs was developed. The rat tier I screen assesses such functions as home cage and open field activity, stimulus reactivity, and neuromuscular function. The dog tier I screen emphasizes evaluation of gait, postural reactions, and reflex function.

Rambert (2000) underlined the importance of some general conditions for general pharmacology of the CNS, such as choice of the animal species, administration route, and controls of the experimental context (surroundings, temperature, schedules of the trials, interference with food intake episodes, influence of noises and smells, expertise of the experimental staff).

Crawley and Paylor (1997), Crawley (2000) proposed a test battery and behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Several neurological and neuropsychological tests are described which can be used as first screen for behavioral abnormalities in mutant mice. Multiple behavioral paradigms are included for several categories, such as neurological reflexes, sensory abilities, motor functions, learning and memory, feeding, sexual behavior, analgesia, aggression, anxiety, depression, schizophrenia, and drug abuse.

Detailed procedure of the Irwin test performed in rats is given by Porsolt (2006).

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Safety Pharmacology Core Battery

Purpose and Rationale

Regulatory agencies (EMA 1997, 2000) have pointed out the necessity to perform safety pharmacology studies before application of a new drug to human beings and to assess most carefully any potential side effects. Organs or systems acutely necessary for life, i.e., the cardiovascular,

respiratory, and central nervous systems, are considered to be the most important to assess in safety pharmacology studies. The following parameters of CNS activity should be assessed appropriately: motor activity, behavioral changes, coordination, sensory/motor reflexes, and body temperature.

Detailed descriptions of tests are found for observation tests:

Observational Assessment

Motor activity and behavior:

Method of Intermittent Observations
Open Field Test
Hole-Board Test
Combined Open Field Test

Coordination:

Inclined Plane
Chimney Test
Grip Strength
Rotarod Method

Sensory/motor reflexes:

Influence on Polysynaptic Reflexes
Masticatory Muscle Reflexes

Follow-up studies for CNS safety pharmacology are recommended, such as behavioral pharmacology; learning and memory; specific ligand binding; neurochemistry; and visual, auditory, and/or electrophysiology examinations. Detailed descriptions of these tests are found in the respective chapters, e.g.,

EEG Analysis From Rat Brain by Telemetry

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Effects on Motility (Sedative or Stimulatory Activity)

General Considerations

A survey on methods to evaluate depressants of the central nervous system has been published by Turner (1965). Many of these tests are still valid in spite of the fact that new classes of drugs have been introduced since that time which have not only augmented the therapeutic armamentarium but also changed the battery of pharmacological tests.

Sedative properties of drugs are tested mostly in mice or rats. Their spontaneous motor activity depends on various factors, such as the social situation (one or more animals), familiarity with the test environment, light, and temperature. The term "spontaneous motor activity" includes different types of movements, such as locomotion, rearing, sniffing, grooming, eating, and drinking. These phenomena can be well recognized by a skilled observer but are difficult to record over long periods of time and to quantitate. Therefore, besides procedures based on observation many methods for automatic registration have been developed. Almost every pharmacologist working in this field has designed his/her own apparatus. Several attempts have been made to measure not only simple locomotion but also rearing and other types of movement. The conditions to characterize drug effects by measuring locomotor activity have been surveyed by Kinnard and Watzman (1966), Geyer (1990).

A special phenomenon, called "thigmotaxis," which means that rats have the tendency to remain close to the walls of the cage, has been described by Barnett (1963). Moreover, methods to measure curiosity have been recommended. Only a few examples of prototypic methods and equipment can be given.

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Method of Intermittent Observations

Purpose and Rationale

The method described by Ther (1953) was designed to study stimulant and sedative drugs. The use of three mice per group implied a special social situation of the animals. For testing sedative activity, the mice are additionally treated with a stimulant, and for testing stimulant activity, with a sedative drug. Each group of mice is observed repeatedly only for a short period of time during 1 h and compared to a control group by a "blind" observer.

Procedure

Mice of either sex with an average weight of 25 g are deprived of food and water for 24 h before the test. To avoid any influence of the circadian rhythm the experiment is performed only between 8:00 and 12:00 A.M. Twelve animals are divided into groups of three mice. One group of three mice serves as control group; the other groups receive different doses of the test drug intraperitoneally. For testing sedative activity, the mice are injected after 10 min subcutaneously with 0.5 mg/kg methamphetamine. For testing stimulant activity, the mice are treated with 800 mg/kg paraldehyde. Ten minutes afterward, each group is placed into a glass jar of 12 cm diameter and 20 cm height which in turn is placed in a wooden box of 130 × 50 × 30 cm. The glass jars are illuminated from above. Ten minutes after administration of methamphetamine or paraldehyde the observation is started.

Evaluation

During 1 h the observer looks every minute for 1 s to each jar and registers if none, one, two, or all three mice show any characteristic change in locomotion, rearing, grooming, or sniffing. The maximum count in 1 h would be 180. Generally, activity decreases within 1 h. Nevertheless, methamphetamine-treated animals have a total count between 120 and 150. Groups treated with an effective sedative drug show a dose-dependent decrease of total counts. The number of counts in the treated groups is calculated as percentage of controls. From dose–response curves ED_{50}-values can be calculated.

Critical Assessment of the Method

The method gives reliable results for sedative drugs and for compounds with central depressant activity, such as antihistaminics, neuroleptics, and hypnotics. The disadvantage of the method lies in the fact that a skilled and trained observer is needed in order to get reproducible results. Therefore, several attempts have been made to automatize the method of intermittent observation.

Modifications of the Method

Schaumann and Stoepel (1961) followed the principle of intermittent observation using a camera mounted above small wire cages (10 × 10 cm). The mice in the cages were photographed with an exposure time of 3 s every 7,5 min over a period of 2,5 h. Mice without movements give clear pictures, calculated as zero. Slight movements induce blurred contours, calculated as 1 point, and major movements give completely blurred pictures, calculated as 2 points. Normal mice show 20 or less points during 20 observations. Mice with more than 20 points are considered to be stimulated, mice with less than 5 points to be

sedated. In this way ED_{50} values can be calculated.

Vogel and Ther (1963) published an apparatus with automatic registration of intermittent observations. Eighteen cages are used which have a freely movable bottom with minimal weight which is supported by springs. A small permanent magnet attached below the floor induces an electrical current in a coil if the bottom is moved. Two mice are brought into each cage. The control device registers during a variable period between 0.5 and 3 s if the bottom of one cage is moved or not. Within 1 min, all 18 cages are registered successively. The number of movements within 1 h is recorded for each cage. The sensitivity of the electric induction system is variable. A calibration is possible which picks up every movement of the animal, however, does not register the movements due to breathing. Dose–response curves can be established for stimulant and sedative drugs.

A similar apparatus was used by Hirabayashi and Tadokoro (1993) and Namina et al. (1999). The ambulatory activity of mice was measured by the tilting cage method using the ambulometer model AMBM1 (O'Hara, Tokyo, Japan).

Meyer (1962) measured the time until the exploration motility decreased to one-third of the initial value.

Koek et al. (1987) used the principle of intermittent observations for various activities of rats such as locomotion, rearing, sniffing, licking, gnawing, grooming, loss of righting, Straub tail, etc. in order to compare the behavioral effects of drugs.

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Open Field Test

Purpose and Rationale

Interruption of light beams as a measure of movements of rats or mice in a cage ("open field") has been used by many authors such as Dews (1953), Saelens et al. (1968), and Nakatsu and Owen (1980). Recently developed devices allow to register not only general motor activity but also locomotion, rearing, and the speed of locomotion (Barros et al. 1991; Ericson et al. 1991).

Procedure

The rats are observed in a square open field arena (68 × 68 × 45 cm) equipped with two rows of eight photocells, sensitive to infrared light, placed 40 and 125 mm above the floor, respectively. The photocells are spaced 90 mm apart, and the last photocell in a row is spaced 25 mm from the wall. Measurements are made in the dark in a ventilated, sound-attenuating box. Interruptions of

photocell beams can be collected by a microcomputer and the following variables evaluated:

- Motor activity: All interruptions of photo beams in the lower rows
- Peripheral motor activity: Activation of photo beams in the lower rows, provided that the photo beams spaced 25 mm from the wall were also activated
- Rearing: All interruption of the photo beams in the upper rows
- Peripheral rearing: Interruption of photo beams in the upper rows, provided that the photo beams spaced 25 mm from the wall were also activated
- Locomotion: Successive interruptions of photocells in the lower rows when the animal is moving in the same direction
- Speed: The time between successive photo beam interruptions during locomotion collected in 0.1 s categories

Adult male Sprague Dawley rats with a weight between 280 and 320 g are used. Drugs are injected subcutaneously 10–40 min. before test. The rats are observed for 15 min, whereby counts per min. are averaged for 3 min intervals.

Evaluation

Dose–response curves can be obtained for sedative and stimulant drugs, whereby the various parameters show different results. The effects of various doses are compared statistically with the values of controls and among themselves.

Critical Assessment of the Method

Measurement of several parameters in an open field device allows to differentiate between various types of sedative or stimulant drugs, but these differences can only be detected if dose–response curves are obtained for each parameter.

Modifications of the Method

Besides interruption of light beams, devices based on capacitance systems such as Animex (Columbus Instruments, Ohio, USA) and Varimexhave been developed and are widely used (Crunelli and Bernasconi 1979; Liu et al. 1985; Laviola and Alleva 1990; Honma et al. 1990; Magnus-Ellenbroek and Havemann-Reinicke 1993; Dauge et al. 1995; Petkov et al. 1995; Surmann and Havemann-Reinicke 1995; Gillies et al. 1996; Irifune et al. 1997; Ghelardini et al. 1998).

Nikodijevic et al. (1991) studied the behavioral effects of A_1- and A_2-selective adenosine agonists and antagonists in mice using a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The monitor included multiple activity monitor cages (40 × 40 × 30.5 cm), each of which was surrounded by horizontal and vertical sensors not detectable by the rodent.

Steiner et al. (1997) found that D_3 receptor-deficient mice enter the center of an open field significantly more than their littermates suggesting an anxiolytic-like effect of the D_3 receptor mutation.

Vorhees et al. (1992) described a locomotor activity system for rodents. The system consists of a black, ventilated test chamber, internally lighted with a ceiling-mounted video camera. The camera's image is transmitted to a contrast-sensitive tracker which maps the point of highest contrast and relays the digitalized coordinates to a PC. Dedicated software stores the information and simultaneously displays a map of the tracked subject.

Rex et al. (1996, 1998) described a modified open field test sensitive to anxiolytic drugs. Food-deprived rats were placed in one corner of the open field containing food in the center. The number of rats beginning to eat within the first 5 min was increased by known anxiolytic drugs.

Several authors used computerized systems, based on interruptions of infrared light beams, on magnetic field or on video analysis such as the VideoMot2 system developed by TSE Systems, Bad Homburg, Germany, or systems available through Bilaney Consultants Ltd., St Julians, Sevenoaks, Kent, UK (Sillaber et al. 1998; Wellmer et al. 2000; Kuzmin et al. 2003; Sienkiewicz-Jarosz et al. 2003; Strekalova et al. 2005).

Ströhle et al. (1997) mounted a video camera connected to a computer directly above six

locomotor boxes (60 × 60 cm each). The distance traveled by the rat in a 20-min interval was determined with a video digitizer (TSE Systems, Bad Homburg, Germany).

Spooren et al. (2000), Stobrawa et al. (2001), Heijtz et al. (2002, 2004), Abo-Salem et al. (2004), Bilkei-Gorzo et al. (2004) measured motor activity (locomotion and rearing) in several animals (rats or mice) simultaneously by means of a multibox detection system (ActiMot; TSE Systems, Bad Homburg Germany). This system use individual photocell activity units (48 × 48 cm) connected to a control unit. Each unit consists of a base frame with two pairs of light-barrier strips (each with 16/32 pairs of infrared transmitter and receiver diodes set at a distance of 28/14 mm; XY-coordinate). An additional pair of light-barrier strips (Z-coordinate) is used to detect rearing activity. Data for the number and sequence of photocell interruptions were collected on a computer.

A novel method for counting spontaneous motor activity in rats was proposed by Masuo et al. (1997). In the "Supermex" system, a sensor detects the radiant body heat of an animal.

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Hole-Board Test

Purpose and Rationale

The evaluation of certain components of behavior of mice such as curiosity or exploration has been attempted by Boissier et al. (1964) and Boissier and Simon (1964). They used an open field with holes on the bottom into which the animals could poke their noses. The "planche à trous" or "hole-board" test has become very well recognized and has been modified and automatized by many authors.

Procedure

Mice of either sex (NMRI strain) with a weight between 18 and 22 g are used. The hole-board has a size of 40 × 40 cm. Sixteen holes with a diameter of 3 cm each are distributed evenly on the floor. The board is elevated so that the mouse poking its nose into the hole does not see the bottom. Nose-poking is thought to indicate curiosity and is measured by visual observation in the earliest description and counted by electronic devices in more recent modifications. Moreover, in the newer modifications motility is measured in addition by counting interruption of light beams. Usually, six animals are used for each dose and for controls. Thirty minutes after administration of the test compound the first animal is placed on the hole board and tested for 5 min.

Calculation

The number of counts for nose-poking of treated animals is calculated as percentage of control animals.

Critical Assessment of the Method

Poking the nose into a hole is a typical behavior of mice indicating a certain degree of curiosity. Evaluation of this component of behavior has been proven to be quite useful. Benzodiazepines tend to suppress nose-pocking at relatively low doses.

Modifications of the Method

A hole-poke measuring system is commercially available from TSE Systems, Bad Homburg, Germany.

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Combined Open Field Test

Purpose and Rationale

The simultaneous determination of locomotion and curiosity by using a modification of the hole-board test and a photo-beam system has been proposed as a relatively simple test (Weischer 1976). Several types of such equipment are commercially available.

Procedure

Male mice (NMRI strain) with an average weight of 30 g are used. Each animal is tested individually in an automated open-field box which consists of a black Plexiglas cage (35 × 35 × 20 cm) with a post (8 × 8 × 20 cm) in the center of the cage. Two evenly spaced photocell beams perpendicular to the wall and 2 cm above the floor divide the box into four compartments. Every photocell beam interruption is registered automatically as an activity count. Each wall of the cage contains four evenly spaced 2 cm diameter holes in a horizontal array 7 cm above the floor. A row of four photocell beams is mounted 1 cm outside of the holes and automatically records every exploratory nose-poke. Thirty min. after intraperitoneal and 60 min. after oral administration of the test compound the animal is placed into the cage and the behavior recorded for a period of 5 min. Ten mice are used for each dose as well as for controls.

Evaluation

Counts for motility (interruption of photocell beams inside the cage) and for curiosity (interruption of photocell beams outside the cage due to nose-poking) are recorded individually. The mean values of the treated groups are expressed as percentage of the control group. Using different doses, dose–response curves can be obtained.

Critical Assessment of the Method

A dissociation between exploratory behavior and locomotion has been found with several drugs. Even well-known stimulants can reduce exploratory behavior with a concomitant increase in locomotion. Depending on the dose, tranquilizers can reduce exploration without affecting locomotion. Due to the modifications of the equipment the results of different authors are often difficult to compare.

The limitations of photocell activity cages for assessing effects of drugs were discussed by Krsiak et al. (1970).

Modifications of the Test

Geyer (1982) described a similar device and reported different effects of amphetamine, caffeine, apomorphine, and scopolamine. Adams and Geyer (1982) used this device to study the LSD-induced alterations of locomotor patterns and exploration in rats.

Geyer et al. (1986) described a behavioral monitor which was designed to assess the spatial and temporal sequences of locomotor movements in rats.

Other authors, e.g., Wolffgramm et al. (1988), used video recording of the movements of mice in the evening hours at low illumination and counted the locomotor activity as the number of field crossings and the exploratory activity as the number of rearings.

Ljungberg and Ungerstedt (1977) designed a testbox for the automatic recording of eight components of behavior in rats including compulsive gnawing induced by apomorphine.

Matsumoto et al. (1990) described a system to detect and analyze motor activity in mice consisting of a doughnut-shaped cage with 36 units of detectors radially arranged from the center of the cage. Each detector unit consisted of four pairs of photosensors (higher-, lower-, inner-, and outer-position sensors).

Schwarting et al. (1993) described a video image-analyzing system for open-field behavior which measures turning behavior, thigmotactic scanning, and locomotion in rats.

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EEG Analysis from Rat Brain by Telemetry

Purpose and Rationale

Field potential analysis of freely moving rats by radioelectroencephalography from different brain areas has been developed as a sensitive method in pharmacology (Dimpfel et al. 1986, 1989, 1990, 1992; Kropf et al. 1991).

Procedure

Male adult rats are implanted with four stainless steel electrodes, mounted on a base plate, into the frontal cortex, striatum, thalamus, and reticular formation. The plate carries a microplug for a four-channel radiotransmitter. During the experimental sessions, which start 2 weeks after surgery, the transmitted field potentials are analyzed in real time using Fast Fourier Transformations. The resulting power density spectra are segmented into six frequency bands, each representing the integrated power over a certain frequency band.

Evaluation

The data from three predrug periods of 15 min each are compared with those from continuously monitored 15-min postdrug periods.

Modifications of the Method

De Simoni et al. (1990) developed a miniaturized optoelectronic system of telemetry for data obtained in freely moving animals by in vivo voltammetry, an electrochemical technique that uses carbon fiber microelectrodes to monitor monoamine metabolism and release continuously (Justice 1987).

Krügel et al. (2001) tested functional recovery after neuronal injury by P2 receptor blockade. The functional changes in the neuronal activity were recorded by telemetric EEG (TSE Systems, Bad Homburg, Germany).

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Tests for Muscle Coordination

Inclined Plane

Purpose and Rationale

The method of Allmark and Bachinski (1949) using an inclined screen was originally developed for testing curare-like agents. Later on, it has been used by many authors (e.g., Randall et al. 1961) for testing compounds for muscle relaxant activity. The principle of an inclined plane has been used by Ther et al. (1959) for differentiating neuroleptics from other centrally active drugs. Rivlin and Tator (1977) also used an inclined plane to assess skeletal muscle relaxation.

Procedure

The plane consists of two rectangular plywood boards connected at one end by a hinge. One board is the base; the other is the movable inclined plane. Two plywood side panels with degrees marked on their surface are fixed on the base. A rubber mat with ridges 0.2 cm in height is fixed to the inclined plane which is set at 65°. Male mice (Charles River strain) with a body weight between 20 and 30 g are used. The test compound or the standard is administered to groups of 10 mice either i.p. or s.c. or orally. After 30, 60, and 90 min, the mice are placed at the upper part of the inclined plane and are given 30 s to hang on or to fall off.

Evaluation

The peak time is determined as the time at which a compound produces the maximum performance deficit. At this time interval, a range of doses is tested using 10 animals per group. ED_{50} values are calculated.

Critical Assessment of the Method

The method has been proven to be a simple assay for muscle relaxant activity. Although the muscle relaxant tests satisfy the criteria of sensitivity and relative potency compared with clinically effective doses, the effects of anxiolytics are not clearly differentiated from neuroleptic and even from neurotoxic compounds.

Modifications of the Method

Instead of an inclined wooden board, an inclined screen has been used by Randall et al. (1961) and Simiand et al. (1989).

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Chimney Test

Purpose and Rationale

The "test de la cheminée" has been introduced by Boissier et al. (1960) as a simple test for tranquilizing and muscle relaxant activity.

Procedure

Male mice (CD1, Charles River) weighing between 16 and 22 g are used in groups of 10 animals per dose. Pyrex glass cylinders 30 cm long are required. The internal diameter varies with the animal's weight: for mice weighing 16–18 g, the diameter is 22 mm, for mice weighing 18–20 g, 25 mm, and for mice weighing 20–22 g, 28 mm. Each tube has a mark 20 cm from its base. Initially, the tube is held in a horizontal position. At the end of the tube, near the mark, a mouse is introduced with the head forward. When the mouse reaches the other end of the tube, toward which it is pushed if necessary with a rod, the tube is moved to a vertical position. Immediately, the mouse tries to climb backward and performs

coordinated movements similar to an alpinist to pass a chimney in the mountains. This gave the name for the test. The time required by the mouse to climb backward out at the top of the cylinder is noted.

Evaluation

The ED_{50} (with 95 % confidence limits), the dose for which 50 % of the animals fail to climb backward out of the tube within 30 s, is calculated by log-probit analysis.

Critical Assessment of the Method

The chimney test can be used as an additional test with other tests determining muscle relaxant activity.

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Grip Strength

Purpose and Rationale

The test is being used to assess muscular strength or neuromuscular function in rodents, which can be influenced not only by sedative drugs and muscle relaxant compounds but also by toxic agents.

Procedure

The method was described as "test de l'agrippement" by Boissier and Simon (1960). Male or female mice with an average weight of 22 g are used. In a preliminary experiment the animals are tested for their normal reactivity. The animals are exposed to a horizontal thin thread or metallic wire suspended about 30 cm into the air, which they immediately grasp with the forepaws. The mouse is released to hang on with its forelimbs. Normal animals are able to catch the thread with the hind limbs and to climb up within 5 s. Only animals that fulfill this criterion are included into the experiment. Ten mice are used in the control group and in the experimental groups. After oral or subcutaneous administration the animals are tested every 15 min. Animals which are not able to touch the thread with the hind limbs within 5 s or fall off from the thread are considered to be impaired. The test is continued for 2 h. The animals are observed for their behavior in the cages. Only if their behavior and their motility in the cage seem to be normal can the disturbance of the grasping reflex be considered as caused by central relaxation.

Evaluation

The percentage of animals losing the catching reflex is calculated. By use of different doses, ED_{50} values are calculated. Likewise, time-response curves can be established.

Critical Assessment of the Method

Only simultaneous observation of the animals under normal conditions gives the possibility to distinguish between central relaxation and toxic effects on neuromuscular function.

Modifications of the Method

Kondziella (1964) described a method for the measurement of muscular relaxation in mice based on the capacity of hanging from a horizontal griddle.

Meyer et al. (1979) described a technique to measure the fore- and hind limb grip strength of rats and mice. The apparatus consists of an adjustable trough and a push-pull strain gauge with a triangular brass ring which is grasped by the animal with its forelimbs. The animal is pulled on the tail until the grip is broken. The animal continues to be pulled along the trough until the hind limbs grasp a T-shaped bar being also attached to

a push-pull strain gauge. The trial is completed when the grip of the hind limbs is also broken. Fore- and hind limb strength are measured. Dose–response curves could be established with various doses of chlordiazepoxide and phenobarbital.

Barclay et al. (1981) described the tightrope test for testing performance in mice.

Simiand et al. (1989) used a test originally described by Fleury (1957). A mouse held by the tail is placed on a small metallic grid which the animal grips with its forepaws. The grid is then loaded with weights until the mouse can no longer support the weight of the grid. The endpoint is the maximal weight supported by the animal at least for 2 s. ED_{50} values can be calculated for various centrally active skeletal muscle relaxants such as the benzodiazepines.

Deacon and Gardner (1984) described the pull-up test in rats. A rat is held by its hind legs in an inverted position. The time taken by the rat to pull itself up and grasp the hand of the experimenter is used as the test parameter.

Novack and Zwolshen (1983) tested muscle relaxants in various models, such as morphine-induced rigidity in rats, decerebrate rigidity in cats, and the polysynaptic linguomandibular reflex in cats.

Montag-Sallaz and Montag (2003), Anderson et al. (2004), and Baier et al. (2004) used a grip strength meter apparatus, designed by TSE Systems, Bad Homburg, Germany. If the animal releases the grip, then the maximum force is shown on a digital display of a connected control unit.

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Rotarod Method

Purpose and Rationale

The test is used to evaluate the activity of drugs interfering with motor coordination. In 1956, Dunham and Miya suggested that the skeletal muscle relaxation induced by a test compound could be evaluated by testing the ability of mice or rats to remain on a revolving rod. This forced motor activity has subsequently been used by many investigators. The dose which impairs the ability of 50 % of the mice to remain on the revolving rod is considered the endpoint.

Procedure

The apparatus consists of a horizontal wooden rod or metal rod coated with rubber with 3 cm diameter attached to a motor with the speed adjusted to two rotations per minute. The rod is 75 cm in length and is divided into six sections by plastic disks, thereby allowing the simultaneous testing of six mice. The rod is in a height of about 50 cm above the tabletop in order to discourage the animals from jumping off the roller. Cages below the sections serve to restrict the movements of the animals when they fall from the roller. Male mice (CD-1 Charles River strain) with a weight between 20 and 30 g undergo a pretest on the apparatus. Only those animals which have demonstrated their ability to remain on the revolving rod for at least 1 min are used for the test. The test compounds are administered intraperitoneally or orally. Thirty minutes after intraperitoneal or sixty minutes after oral administration the mice are placed for one minute on the rotating rod. The number of animals falling from the roller during this time is counted.

Using different doses, ED_{50} values can be calculated. Moreover, testing at various time intervals, time-response curves can be obtained.

Calculation

Percent animals falling from the rotarod within the test period is calculated for every drug concentration tested. ED_{50} is defined as the dose of drug at which 50 % of the test animals fall from the rotarod.

Critical Assessment of the Test

Many central depressive drugs are active in this test. Benzodiazepines, such as diazepam and flurazepam, have ED_{50} values below 1 mg/kg i.p. The activity of neuroleptics, such as chlorpromazine or haloperidol, is in the same range. In this way, the test does not really differentiate between anxiolytics and neuroleptics but can evaluate the muscle relaxant potency in a series of compounds such as the benzodiazepines. Moreover, the test has been used in toxicology for testing neurotoxicity.

Modifications of the Method

A comparison of the rotarod method in rats with other tests such as blockade of morphine-induced rigidity in rats, decerebrate rigidity in cats, and polysynaptic-monosynaptic reflex preparations in cats was published by Novack and Zwolshen (1983).

Capacio et al. (1992) used the accelerating rotarod to assess motor performance decrement in rats after administration of candidate anticonvulsant compounds in nerve agent poisoning.

The accelerating rotarod system (TSE Systems, Bad Homburg, Germany) was also used by Augustin et al. (2001), Hosseinzadeh and Asl (2003), Dere et al. (2003), and Vitali and Clarke (2004).

Rozas and Labandeira-Garcia (1997) described a drug-free rotarod test that was used to evaluate the effects of unilateral 6-hydroxydopamine lesions, nigral grafts, and subrotational doses of apomorphine. The rotarod unit was automated and interfaced with a personal computer allowing automatic recording of the time that each rat was able to stay on the rod at different rotational speeds.

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Treadmill Performance

Purpose and Rationale

Treadmill experiments have been used in pharmacology and physiology for various purposes:

1. To evaluate the influence of drugs on endurance (Snider et al. 1983; Matthew et al. 1988; Ahlenius et al. 1997a, b; Soares et al. 2003; Minami et al. 2004)
2. To study muscle metabolism (Balon and Nadler 1997; Zhou and Dohm 1997; Juel 1998; Foianini et al. 2000; Gosmanov et al. 2002; Saengsirisuwan et al. 2002; Steinberg et al. 2004)
3. To study influence on the cardiovascular system (Koller et al. 1995; Roth et al. 1998; Spier et al. 1999; Sun et al. 1994, 2002; Villanueva et al. 2003)
4. To study the effect of diet (Oudot et al. 1996) and of circadian rhythm (Marchant and Mistlberger 1996)

Procedure

Minami et al. (2004) studied the effect of a high-salt diet or chronic captopril treatment on exercise capacity in normotensive rats. Male Sprague Dawley rats were submitted to stepwise increasing exercise on a motor treadmill at a speed of 10, 20, and 30 m/min, 0-grade incline, for 4 min with 2-min rest intervals. Exercise was stopped when rats were unable to continue running with exhaustion despite contact with a shock at the rear of the treadmill belt. Mean arterial pressure (MAP) and heart rate (HR) were recorded continuously at rest and during the three different intensities of exercise.

Evaluation

Values obtained for MAP and HR during the 10 s before the beginning of exercise, when all values reached a stable state, and during the last 10 s at each exercise level were used for comparison. All results are expressed as mean ± SEM. One-way ANOVA was used to assess significant differences in means across groups at rest and at each exercise level.

Treadmill Systems

A fully computerized electronically controlled treadmill system for small laboratory animals is provided by TSE Systems, Bad Homburg, Germany: The apparatus basically consists of a rotating running belt driven by a servo-controlled

motor, which provides precise operator-defined tread speed. Separating panels divide the running surface into separate exercise lanes each suited for an individual animal. The floor grids can be used to apply an electric stimulus of variable length and intensity. The incline of the running surface can be steplessly adjusted to control the labor of the animal.

Columbus Instruments, Columbus, OH, offers the animal treadmill Exer 3/6, which is a general-purpose treadmill for three rats or six mice utilizing a single belt construction with dividing walls suspended over the tread surface supplied with or without a stimulus assembly. Speed of the belt can be adjusted in the range of 6–100 m/m.

Modifications of the Method

Treadmill experiments in **cats** were performed by Douglas et al. (1993).

Nakai et al. (2003) and Liu et al. (2004) used treadmill experiments to study walking dysfunction in the **rat neuropathic intermittent claudication model**. In this model, male Sprague Dawley rats were anesthetized with pentobarbital. L4–L6 vertebrae were surgically exposed and two small holes drilled, one between L4 and L5 and the second between L6 and L6 intervertebral spaces. Two rectangular silicone rubber strips (1.25 × 4 mm, thickness: 1 mm) were then placed in the L4–L6 epidural spaces. The incision was closed in two layers and penicillin administered systemically.

Antri et al. (2005) used treadmill experiments to study long-lasting recovery of locomotor function in chronic spinal rats following combined pharmacological stimulation of serotonergic receptors with 8-OHDPAT and quipazine.

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Influence on Polysynaptic Reflexes

Purpose and Rationale

Inhibition of polysynaptic reflexes is considered to be the major mode of action of muscle relaxants. Polysynaptic transmission can be measured by the flexor reflex of the hind paw in anesthetized rats, whereas the monosynaptic Hoffmann reflex is measured by electromyographic recordings from the plantar foot muscle (Block and Schwarz 1994; Schwarz et al. 1994, 1996).

Procedure

Male Wistar rats (250–280 g) are anesthetized with urethane (400 mg/kg i.p.) and α-chloralose (80 mg/kg i.p.). For the flexor reflex a hind paw is stimulated with a pair of fine subcutaneous needle electrodes (5 square-wave shocks at 500 Hz, 0.2 ms duration and 3.0 reflex threshold). Electromyographic recordings are made with a pair of fine needle electrodes inserted into the ipsilateral tibialis muscle. Seven consecutive electromyographic recordings are amplified, and band-pass filtered (8–10 Hz), collected at a sample rate of 10 Hz, averaged and evaluated using a signal averager (CED, Cambridge, UK) on an IBM-compatible personal computer. Ten consecutive responses are averaged before and 20 min after i.p. application of drug.

For stimulation of the Hoffmann reflex a pair of needle electrodes is transcutaneously inserted into the surrounding of the tibial nerve (single square-wave shocks, 0.2 ms duration, at 2.0 threshold). Electromyographic recordings are made with a

pair of skin clip surface electrodes from the plantar foot muscle. Low-intensity electrical stimulation of the tibial nerve elicits a reflex response similar to the human Hoffmann (H) reflex, which has been attributed to monosynaptic excitation of spinal α-motoneurons predominantly by primary muscle spindle afferent fibers. With increasing stimulus strength, the H-wave is preceded by an electromyographic wave, the M wave, which is due to a direct excitation of axons of α-motoneurons.

In all reflex experiments, values measured after solvent or drug application are expressed as a percentage of the corresponding preinjection value.

Evaluation

Statistical evaluation of group differences is performed using the Mann–Whitney *U*-test. Statistical analysis for dose dependency of drug effects is carried out by the Kruskal-Wallis test.

Modifications of the Method

Ono et al. (1990), Farkas and Ono (1995), Hasegawa and Ono (1996), andOtsu et al. (1998) recorded spinal reflexes from spinalized and nonspinalized rats anesthetized with α-chloralose and urethane. Laminectomy was performed in the lumbosacral region. Ventral and dorsal roots of the segments L4 and L5 were isolated. A skin pouch was formed at the site of the dissection to cover the exposed tissues with liquid paraffin kept at 36 °C. The dorsal root of L5 was placed on bipolar silver wire electrodes for stimulation (0.2 Hz, 0.05 ms, supramaximal). The ipsilateral ventral root of L5 and the dorsal root of L4 were placed on bipolar wire electrodes for recording. Monosynaptic and polysynaptic reflexes and dorsal root–dorsal root reflexes were evoked in the L5 ventral root and in the L4 dorsal root, respectively. These reflex potentials were amplified, displayed on an oscilloscope, and averaged eight times by an averaging computer.

Turski and Stephens (1993) recorded monosynaptic Hoffman reflexes in NMRI **mice** anesthetized with 80 mg/kg α-chloralose i.p. +400 mg/kg urethane i.p. The tibial nerve was stimulated with single square-wave pulses, 0.2 ms duration, at 1.2–1.6 times the nerve threshold. Electromyogram recordings were made with a pair of skin clip surface electrodes from the plantar foot muscle. For recording polysynaptic flexor reflexes, the tibial nerve was stimulated with five square-wave pulses at 500 Hz, 0.2 ms duration, at 3.0 times the nerve threshold. Electromyogram recordings were made with a pair of wire electrodes inserted percuteneously into the ipsilateral tibial muscle.

Furthermore, these authors used **genetically spastic rats**. A mutant strain of Wistar rats, which carries an autosomal recessive gene defect, is characterized by a progressive paresis of the hind limbs with increased tone on the extensor muscles (Pittermann et al. 1976). This genetically determined syndrome of spasticity in the rat permits quantitative evaluation of the effect of drugs on muscle tone by recording activity in the electromyogram from a hind limb extensor muscle (Klockgether et al. 1985; Turski et al. 1990).

Farkas et al. (1989) and Tarnava et al. (1989) studied the effects of drugs on the reflex potentials evoked by afferent nerve stimulation and recorded from the spinal roots in unanesthetized spinal **cats**. An analog integrating method was used for quantitative evaluation of the reflex potentials. The amplified and band-pass-filtered signals from the ventral root (monosynaptic reflex and polysynaptic reflex) and from the dorsal root (dorsal root reflex and dorsal root potential) were fed into signal selectors, which transmitted the input signals only within the chosen poststimulus intervals. Thus, the various components of the reflex potentials were separated according to their latencies.

Shakitama et al. (1997) recorded ventral root reflex potential in anesthetized rats and ventral and dorsal root potentials in anesthetized intact and spinalized cats.

Suzuki et al. (1995) studied the recovery of reflex potentials after spinal cord ischemia produced by occlusion of the thoracic aorta and the bilateral internal mammary arteries for 10 min in cats.

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Masticatory Muscle Reflexes

Purpose and Rationale

The temporomandibular joint dysfunction syndrome, characterized by pain and clicking in the temporomandibular joint and limitation of function, involves a condition caused by hypertonia combined with parafunctional habits, such as clenching or grinding of teeth and hyperreflexia (Laskin and Block 1986), which may be treated by muscle relaxants. Ozawa et al. (1996) studied the effects of a centrally acting muscle relaxant on *masticatory* muscle reflexes in rats. Both monosynaptic and polysynaptic reflexes can be studied using this model.

Procedure

For recording the **monosynaptic tonic vibration reflex** of the masseter muscle, Wistar rats are anesthetized with ether, intubated with a tracheal cannula, and fixed into a stereotactic apparatus. Decerebration is performed by radiofrequency lesion of the midbrain using a Lesion Generator

(Radionics, RFG-4, USA) and a lesioning electrode inserted into the midbrain. After lesioning, ether anesthesia is discontinued. The tonic vibration reflex of the masseter muscle, recorded as electromyogram, is induced every 10–12 s by a sinusoidal vibration (100–500 Hz, 2 s) which is applied to the mandibula, and is delivered by a vibration generator driven by a low-frequency oscillator and an amplifier. The evoked electromyogram is amplified by a biophysical amplifier and recorded on a thermal array recorder. The root mean square of the electromyogram is also recorded through an integrator.

For recording the **polysynaptic jaw opening reflex**, the animals are anesthetized by intraperitoneal pentobarbital-Na (50 mg/kg), fixed into position on their back, and intubated with a tracheal cannula. Intrapulpal stimulation (0.5 Hz, 0.2 s in pulse duration, supramaximal intensity) delivered by an electrical stimulator is performed via a dental reamer inserted into the dental pulp of the mandibula. The jaw opening reflex recorded as phasic component of the electromyogram evoked in the ipsilateral digastric muscle is amplified by a biophysical amplifier and recorded on a thermal array recorder.

For recording of the **polysynaptic tonic periodontal masseteric reflex** (Funakoshi and Amano 1974) the animals are anesthetized by intraperitoneal injection of pentobarbital-Na (35 mg/kg), which is supplemented as required, intubated with a tracheal cannula, and fixed onto a stereotaxic apparatus. The maxillary incisor is stimulated by pressing for 5 s every 5 min using a vibration generator driven by a trapezoid generator. The electromyogram responses to this stimulation are amplified by a biophysical amplifier and recorded on a thermal array recorder. The evoked electromyogram is transformed into square-wave pulses, fed into a staircase generator, and recorded on a thermal array recorder.

Evaluation

The significance of differences between the control and the drug-treated groups is evaluated with Dunnett's test.

Modifications of the Method

Boucher et al. (1993) performed microinfusions of excitatory amino acid antagonists into the trigeminal sensory complex of freely moving rats while recording the long-latency jaw opening reflex elicited by electrical stimulation of the dental pulp.

Bakke et al. (1998) studied neurokinin receptor mechanisms in the increased jaw muscle activity in anesthetized rats which can be evoked by injection of the small fiber excitant and inflammatory irritant mustard oil into the temporomandibular joint region.

Alia et al. (1998) preformed intraoral administration of a NK_1 antagonist in freely moving guinea pigs while recording the short- (6–10 ms) and long-latency (18–26 ms) jaw-opening reflex elicited by electrical stimulation of the lower incisor tooth pulp.

Huopaniemi et al. (1988) determined the threshold of the tooth pulp–evoked jaw-opening reflex after naloxone in barbiturate-anesthetized cats.

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Tests for Anxiolytic Activity

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Contents

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General Considerations

Definitions in psychopharmacology have been coined by the activity of special compounds or chemical classes found in patients. This is not only true for the term "neuroleptic" but also for the term "anxiolytic." Other terms have been "ataractic" or "psycholeptic." Anxiolytics are derived from "tranquilizers," such as meprobamate, which was used widely until the advent of benzodiazepines. The property which these drugs have in common is the alleviation of anxiety, thus explaining the term "anxiolytic." These agents are used for the relatively minor disorders of the nonpsychotic or neurotic type, whereas the antipsychotic agents (phenothiazines, butyrophenones) are given mainly to combat the more severe psychotic or schizophrenic reactions. Thus, the terms "antianxiety" and "antipsychotic" indicate a qualitative distinction in the clinical use and mode of action of the drug. Pathological anxiety in man has been defined by its interference with normal functions, by manifestations of somatic disorders, emotional discomfort, interference with productivity at work, etc. This complex characterization of anxiety in man already indicates the difficulties to find appropriate pharmacological models. Therefore, several tests have to be performed to find a spectrum of activities which can be considered to be predictive for therapeutic efficacy in patients. For in vivo studies, most investigators use a battery of anticonvulsive tests, antiaggressive tests, and evaluation of conditioned behavior.

Most of the actions of benzodiazepines are thought to be mediated by potentiation of g-amino-butyric acid (GABA). Two subtypes of GABA receptors ($GABA_A$ and $GABA_B$) have been described. Moreover, specific binding sites for benzodiazepines have been discovered near these GABA receptors in various areas of the brain. These sites occur in a macromolecular complex that includes GABA receptors, benzodiazepine receptors and receptors for other drugs, and a chloride channel. The benzodiazepines potentiate

the neurophysiological actions of GABA at the chloride ion channel by increasing the binding of GAB_A to $GABA_A$ receptors. This implies that the $GABA_A$ receptor is involved in anxiety and that its direct activation would have an anxiolytic effect. Based on these findings various in vitro tests have been developed.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids in particular glutamates. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA), and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). Some methods are described in chapter "► Anti-Epileptic Activity."

Serotonin may play a role in anxiety, since treatment with drugs that reduce serotoninergic function, including benzodiazepines, has anxiolytic effects in animal models (Dourish et al. 1986). Several subtypes of serotonin receptors have been elucidated, e.g., 5-HT_{1A}, 5-HT_{1B}. 5-HT_{1C}, 5-HT_{1D}, 5-HT_2, 5-HT_3. Further differentiation is underway. 5-HT_{1A}, 5-HT_{1B}, and 5-HT_3 receptors are considered to be involved in the effect of antianxiety and novel antipsychotic drugs (Peroutka 1988; Costall et al. 1988). Some in vitro methods are described in chapters "► Antidepressant Activity" and "► Neuroleptic Activity."

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In Vitro Methods

GABA Receptor Binding

General Considerations

γ-Aminobutyric acid (GABA) is known to be an important inhibitory neurotransmitter in the brain. Abnormalities in the GABA system have been found in neurological and psychiatric diseases such as Huntington's chorea, anxiety, panic attacks, schizophrenia, and epilepsy. GABA is also implicated in the mechanism of benzodiazepines and related central nervous system (CNS) drugs.

GABA receptors have been divided into $GABA_A$, $GABA_B$, and $GABA_C$ receptor subtypes which by themselves form receptor families

(Enna and Möhler 1987; Matsumoto 1989; Möhler 1992).

Without doubt, GABA is the most important inhibitory transmitter in the CNS. One may expect important new developments from attempts to influence the GABAergic system specifically by synthetic compounds. In general, $GABA_A$ receptor agonists are CNS depressants, muscle relaxants, and possess some nociceptive properties, whereas the receptor antagonists are convulsants. Most benzodiazepine-binding sites seem to be associated with $GABA_A$ receptors combined with a chloride channel. Among $GABA_B$ receptor agonists, baclofen has achieved success in clinical use. Results of ongoing research with receptor subtypes will result in the development of new therapeutic agents.

Studies by Urwyler et al. (2001) demonstrated that the $GABA_B$ receptor can be modulated in an allosteric manner. These compounds are not receptor agonists but appear to act on the heptahelical region of $GABA_{B2}$ to enhance the action of the receptor agonists GABA and baclofen.

$GABA_C$ receptors are ligand-gated chloride channels that are present in many parts of the brain including the superior colliculus, cerebellum, hippocampus, and, most prominently, the retina (Ragozzino et al. 1996; Bormann 2000; Qian and Ripps 2001; Chehib 2004; Schlicker et al. 2004; Yang 2004; Pan et al. 2005; Wang and Slaughter 2005).

The formation and plasticity of GABAergic synapses, their physiological mechanisms, and pathophysiological implications are discussed by Fritschy and Brünig (2003).

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In Vitro Assay for GABAergic Compounds: [^{3}H]-GABA Receptor Binding

Purpose and Rationale

Radiolabeled GABA is bound to synaptic membrane preparations of mammalian brains. The labeling of the synaptic receptor with

^{3}H-GABA requires careful attention to possible interference from nonsynaptic binding since ^{3}H-GABA can also bind nonspecifically to plasma membranes. The most prominent of them is the sodium-dependent binding of GABA to brain membranes, a process which appears to be associated with the transport (uptake) sites of GABA. Sodium-independent binding of ^{3}H-GABA has characteristics consistent with the labeling of GABA receptors. In addition, the relative potencies of several amino acids in competing for these binding sites parallel their abilities to mimic GABA neurophysiologically. Therefore, the sodium-independent binding of ^{3}H-GABA provides a simple and sensitive method to evaluate compounds for GABA-mimetic properties.

Procedure

Reagents

- 0.05 M Tris-maleate buffer (pH 7.1).
- 6.05 g of Tris-base are dissolved in distilled water and made up to 1000 ml. 5.93 g of Tris-maleate are dissolved in 500 ml of water. The 0.05 M Tris maleate, pH 7.1 buffer is prepared by slowly adding Tris maleate to the Tris-base solution until the pH reaches 7.1.
- 0.32 M Sucrose: 109.5 g of sucrose are dissolved in distilled water and filled up to 1000 ml. The solution is stored at 4 °C.
- ^{3}H-GABA (specific activity approximately 40 Ci/mmol) is made up to a concentration of 780 nmol in distilled water and 20 μl added to each test tube (yielding a final concentration of 15 nmol in the assay). Isoguvacine or muscimol is prepared by dissolving 8.35 mg of isoguvacine or 6.40 mg of muscimol in 10 ml water. Twenty microliter of these solutions when added to one milliliter of incubation medium give a final concentration of 0.1 mM isoguvacine or muscimol.
- Test drugs: 1 mM stock solutions are initially prepared. These are serially diluted to the required concentrations prior to the addition to the incubation mixture. Final concentrations are usually from 2×10^{-8} to 1×10^{-5} M.

Tissue Preparation

Male Charles-River rats (100–150 g) are decapitated and their whole brains rapidly removed and homogenized in 15 vol of ice-cold 0.32 M sucrose. The homogenate is centrifuged at 1000 *g* for 10 min. Thepellet (nuclear fraction) is discarded and the supernatant fluid recentrifuged at 20,000 *g* for 20 min. The supernatant is discarded and the crude mitochondrial pellet resuspended in 15 volume distilled water using a Tekmar homogenizer. The suspension is centrifuged at 8000*g* for 20 min. The supernatant is collected and used to carefully resuspend, using a gentle squirling motion, the pellet's soft, upper, buffy layer. This suspension is then centrifuged at 48,000 *g* for 20 min. The final crude synaptic membrane pellets are resuspended (without homogenization) in 15 volumes of distilled water and centrifuged at 48,000 *g* for 20 min. The supernatant is discarded, and the centrifuge tubes containing the pelleted membranes are capped with parafilm and stored frozen at −70 °C.

Assay Procedure

A frozen membrane pellet from one whole rat brain is resuspended in 15 volumes of 0.05 M Tris-maleate buffer (pH 7.1) by homogenization at 4 °C. Triton X-100 is added to a final concentration of 0.05 %. This suspension is then incubated at 37 °C for 30 min followed by centrifugation at 48,000 *g* for 10 min. The supernatant is discarded and the pellet resuspended by homogenization in the same volume of 0.05 Tris-maleate buffer (pH 7.1) at 4 °C. The preincubation with Triton enhances specific GABA receptor binding while lowering nonspecific binding.

For the standard Na-independent ^{3}H-GABA binding assay procedure, aliquots of the previously frozen, Triton-treated crude synaptic membranes are incubated in triplicate at 4 °C for 5 min in 0.05 M Tris-maleate buffer (pH 7.1) containing 15nM ^{3}H-GABA alone or in the presence of 0.1 mM isoguvacine or muscimol or the test drug.

The procedure is as follows:

- 1 ml of the 0.05 M Tris-maleate homogenate
- 20 μl of ^{3}H-GABA
- 20 μl of test drug or 20 ml of 0.1 mM isoguvacine or muscimol

After incubation at 4 °C for 5 min, the reaction is terminated by centrifugation for 15 min at 5000 rpm. The supernatant fluid is aspirated and the pellet washed twice with 1 ml of the Tris-maleate buffer. Two milliliter of liquiscint are added to each tube which is then vigorously vortexed. The contents of the tubes are transferred to scintillation vials and the tubes rinsed with an additional 2 ml of cocktail. An additional 6 ml of liquiscint are added to each scintillation vial. The radioactivity is measured by liquid scintillation photometry.

Evaluation

Specific ^{3}H-GABA binding is defined as the radioactivity which can be displaced by a high concentration of unlabeled GABA and is obtained by subtracting from the total bound radioactivity the amount of radioactivity bound in the presence of 0.1 mM isoguvacine. Results are converted to percent of specifically bound ^{3}H-GABA displaced by a given concentration of test drug. IC_{50} values with 95 % confidence limits are then obtained by computer-derived linear regression analysis.

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$GABA_A$ Receptor Binding

Purpose and Rationale

Two subtypes of GABA receptors have been identified:

1. $GABA_A$ receptor, for which muscimol is the typical agonist, whereas bicucculine, picrotoxin, and SR 95531 are antagonists
2. $GABA_B$ receptor, for which baclofen is the typical agonist

The $GABA_A$ receptor directly gates a Cl^- ionophore and has modulatory binding sites for benzodiazepines, barbiturates, neurosteroids, and ethanol. By contrast, $GABA_B$ receptors couple to Ca^{2+} and K^+ channels via G proteins and second messenger systems; they are activated by baclofen and are resistant to drugs that modulate $GABA_A$ receptors.

Several subtypes of $GABA_A$ receptors have been identified by ligand binding studies (Kleingoor et al. 1991; Turner et al. 1991; Gusti et al. 1993). Molecular biology techniques revealed the $GABA_A$ receptor to be assembled as a pentameric structure from different subunit (α, β, γ, and δ subunit) families making it possible that a very large number of such heteromeric $GABA_A$ receptors exist in the mammalian central and peripheral nervous system (Krogsgaard-Larsen et al. 1994; Lambert et al. 1995; Smith and Olsen 1995; Costa 1998). A total of 19 genes encoding $GABA_A$ receptor subunits are known, while several additional isoforms can occur as splicing variants of some of these (Barnard 1998). Probably more than 500 distinct $GABA_A$ receptor subtypes exist in the brain (Sieghart 2000). Sequences of six α, three β, three γ, one δ, three ρ, one ε, one π, and one θ $GABA_A$ receptor subunits have been reported in mammals (Barnard 2000; Alexander et al. 2001). More insight into the pharmacological functions of $GABA_A$ receptor subtypes is expected from

studies in gene-knockout mice and by knock-in point mutations (Rudolph et al. 2001).

$GABA_C$ receptors were described as a pharmacologically distinct group by Bormann and Feigenspan (1995), Johnston (1996), Bormann (2000), and Zhang et al. (2001). These receptors are Cl^- pores that are insensitive to both bicucculine and baclofen. An IUPHAR Committee has recommended in 1998 that the term $GABA_C$ should be avoided and bicucculine and baclofen-insensitive GAB_A receptors classified as a minor subspecies of $GABA_A$ receptors of the "AO" type.

Subtypes of $GABA_A$ receptors were classified on the basis of subunit structure and receptor function by Barnard et al. (1998).

In rat brain membranes, only the $GABA_A$ receptor is labeled with the $GABA_A$ selective radioligand ^{3}H-SR 95531 in the given concentration range. The assay allows specifically the estimation of the test drug's binding characteristics to the $GABA_A$ receptor subpopulation.

Procedure

Materials

Radioligand: ^{3}H-SR 95531 (^{3}H-2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium) bromide (New England Nuclear, Boston).

Membrane Preparation

Rats are killed by decapitation, the brains quickly dissected and after separation from the cerebellum placed into ice-cold sucrose solution. The brains (approximately 20 g wet weight) are then homogenized in a glass Teflon potter (1 g brain weight/15 ml 320 mM D(+)-sucrose solution) and centrifuged at 1000*g* at 4 °C for 10 min. The pellets are discarded and the supernatants centrifuged at 20,000*g* for 20 min. The resulting supernatants are discarded and the pellets lysed by hypoosmotic shock (addition of 20 volumes of ice-cold bidistilled water). After homogenization in a glass Teflon homogenizer, the suspension is stirred under cooling for 20 min and centrifuged at 48,000*g* for 20 min. The resulting pellets are resuspended in ice-cold bidistilled water, the suspension stirred and recentrifuged as before. The final pellets are resuspended in the incubation buffer (50 mM Tris–HCl and 100 mM $MgCl_2 \times 6H_2O$, pH 7.4) corresponding to 1 g brain wet weight/1 ml buffer. The membrane suspension is immediately stored in aliquots of 1 ml at −20 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

On the day of the experiment, the required volume of the membrane suspension is slowly thawed and diluted 1:20 with bidistilled water. After stirring for 10 min, the membrane suspension is centrifuged at 50,000 *g* for 10 min. The resulting pellets are resuspended in ice-cold incubation buffer, yielding a membrane suspension with a protein content of 1 mg/ml.

Assay

For each concentration, assays are performed in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

Total binding:

- 50 ml ^{3}H-SR 95531 (12 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml incubation buffer

Nonspecific binding:

- 50 ml ^{3}H-SR 95531 (4 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml (+) bicucculine (10^{-4} M)

Competition Experiments

- 50 ml ^{3}H-SR 95531 (1 constant concentration, 8–10 $\times 10^{-9}$ M)
- 50 ml incubation buffer without or with nonlabeled test drug (15 concentrations, 10^{-10} to 10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample. The samples are incubated for 30 min at 4 °C. The reaction is stopped by subjecting the total

incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free radioactivity. Filters are washed immediately with approximately 20 ml ice-cold rinse buffer (50 mM Tris HCl, pH 7.4) per sample. The membrane-bound radioactivity is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding
- Nonspecific binding
- Specific binding = total binding – nonspecific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-SR 95531 versus nonlabeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3\mathrm{H} \times \mathrm{IC}_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50 % of specifically bound ^{3}H-SR 95531 in the competition experiment.

$[^3H]$ = concentration of ^{3}H-SR 95531 in the competition experiment.

$K_D{}^3H$ = dissociation constant of ^{3}H-SR 95531, determined from the saturation experiment.

The K_i-value of the test drug is that concentration at which 50 % of the receptors are occupied by the test drug.

Modifications of the Method

Binding to the agonist site of the $GABA_A$ receptor can be measured with [^{3}H]muscimol (Snodgrass 1978; Williams and Risley 1978; Martini et al. 1983).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1 nM [^{3}H]muscimol for 10 min at 0 °C. Nonspecific binding is estimated in the presence of 100 nM muscimol. Membranes are filtered and washed three times and the filters counted to determine specifically bound [^{3}H]muscimol.

The $GABA_A$ receptor chloride channel can be studied by binding with [^{3}H]t-butylbicycloorthobenzoate ([^{3}H]TBOB) (Schwartz and Mindlin 1988; Lewin et al. 1989).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. 0.4 mg of membrane preparation is incubated with 3 nM [^{3}H] TBOB for 15 min at 15 °C. Nonspecific binding is estimated in the presence of 200 μM picrotoxin. Membranes are filtered and washed three times and the filters counted to determine specifically bound [^{3}H] TBOB.

Cromer et al. (2002) presented a model for the extracellular, ligand-binding domain of the $GABA_A$ receptor that is based on the structure of a soluble acetylcholine-binding protein.

Kittler and Moss (2003) discussed modulation of $GABA_A$ receptor activity by phosphorylation and receptor trafficking and the implications for the efficacy of synaptic inhibition.

Lambert et al. (2003) reviewed neurosteroid modulation of $GABA_A$ receptors with special emphasis on pregnane steroids.

Lüscher and Keller (2004) described regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses.

Macdonald et al. (2004) reviewed mutations of the $\alpha 1$, $\gamma 2$, and δ subunits of the $GABA_A$ receptor that are associated with different idiopathic generalized epilepsy syndromes.

Mody and Pearce (2004) reviewed the diversity of inhibitory neurotransmission through $GABA_A$ receptors.

Rudolph and Möhler (2004) reported analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Whereas the knockout mice have provided information primarily with respect to the regulation of subunit gene transcription, receptor

assembly, and some physiological functions of individual receptor subtypes, the point-mutated knockin mice in which specific $GABA_A$ receptors are insensitive to diazepam or some general anesthetics have revealed the specific contribution of individual receptor subtypes to the pharmacological spectrum of diazepam and cerebral anesthetics.

Among $GABA_A$ agonists and partial antagonists, Krogsgaard-Larsen et al. (2004) characterized THIP (Gaboxadol) as a nonopioid analgesic and a novel type of hypnotic.

Boehm et al. (2004) described γ-Aminobutyric acid receptor subunit mutant mice, which offer new perspectives on alcohol actions.

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$GABA_B$ Receptor Binding

Purpose and Rationale

Baclofen, as an analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), binds as agonist to the subtype B of the GABA receptor. Baclofen is effective in the treatment of spasticity caused by multiple sclerosis or other diseases of the spinal cord, particularly traumatic lesions. Studies of similar compounds may lead to other effective antispasmodic drugs.

In rat cerebellar membranes, only the $GABA_B$ receptor is labeled in the given concentration range of the $GABA_B$ selective agonist ^{3}H-(−) baclofen. The assay allows specifically the estimation of the test drug's binding characteristics at the $GABA_B$ subtype receptor population.

The receptor on which baclofen acts is coupled via G_i/G_o proteins to Ca^{2+} and K^+ channels as well as adenyl cyclase in neurons and hence is classified as a metabotropic receptor.

As in other receptor families, heterogeneity of the $GABA_B$ receptor has been found (Scherer et al. 1988; Bittiger et al. 1992; Bonanno and Raiteri 1992, 1993a, b; Bowery 1993; Lanza et al. 1993). At least three distinct subtypes have been identified:

(a) The postsynaptic receptor linked via a G-protein to a K^+ channel which upon stimulation by GABA hyperpolarizes the neuron
(b) The presynaptic autoreceptor at GABA nerve endings; blockade of this receptor augments the release of GABA in electrically stimulated rat cortical slices
(c) The presynaptic heteroreceptor at glutamate nerve endings; blockade with GABAB antagonists increases release of glutamate from K^+ stimulated cortical slices

The structure of $GABA_B$ receptors was identified when isoforms were detected (Kaupmann et al. 1997). Potent $GABA_B$ antagonists were described (Bittiger et al. 1992, 1993; Froestl et al. 1996).

Procedure

Materials

Radioligand: ^{3}H-(−)baclofen, specific activity 1.11– 1.85 TBq/mmol (30–50 Ci/Mmol) New England Nuclear, Boston

Membrane Preparation

Rats are killed by decapitation, the cerebella quickly removed, and placed into ice-cold preparation buffer (50 mM Tris–HCl, pH 7.4). Approximately 5 g wet weight of the cerebella are homogenized using a glass Teflon potter, corresponding to 1 g cerebellum wet weight/50 ml buffer, and centrifuged at 48,000 *g* at 4 °C for 10 min. The pellets are resuspended in approximately 270 ml preparation buffer and centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebellum wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at −77 °C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

On the day of the experiment, the required volume of the membrane suspension is slowly thawed, and centrifuged at 50,000 *g* for 20 min. The pellets are resuspended in the same volume of incubation buffer and stirred for 45 min at room temperature. The suspension is recentrifuged as before. This washing step is repeated three times. The resulting pellets are resuspended in ice-cold incubation buffer in a volume, yielding a membrane suspension with a protein content of 1 mg/ml. The membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Assay

For each concentration samples are used in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates). The concentration

of ^{3}H-(−)baclofen is constant in all samples (1.8–2 × 10^{-8} M).

Saturation Experiments

Total Binding

- 50 µl ^{3}H-(−)baclofen
- 50 µl nonradioactive racemic baclofen (15 concentrations, 0–1.2 × 10^{-6} M)

Nonspecific Binding

The measurement of the nonspecific binding is performed at the lowest concentration of the saturation range, i.e., 1.8 × 10^{-8} M of the ^{3}H-(−) baclofen without nonradioactive racemic baclofen.

- 50 µl 3H-(−)baclofen
- 50 µl gamma-aminobutyric acid

Competition Experiments

- 50 µl ^{3}H-(−)baclofen
- 50 µl incubation buffer without or with labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1 mg protein/1 ml). The samples are incubated for 60 min at 4 °C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane bound is separated from the free radioactivity. The filters are washed with approximately 20 ml ice-cold buffer. The retained membrane bound radioactivity on the filter is measured after addition of 0.3 ml ethylene glycol monomethyl ether and 2 ml liquid scintillation cocktail per sample and an equilibration time of 1 h in a Packard liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding
- Nonspecific binding
- Specific binding = total binding − nonspecific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H(−)baclofen versus nonlabeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D{}^3\mathrm{H} \times \mathrm{IC}_{50}}{K_D{}^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50 % of specifically bound ^{3}H-(−)baclofen in the competition experiment.

[^{3}H] = concentration of ^{3}H-(−)baclofen in the competition experiment.

$K_D{}^3$H = dissociation constant of ^{3}H-(−)baclofen, determined from the saturation experiment.

The *K*i-value of the test drug is the concentration at which 50 % of the receptors are occupied by the test drug.

Modifications of the Method

Kaupmann et al. (1998) showed that $GABA_B$ receptor subtypes assemble into functional heteromeric complexes.

Bowery and Enna (2000) demonstrated that $GABA_B$ receptors are functional metabotropic heterodimers.

Molecular structure and physiological functions of $GABA_B$ receptors are reviewed by Bettler et al. (2003) and Bowery et al. (2003).

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Benzodiazepine Receptor: [^{3}H]-Flunitrazepam Binding Assay

Purpose and Rationale

Experiments using ^{3}H-diazepam or ^{3}H-flunitrazepam have demonstrated specific binding sites in CNS membrane preparations that satisfy the criteria for pharmacological receptors, e.g., saturability, reversibility, stereoselectivity, and significant correlation with in vivo activities of the drugs in this class.

Heterogeneity of benzodiazepine receptors has been reported (Klepner et al. 1979; Supavilai and Karobath 1980; Hafely et al. 1993; Davies et al. 1994). There are four classes of benzodiazepine and nonbenzodiazepine high-affinity ligands for benzodiazepine recognition sites associated with $GABA_A$ receptors: The first class (e.g., diazepam, flunitrazepam, alprazolam) facilitates the action of GABA, increasing the opening frequency of Cl^- channels. These ligands are called full positive allosteric modulators, or full agonists. A second class of ligands, which includes the β-carbolines, can decrease the opening frequency of Cl^- channels. These ligands are known as full negative allosteric modulators, or full inverse agonists. A third class (e.g., flumazenil) binds with high affinity to benzodiazepine recognition sites, but it can also prevent the GABA modulations elicited by positive or negative allosteric modulators; this class is called a modulator antagonist. A fourth class of ligands for benzodiazepine recognition sites is known to elicit either partial amplification or partial attenuation of GABA action at various $GABA_A$ receptors and comprises the class called partial positive and partial negative allosteric modulators or partial agonists and partial inverse agonists, respectively.

The names **ω_1, ω_2, and ω_3 receptor subtypes** have been proposed to replace the nomenclature of benzodiazepine BZ_1, BZ_2, and BZp receptors (Langer and Arbilla 1988; Langer et al. 1990; Griebel et al. 1999a, b).

Procedure

Reagents

- [Methyl-3H]-Flunitrazepam (70–90 Ci/mmol) can be obtained from New England Nuclear.
- Clonazepam HCl can be obtained from Hoffmann La Roche.

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. The cerebral cortices are removed, weighed, and homogenized with a Potter-Elvejhem homogenizer in 20 volumes of ice-cold 0.32 M sucrose. This homogenate is centrifuged at 1000*g* for 10 min. the pellet is discarded, and the supernatant is centrifuged at 30,000 *g* for 20 min. The resulting membrane pellet is resuspended in 40 volumes of 0.05 M Tris buffer, pH 6.9.

Assay

1 ml 0.05 Tris buffer, pH 6.9
560 µl H_2O
70 µl 0.5 M Tris buffer, pH 6.9
50 µl ^{3}H-Flunitrazepam

20 µl vehicle (for total binding) or 0.1 mM Clonazepam (for nonspecific binding) or appropriate drug concentrations
300 µl tissue suspension

The tubes containing ^{3}H-flunitrazepam, buffer, drugs, and H_2O are incubated at 0–4 °C in an ice bath. A 300 µl aliquot of the tissue suspension is added to the tubes at 10-s intervals. The timer is started with the addition of the mixture to the first tube. The tubes are then incubated at 0–4 °C for 20 min and the assay stopped by vacuum filtration through Whatman GF/B filters. This step is performed at 10-s intervals. Each filter is immediately rinsed with three 5-ml washes of ice-cold buffer, pH 6.9. The filters are counted in 10 ml of liquid scintillation counting cocktail.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of clonazepam. Specific binding is approximately 97 % of total ligand binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} calculations are performed using log-probit analyses.

Critical Assessment of the Method

Binding to the benzodiazepine receptor is not absolutely predictive for anxiolytic activity. A range of compounds have been discovered that do not have the benzodiazepine structure but that do interact with the benzodiazepine receptors (Gardner 1988; Byrnes et al. 1992). They may have a different pharmacological profile in vivo.

Modification of the Method

Takeuchi et al. (1992) developed a nonisotopic receptor assay for benzodiazepine drugs using the biotin-1012-S conjugate. The free conjugate in the supernatant was determined with a solid-phase avidin-biotin binding assay.

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Serotonin Receptor Binding

General Considerations

Several surveys on 5-HT receptors and their classification have been published, e.g., by Humphrey et al. (1993), Peroutka (1993), Boess and Martin (1994), Hoyer et al. (1994), Kebabian and Neumeyer (1994), Martin and Humphrey (1994), Saxena (1994), Brancheck (1995), Sleight et al. (1995), Bockaert et al. (1997), Branchek and Zgombick (1997), Briley et al. (1997), Costal and Naylor (1997), Glennon and Dukat (1997), Göthert and Schlicker (1997), Hamon (1997), Hartig (1997), Hoyer and Martin (1997), Jacobs and Fornal (1997), Roth and Hyde (1997), Uphouse (1997), Martin (1998), Martin and Eglen (1998), Saxena et al. (1998), Barnes and Sharp (1999), Pauwels (2000), and Gershon (2004).

The classification has evolved from a scheme recognizing 3 classes (5-HT_{1like}, 5-HT_2, and 5-HT_3) to one accepted by the NC-IUPHAR subcommittee for 5-hydroxytryptamine (serotonin) receptors in which 7 classes embrace 14 distinct receptor subtypes (Martin 1988; Martin and Eglen 1998). Some revisions of the nomenclature were made:

Renaming the 5-HT_{1C} receptor to 5-HT_{2C} on the basis of recognitory, transductional, and structural identity with the 5-HT_2 family.

Alignment of the classification scheme with the human genome, meaning that human receptors are given preeminence in the nomenclature.

Renaming the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes to 5-HT_{1D} and 5-HT_{1B}, respectively.

Recognition that the "5-HT_{1like}" positively coupled to adenylate cyclase and mediating smooth muscle relaxation is the 5-HT_7 receptor.

Use of a lower-case notation to describe a putative receptor defined only by gene product, with "promotion" to an upper-case notation when the receptor is fully defined in terms of operational, recognitory, and structural properties.

Up to seven functional isoforms of the 5-HT_{2C} receptor, two functional isoforms of the 5-HT_4 receptor, and four isoforms of the 5-HT_7 receptor were recognized.

Murphy et al. (1999) reviewed molecular biology–based alterations in 5-HT receptors including altered characteristics of mice lacking different 5-HT receptors, e.g., 5-HT_{1B}-receptor-deficient mice, 5-HT_{2C} receptor-deficient mice, 5-HT_{1A}-receptor-deficient mice, 5-HT cell-membrane-transporter-deficient mice, and vesicular monoamine-transporter-deficient mice.

The role of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2B}, and 5-HT_7 receptors in cardiovascular physiology and pharmacology was discussed by Watts and Cohen (1999).

5-HT Receptor Types and Subtypes

5-HT_1 Subtypes. At least five *5-HT_1 subtypes* are described (5-*HT_{1A}*, 5-*HT_{1B}*, 5-HT_{1D}, 5-ht_{1E}, 5-ht_{1F}), which share 41–63 % overall sequence identity and couple preferentially to Gi/o to inhibit cAMP formation.

Hartig et al. (1996) suggested a revised nomenclature for 5-HT_{1B}, 5-$HT_{1D\alpha}$, and 5-$HT_{1\beta}$p receptor subtypes.

5-HT_{1A} receptor agonists were described by Sills et al. (1984), Porsolt et al. (1992), Foreman et al. (1994), Wolff et al. (1997), and Hamon (1997), 5-HT_{1A} receptor antagonists by Allen et al. (1997), 5-$HT_{1B/D}$ receptor antagonists by de Vries et al. (1997), 5-HT_{1D} receptor agonists by Macor et al. (1994), van Lommen et al. (1995), and Valentin et al. (1996), and 5-HT_{1D} receptor antagonists by Clitherow et al. (1994), de Vries et al. (1996), Rollema et al. (1996), and Briley et al. (1997).

Ramboz et al. (1998) recommended serotonin receptor 1α knockout mice as an animal model of anxiety-related disorder.

Presynaptic receptors may be preferably involved in the anxiolytic effects of 5-HT_{1A} receptor agonists, whereas in the antidepressant effects postsynaptic receptors are strongly involved (de Vry et al. 1991; de Vry 1995).

5-HT1 receptors are involved in learning and memory processes.

An endogenous peptide interacting specifically with the serotonergic 1B receptor subtypes was identified (Rousselle et al. 1996).

5-HT autoreceptors, mainly of the 5-HT_{1D} subtype, were studied by Starke et al. (1989), Fink et al. (1995), Bühlen et al. (1996), Glennon et al. (1996), Roberts et al. (1996), and Price et al. (1996).

Cushing et al. (1994) studied the role of a 5-HT_{1D}-like receptor in serotonin-induced contraction of canine coronary artery and saphenous vein.

Wainscott et al. (2005) and Lucaites et al. (2005) described [^{3}H]Y334370 as a radioligand for the 5-HT_{1F} receptor.

See sections "Serotonin (5-HT_{1A}) Receptor: Binding of [3H]-8-Hydroxy-2-(di-n-Propylamino)-Tetralin ([3H]-DPAT)" and "Serotonin (5-HT1B) Receptors in Brain: Binding of [3H]5-Hydroxytryptamine ([3H]5-HT)" for binding assays of 5-HT_1 receptors.

5-HT_2 Receptors. *5-HT_2 receptors* have been subdivided into 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, which exhibit 46–50 % overall sequence identity and couple preferentially to $G_{q/11}$ to increase the hydrolysis of inositol phosphates and elevate cytosolic [Ca^{2+}]. (Humphrey et al. 1993; Hoyer et al. 1994; Kebabian and Neumeyer 1994; Martin and Humphrey 1994; Saxena 1994; Shi et al. 1994; Tricklebank 1996; Martin 1998). The human 5-HT_2 receptors were cloned and characterized (Chen et al. 1992; Carey et al. 1996). Species differences in receptors were described (Johnson et al. 1995). 5-HT_2 receptors play a role in the action of antipsychotics and hallucinogens.

Saucler and Albert (1997) identified an endogenous 5-hydroxytryptamine$_{2A}$ receptor in NIH-3 T3 cells.

Wolf and Schutz (1997) found that the serotonin 5-HT$_{2C}$ receptor is a prominent serotonin receptor in basal ganglia.

Bonhaus et al. (1995) described the pharmacology and distribution of human 5-hydroxytryptamine$_{2B}$ (5-HT$_{2B}$) receptor gene products and compared this with 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors.

Chagraoui et al. (2003) reported that agomelatine (S20088) antagonizes the penile erections induced by the stimulation of 5-HT$_{2C}$ receptors in Wistar rats.

See chapter "▶ Neuroleptic Activity" for binding assays to 5-HT$_2$ receptors.

5-HT$_3$ Receptor. The *5-HT$_3$ receptor* is a pentameric, ligand-gated ion channel, activation of which promotes entry of Na^+ and $C\alpha^{2+}$, egress of K^+, and hence neuronal depolarization. It belongs to the transmitter-gated cation superfamily of receptors and appears to be located exclusively in neuronal tissue where it mediates fast depolarization (Malone et al. 1991). Responses are blocked by a wide range of potent antagonists, which are highly selective with respect to other 5-HT receptors (Silverstone and Greenshaw 1996). The Bezold-Jarisch reflex can be elicited by 5-HT$_3$ receptor agonists and be blocked by 5-HT$_3$ antagonists. 5-HT$_3$ receptor antagonists are used as antimigraine drugs. 5-HT$_3$ receptors are involved in feeding behavior (see chapter "▶ Assays of Anti-Obesity Activity"). Furthermore, gastric emptying (see chapter "▶ Pharmacological Effects on Intestinal Functions") and emesis (see chapter "▶ Emetic and Anti-Emetic Activity") can be influenced by 5-HT$_3$ antagonists.

5-HT$_3$ receptor antagonists, such as **ondansetron** (Koulu et al. 1990; Ishizuka et al. 1993; Miyata et al. 1995; Cappelli et al. 1996), **granisetron** (Sanger and Nelson 1989; Ito et al. 1995; Endo et al. 1999; Yan and White 2005), **tropisetron** (Macor et al. 2001; de la Vega et al. 2005), **zacopride** (Waeber et al. 1990; Kidd et al. 1992), and **alosetron** (Clayton et al. 1999) are used clinically in the prevention of cisplatin-induced emesis (Karim et al. 1996; Roila et al. 1997).

The synthesis and pharmacological profile of several selective 5-HT$_3$ receptor antagonists have been reported (Fitzpatrick et al. 1990; Rosen et al. 1990; Swain et al. 1991; Ito et al. 1992; Bachy et al. 1993; Heidempergher et al. 1997; Rival et al. 1998; Modica et al. 2004).

Boess et al. (1997) analyzed the ligand binding site of the 5-KT$_3$ receptor using site direction mutagenesis.

See section "5-HT$_3$ Receptor in Rat Entorhinal Cortex Membranes: Binding of [3H]GR 65630" for binding assays to the 5-HT$_3$ receptor.

5-HT$_4$ Receptors. The *5-HT$_4$ receptors* couple preferentially to GS protein and activate adenylate cyclase, thereby increasing intracellular cAMP levels (Martin 1998). This induces long-term modulation of ion channel activity, which is fundamental in learning and memory (Eglen et al. 1995; Eglen and Hegde 1966). Excitatory responses were found in guinea pig ileum and colon and inhibitory responses in rat esophagus (Ford and Clarke 1993).

The tunica muscularis mucosae preparation of the rat esophagus has been recommended for evaluation of 5-HT$_4$ receptor ligands since it possesses a homogeneous population of 5-HT4 receptors which mediates a well-defined relaxant response to 5-HT (see chapter "▶ Tests on Esophagus").

5-HT$_4$ receptors may mediate arrhythmias (Kaumann 1994). Two splice variants have been identified (Gerald et al. 1995). Several selective 5-HT$_4$ receptor agonists and antagonists were described (Gaster et al. 1995; Eglen and Hegde 1966; Eglen 1967).

Dumuis et al. (1992) characterized DAU 6285, a novel 5-HT$_4$ receptor antagonist of the azabicycloalkyl benzamidazolone class.

Wardle et al. (1994) described the effects of SB 204070, a highly potent and selective 5-HT$_4$ receptor antagonist, on guinea pig distal colon.

The 5-HT4 binding site in human brain was characterized by Arranz et al. (1998).

López-Rodríguez et al. (1999) reported the synthesis and structure-activity relationships of new benzimidazole-4-carboxamides and carboxylates as potent and selective 5-HT$_4$ receptor antagonists.

Radioligand binding assays for 5-HT_4 receptors using [^{3}H]-GR113808 have been described by Grossman et al. (1993), Domenech et al. (1994), Schiavi et al. (1994), Katayama et al. (1995), and Ansanay et al. (1996).

Brattelid et al. (2004) reported cloning, the pharmacological characterization and tissue distribution of a novel 5-HT_4 receptor splice variant, 5-HT_{4i}.

A survey on molecular biology and potential functional role of **5-HT_5, 5-HT_6, and 5-HT_7 receptors** was given by Branchek and Zoombick (1997).

A review on 5-HT_5 receptors was given by Nelson (2004).

5-HT_6. The rat *5-HT_6* receptor has been cloned by Ruat et al. (1993). The distinguishing features of this receptor are the high affinity for a series of antipsychotic compounds as well as affinity for a number of cyclic antidepressants (Branchek 1995).

Bourson et al. (1995) used antisense oligonucleotides to determine the role of the 5-HT_6 receptor in the rat brain.

Boess et al. (1998) reported labeling of 5-hydroxytryptamine binding sites in rat and porcine striatum by the 5-HT_6 receptor-selective ligand [^{3}H]Ro 63-0563.

Branchek and Blackburn (2000) reviewed 5-HT_6 receptors as emerging targets for drug discovery.

A review on 5-HT_6 receptors was given by Wooley et al. (2004).

Routledge et al. (2000) characterized SB-271046 as a potent, selective, and orally active 5-HT_6 receptor antagonist.

Wooley et al. (2003) reported the reversal of cholinergic-induced deficit in a rodent model of recognition memory by the selective 5-HT_6 receptor antagonist Ro 04-6790.

5-HT_7. Lovenberg et al. (1993), Shen et al. (1993), Gobbi et al. (1996), Villalón et al. (1997) described an adenyl cyclase-activating serotonin receptor (*5-HT_7*) implicated in the regulation of mammalian circadian rhythms.

A receptor autoradiographic and hybridization analysis of the distribution of the 5-HT_7 receptor in rat brain was reported by Gustafson et al. (1996).

Stowe and Barnes (1998) used [^{3}H]5$^-$ carboxamidotryptamine for selective recognition sites in rat brain.

Three different splice variants of the 5-HT_7 receptor have been described both in rat and human tissues (Vanhoenacker et al. 2000).

Roth et al. (1994) described the binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors.

Hedlund and Sutcliffe (2004) reviewed the functional, molecular, and pharmacological advances in 5-HT_7 receptor research.

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Serotonin (5-HT_{1A}) Receptor: Binding of [^{3}H]-8-Hydroxy-2-(di-n-Propylamino)-Tetralin ([^{3}H]-DPAT)

Purpose and Rationale

Determination of the affinity of test compounds for the 5-HT_{1A} receptor in brain may be useful for predicting compounds with novel anxiolytic or atypical antipsychotic profiles.

The existence of at least two populations of 5-HT_1 receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated as the 5-HT_{1A} subtype and the insensitive receptors referred to as the 5-HT_{1B} subtype (Middlemis and Fozard 1983). Other 5-HT binding sites (5-HT_{1C}, 5-HT_{1D}, 5-HT_3, and 5-HT_4) have subsequently been identified in various species, based on differential sensitivity to 5-HT antagonists (Peroutka 1988).

Schlegel and Peroutka (1986) identified [^{3}H]DPAT as a selective ligand for the 5-HT_{1A} receptor. These authors reported that [^{3}H]DPAT labeled an autoreceptor. Lesion studies suggest that [^{3}H]DPAT labeled receptors are not terminal autoreceptors but may be somatodendritic autoreceptors (Gozlan et al. 1983). Although DPAT decreases the firing rate in the raphe nucleus and inhibits 5-HT release, the actual location and function is somewhat controversial (Verge et al. 1986). These studies and the sensitivity of [^{3}H]DPAT binding to guanine nucleotides and effects on adenylate cyclase suggest that DPAT acts as an agonist at the 5-HT_{1A} receptor (Schlegel and Peroutka 1986).

Serotonin may play a role in anxiety, since drugs which reduce serotoninergic function have anxiolytic effects in animal models (Dourish et al. 1986). Since buspirone and its analogues have relatively higher affinity for the 5-HT_{1A} receptor than other receptors and no effect on the benzodiazepine site, their anxiolytic properties are attributed to activity at the 5-HT_{1A} receptor (Verge et al. 1986; Iversen 1984; Traber and Glaser 1987).

Besides 5-HT_{1A} receptor agonists (Misslin et al. 1990; Griebel et al. 1992; Hascoet et al. 1994; Stanhope and Dourish 1996), 5-HT_{1A} receptor antagonists (Traber and Glaser 1987; Fletcher et al. 1996; Johansson et al. 1997; Cao and Rodgers 1998), 5-HT_{2A} receptor antagonists (Griebel 1996), 5-HT_{2C} receptor antagonists (Jenck et al. 1998), mixed receptor agonists/antagonists (Kleven et al. 1997), 5-HT_3 receptor antagonists (Artais et al. 1995; Roca et al. 1995), and 5-HT_4 receptor antagonists (Kennett et al. 1997) exhibit anxiolytic properties (Handley and McBlane 1993).

Fletcher et al. (1995) described visualization and characterization of 5-HT receptors and transporters in vivo and in man.

Several 5-HT_{1A} receptor antagonists were described in preclinical studies, such as **NAN-190** (Deans et al. 1989; Rydelek-Fitzgerald et al. 1990; Neckelmann et al. 1996), (−)-**alprenolol** (Bjorvatn et al. 1992), **pindolol** (Bel et al. 1994; Dreshfield et al. 1996; Seifritz et al. 1997), **BMY 7378** (Sharp et al. 1990; Grasby et al. 1992), **WAY100635** (Fletcher et al. 1996; Trillat et al. 1998; Harder and Ridley 2000; Smart and Biello 2001), and (−)**tertatolol** (Jolas et al. 2004).

Millan et al. (1999, 2001), Mattson et al. (2003), and Gilbert et al. (2004) described specific 5-HT_{1A} receptor antagonists.

Procedure

Reagents

1. Tris buffers, pH 7.7
   (a) 57.2 g Tris HCl
   16.2 g Tris base
   q.s. to 1 l with distilled water (0.5 M Tris buffer, pH 7.7)
   (b) Make a 1:10 dilution in deionized H_2O (0.05 M Tris buffer, pH 7.7)
   (c) 0.05 M Tris buffer, pH 7.7 containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1 % ascorbic acid
   0.49 mg pargyline HCl
   111 mg CaCl2
   250 mg ascorbic acid
   q.s. to 250 ml with 0.05 M Tris buffer, pH 7.7 (reagent 1b)
2. [^{3}H]-DPAT (2-(N,N-Di[2,3(n)$^{-3}$H] propylamino)-8-hydroxy-1,2,3,4-tetrahydro-naphthalene) (160–206 Ci/mmol) was obtained from Amersham. For IC_{50} determinations: a 10 nM stock solution is made up and 50 μl added to each tube (final concentration = 0.5 nM).
3. Serotonin creatinine sulfate. 0.5 mM stock solution is made up in 0.01 N HCl and 20 μl added to three tubes for determination of nonspecific binding (final concentration = 10 μM).
4. Test compounds:
   For most assays, α 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Hippocampi are removed, weighed, and homogenized in 20 volumes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min and the supernatant discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37 °C for 10 min, and recentrifuged at 48,000 *g* for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM $CaCl_2$, 0.1 % ascorbic acid, and 10 μM pargyline.

Assay

800 μl Tissue
130 μl 0.05 M Tris + $CaCl_2$ + pargyline + ascorbic acid
20 μl vehicle/5-HT/drug
50 μl [^{3}H]DPAT

Tubes are incubated for 15 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed two times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 10 μM 5-HT. IC_{50} values are calculated from the percent specific binding at each drug concentration. The *K*i value may then be calculated by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

The K_D value for [^{3}H] DPAT binding was found to be 1.3 nM by Scatchard analysis in a receptor saturation experiment.

Modification of the Method

Yocca et al. (1987) described BMY 7378, a buspirone analogue with high affinity, selectivity, and low intrinsic activity at the 5-HT_{1A} receptor in rat and guinea pig hippocampal membranes.

Instead of the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (DPAT), selective antagonists to the 5-HT_{1A} receptor, 4-(methoxyphenyl)1-[2′-(n-2″ -pyridinyl)-p-iodobenzamido]-ethyl-piperazine ([^{125}I]p-MPPI), and [^{3}H]p-MPPF = 4-(2′methoxy-)-phenyl-1-[2′-(A-2″-pyridyl)-*p*-fluorobenzamido] ethyl-piperazine have been recommended (Kung et al. 1994a, b, 1995, 1996).

Several other selective 5-HT_{1A} receptor radioligands were recommended:

[^{3}H]lisuride (Sundaram et al. 1995); [^{3}H]WAY-100635 ([*O*-methyl-^{3}H]-N-(2-(4-(2-methoxyphenyl)1-piperazinyl)ethyl)-*N*-(2-piridinyl)cyclohexane carboxamide trihydrochloride) (Laporte et al. 1994; Gozlan et al. 1995; Hume et al. 1995; Khawaja 1995; Khawaja et al. 1997); [^{3}H] Alnespirone (Fabre et al. 1997); [Carbonyl ^{11}C]-Desmethyl-WAY 100635 (Pike et al. 1998); [^{3}H]S 15535 (4-(benzodioxan-5-yl)-1-(indan-2-yl)piperazine) (Newman-Tancredi et al. 1998a); NAD-299 (= (R)-3-N,*N*-dicyclobutylamino-8-fluoro[6−^{3}H]-3,4-dihydro-2H-1-benzopyran-carboxamide) (Jerning et al. 1998; Sanell et al. 1999).

Newman-Tancredi et al. (1998b) performed autoradiographic studies with [35S]-GTPγS and the selective radioligand [^{3}H]S 15535 for parallel evaluation of localization and functionality of the 5-HT$_{1A}$ receptor.

Agonist-Stimulated [^{35}S]-GTPγ S Binding

Agonist-stimulated [35S]-GTPγ S binding is the initial step in signal transduction following G protein activation (Elmendorf et al. 1998; Standaert et al. 1998; Lee et al. 1999). It can be used as an index of receptor stimulation, such as 5-HT$_{1A}$ receptors (Alper and Nelson 1998, 2000; Hughes et al. 2005).

Procedure

Sprague Dawley rats were killed by decapitation, the brains placed in ice-cold 0.9 % NaCl for 1–3 min, the hippocampi dissected free-hand and placed in a vial on ice. The tissue was weighed and frozen at −70 °C. Upon defrosting the tissue was homogenized using a Tekmar tissue homogenizer in cold Tris buffer (50 mM Tris base, pH 7.4). The homogenate was centrifuged (39,800 *g*, 4 °C for 10 min), the supernatant decanted, and the remaining pellet resuspended in the same Tris buffer. After homogenization the suspension was incubated at 37 °C for 10 min in a shaking water bath. The suspension was then centrifuged, the supernatant decanted, and the pellet again washed with cold Tris buffer. After one final centrifugation, the remaining pellet was homogenized in the same Tris buffer at a final concentration of approximately 100 mg tissue/ml. This suspension was frozen in aliquots at −70 °C for later use. On the day of the assay, the frozen tissue suspension was defrosted, resuspended in approximately 35 ml Tris buffer, and centrifuged as above. This final membrane pellet was suspended in assay buffer (4 mM $MgCl_2$, 160 mM NaCl, 0.267 mM EGTA, 67 mM Tris base, pH 7.4) to produce a protein concentration approximating 200 μg/ml.

GTPγ S Binding Assay

To each assay tube was added 200 μl deionized water or drug, 200 μl [35S]GTPγ S (0.1 nM) in ligand buffer (assay buffer containing 1200 μM GDP to produce a final concentration of 300 μM), 200 μl assay buffer, and 200 μl tissue homogenate (approximately 30–50 μg protein). The assay tubes were incubated for 30 min in a shaking water bath. All assays were conducted at 37 °C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters prewet with 20 mM $Na_4P_2O_7$. $10H_2O$ and precooled with three washes of 2 ml cold 50 mM Tris buffer. After filtering the reaction mixture, the filters were washed four times with 1 ml cold 50 mM Tris buffer, placed into 7-ml scintillation vials, and 5 ml Ready Protein scintillation cocktail added. The samples were shaken, allowed to sit for a minimum of 2 h, shaken again, and counted (2 min/sample) in a Beckman scintillation counter. Nonspecific binding was determined by the amount of [^{35}S]-GTPγ S bound in the presence of 10 μM unlabeled GTPγ S and was subtracted from all samples. Basal GTPγ S binding is defined as the specific binding when 200 μl water containing no ligand was added to the assay tube. This is also referred to as agonist-independent binding.

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Serotonin (5-HT_{1B}) Receptors in Brain: Binding of [^{3}H]5-Hydroxytryptamine ([^{3}H]5-HT)

Purpose and Rationale

The purpose of this assay is to determine the affinity of test compounds for the serotonin (5-HT_{1B}) receptor in brain.

The existence of two populations of 5-HT_1 receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated as the 5-HT_{1A} subtype and the insensitive receptors referred to as the 5-HT_{1B} subtype (Middlemiss and Fozard 1983). The 5-HT1B subtype has been identified in the brain of rats and mice (Peroutka 1986) and can be selectively labeled by 5-HT in rat striatum when spiroperidol is included to mask the 5-HT_{1A} and 5-HT_2 receptors. In contrast to the situation in rats and mice, [^{3}H]5-HT binding in the basal ganglia of other mammals displays a pharmacological profile characteristic of 5-HT_{1D} sites. The distribution of 5-HT_{1B} sites in rat brain is similar to that of 5-HT_{1D} sites in human brain (Segu et al. 1991; Boulenguez et al. 1992; Palacios et al. 1992). Several 5-HT_{1B} antagonists have been described (Price et al. 1997; Selkirk et al. 1998).

By comparing the results in the 5-HT_{1B} assay with those in the 5-HT_{1A}, 5-HT_2, and the 5-HT_3 receptor binding assays the relative affinity of a test compound for the major subclasses of 5-HT receptors in the rat brain can be determined.

Procedure

Reagents

1. Tris buffers, pH 7.7
   (a) 57.2 g Tris HCl
   16.2 g Tris base
   q.s. to 1 l with distilled water (0.5 M Tris buffer, pH 7.7)
   (b) Make a 1:10 dilution in deionized H_2O (0.05 M Tris buffer, pH 7.7)

(c) 0.05 M Tris buffer, pH 7.7 containing 10 m Mpargyline, 4 mM $CaCl_2$ and 0.1 % ascorbic acid.
0.49 mg pargyline HCl
110.99 mg $CaCl_2$
250 mg ascorbic acid
q.s. to 250 ml with 0.5 M Tris buffer, pH 7.7.

2. 5-Hydroxy[G-^{3}H]tryptamine creatinine sulfate (17–20 Ci/mmol) (Amersham). For IC_{50} determinations: a 40 nM stock solution is made up and 50 ml added to each tube (final concentration = 2.0 nM).
3. Serotonin creatinine sulfate. A 0.5 mM stock solution is made up in 0.01 N HCl and 20 ml added to three tubes for determination of nonspecific binding (final concentration = 10 µM).
4. Spiroperidol is dissolved in dilute glacial acetic acid. A 20 µM stock solution is prepared and 50 µl added to each tube to prevent binding to 5-HT_{1A} and 5-HT_2 receptors (final concentration in the assay = 1 µM).
5. Test compounds:
For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed, and homogenized in 20 volumes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min and the supernatant discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37 °C for 10 min, and recentrifuged at 48,000 *g* for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM $CaCl_2$, 0.1 % ascorbic acid, and 10 mM pargyline.

Assay

800 µl tissue
80 µl 0.05 M Tris + $CaCl_2$ + pargyline + ascorbic acid
20 µl vehicle/5-HT/drug
50 µl [^{3}H]5-HT
50 µl spiroperidol

Tubes are incubated for 15 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed two times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 10 µM 5-HT. IC_{50} values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

The K_D value for [^{3}H] 5-HT binding was found to be 16.5 nM by Scatchard analysis of a receptor saturation experiment.

Modification of the Method

[^{3}H]CP-96,501 = 3-(1,2,5,6-Tetrahydro-4-pyridyl-5-npropoxyindole was recommended as a selective 5-HT_{1B} receptor radioligand (Koe et al. 1992; Lebel and Koe 1992).

Domenech et al. (1997) characterized human serotonin 1D and 1B receptors using [^{3}H]-GR-125743, a novel radiolabeled serotonin 5-$HT_{1D/1B}$ receptor antagonist.

[^{3}H]mesulergine has been used as radioligand for 5-HT1C receptor binding (Jenck et al. 1993, 1994).

[^{3}H]-5-carboxytryptamine was recommended as label for 5-HT_{1D} binding sites (Mahle et al. 1991; Nowak et al. 1993).

Massot et al. (1998) described molecular, cellular, and physiological characteristics of

5-HT-moduline, a tetrapeptide (Leu-Ser-Ala-Leu), acting as endogenous modulator of the 5-HT_{1B} receptor subtype.

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5-HT_3 Receptor in Rat Entorhinal Cortex Membranes: Binding of [^{3}H]GR 65630

Purpose and Rationale

The purpose of this assay is to determine the affinity of test compounds for the 5-HT_3 binding site in the brain. This assay may be useful for predicting the potential of compounds to exhibit antiemetic, anxiolytic, or atypical antipsychotic profiles.

The 5-HT_3 binding site has also been characterized on operational, transductional, and structural characteristics. Originally it was believed that 5-HT_3 binding sites existed only in the periphery (Costall et al. 1988). However, with the introduction of potent and selective 5-HT_3 antagonists such as GR65630, zacopride, ICS 205 930, and MDL 72222 and agonists, data from binding studies have indicated that 5-HT_3 binding sites are also located in selected areas of the brain (Kilpatrick et al. 1987, 1989; Barnes et al. 1988, 1990, 1992; Watling et al. 1988; Miller et al. 1992). The highest levels of 5-HT_3 binding sites have been detected in limbic and dopamine-containing brain areas (entorhinal cortex, amygdala, nucleus accumbens, and tuberculum olfactorium) (Costall et al. 1988). Besides possessing selective binding in dopamine-rich areas, 5-HT_3 antagonists have been reported to block behavioral effects associated with certain drugs of abuse (nicotine and morphine) and to be active in behavioral tests predictive of anxiolytic activity. Based on these selective regional binding results and behavioral studies, it has been speculated that 5-HT_3 antagonists may have a therapeutic benefit in disease states believed to be associated with excessive dopaminergic activity; i.e., schizophrenia, anxiety, and drug abuse.

Several authors described synthesis, pharmacology, and therapeutic potential of H_3 receptor agonists and antagonists (Leurs et al. 1995, 1998; Stark et al. 1996).

Procedure

Reagents

1. 0.05 MKrebs-HEPES buffer, pH 7.4
   11.92 g HEPES
   10.52 g NaCl
   0.373 g KCl
   0.277 g $CaCl_2$
   0.244 g $MgCl_2.6H_2O$
   q.s. to 1 l with distilled H_2O bring pH up to 7.4 (at 4 °C) with 5 N NaOH
2. [^{3}H]GR65630 (87.0 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations: [^{3}H]GR65630 is made up to a concentration of 1.0 nM in Krebs-HEPES buffer such that when 100 µl is added to each tube a final concentration of 0.4 nM is attained in the 250 µl assay.

3. Ondansetron HCl (GR 38032 F) is made up to a concentration of 500 μM in Krebs-HEPES buffer. 50 μl is added to each of three tubes for the determination of nonspecific binding (yielding a final concentration of 100 μM in the 250 μl assay.
4. Test compounds. For most assays, a 50 μM stock solution is made up in a suitable solvent and serially diluted with Krebs-HEPES buffer such that when 50 μl of drug is combined with the total 250 μl assay, a final concentration from 10^{-5} to 10^{-8} M is attained. Characteristically seven concentrations are studied for each assay; however, higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats (150–200 g) are decapitated and the entorhinal cortex removed, weighed, and homogenized in 10 volumes of ice-cold 0.05 M Krebs-HEPES buffer, pH 7.4. The homogenate is centrifuged at 48,000 *g* for 15 min at 4 °C. The resulting pellet is rehomogenized in fresh Krebs-HEPES buffer and recentrifuged at 48,000 *g* for 15 min at 4 °C. The final pellet is resuspended in the original volume of ice-cold KrebsHEPES buffer. This yields a final tissue concentration of 1.2–1.6 mg/ml with the addition of 100 ml to the assay. Specific binding is approximately 55–65 % of total bound ligand.

Assay

100 μl Tissue suspension
100 μl [^{3}H]GR65630
50 μl Vehicle (for total binding) or 500 mM ondansetron HCl (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept on ice for additions, then vortexed and incubated with continuous shaking for 30 min at 37 °C. At the end of the incubation period, the incubate is diluted with 5 ml of ice-cold Krebs-HEPES buffer and immediately vacuum filtered through Whatman GF/B filters, followed by two 5-ml washes with ice-cold Krebs-HEPES buffer. The filters are dried and counted in 10 ml of liquid scintillation cocktail.

Evaluation

Specific GR65630 binding is defined as the difference between the total binding and that bound in the presence of 100 μM Ondansetron HCl. IC_{50} calculations are performed using computer-derived log-probit analysis.

Modification of the Method

Dunn et al. (1991) found that preclinical data with 5-HT3 antagonists predict anxiolytic rather than antipsychotic activity.

Davies et al. (1999) found that the 5-HT_{3B} subunit is a major determinant of serotonin receptor function.

Reiser and Hamprecht (1989) reported that substance P and serotonin (via 5-HT_3 receptors) act synergistically to activate the cation permeability as measured by [^{14}C]guanidinium uptake in neuroblastoma × glioma hybrid cells.

Bönisch et al. (1993) studied the 5-HT_3 receptor-mediated cation influx into N1E-115 mouse neuroblastoma cells by the use of the organic cation [^{14}C]guanidinium.

Emerit et al. (1993) assessed the [^{14}C] guanidium accumulation in cells of the hybridoma (mouse neuroblastoma × rat glioma) clone NG 108–15 exposed to serotonin 5-HT_3 receptor ligands and substance P.

Bonhaus et al. (1993) characterized the binding of [^{3}H]endo-N-(8-methyl-8-azabicyclo[3.2.1.]oct-3-yl)2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazolone-1 carboxamide hydrochloride ([^{3}H]BIMU-1), a benzimidazolone with high affinity to 5-HT_3 and 5-HT_4 receptors in NG 108 cells and guinea pig hippocampus.

Kooyman et al. (1994) studied the specific binding of [^{3}H]GR65630 to 5-HT_3 recognition sites in cultured N1E-115 mouse neuroblastoma cells.

Several other selective 5-HT_3 receptor radioligands were recommended:

[^{3}H]Quipazine (Perry 1990); [^{3}H]GR67330 (Kilpatrick et al. 1990); tritium-labeled 1-methyl-N-[8]-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1H-indazole-3-carboxamide (Robertson et al. 1990); [^{125}I]-(S)-iodozacopride (Gehlert et al. 1993); [^{3}H]-BRL46470 (Steward et al. 1995); [^{3}H]YM060 (Azukawa et al. 1995);

(^{125}I)iodophenpropit (Jansen et al. 1994), (^{125}I) iodoproxyfan (Ligneau et al. 1994), (S)-Des-4-amino-3-[^{125}I]iodozacopride (Mason et al. 1996; Hewlett et al. 1998,1999).

Tairi et al. (1998) and Hovius et al. (1999) studied ligand binding to the serotonin 5-HT$_3$ receptor with a novel fluorescence ligand and developed a total internal reflection fluorescence assay which is suitable for high-throughput screening.

Thompson et al. (2005) reported locating an antagonist in the 5-HT$_3$ receptor binding site using modeling and radioligand binding.

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Histamine H_3 Receptor Binding in Brain

Purpose and Rationale

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H_1 receptor couples mainly to $G_{q/11}$, thereby stimulating phospholipase C, whereas the H_2 receptor interacts with Gs to activate adenylyl cyclase. The histamine H_3 and H_4 receptors couple to Gi proteins to inhibit adenylyl cyclase and to stimulate MAPK. The histamine H_3 receptor is regarded as a therapeutic target for cognitive and sleep disorders (Leurs et al. 1991, 1998, 2005; Passani et al. 2004).

Histamine modulates its own synthesis and release from depolarized brain slices or synaptosomes by interacting with H_3 autoreceptors with a pharmacology distinct from that of H_1 and H_2 receptors (Arrang et al. 1985, 1987, 1990; Hill 1990, 1992; Hill et al. 1997; Leurs et al. 1991, 1998). The R-isomer of α-methylhistamine (α-MeHA) was identified as a highly selective H_3-receptor agonist active at nanomolar concentrations. Furthermore, this compound in ^{3}H-labeled form is a suitable probe for the H_3-receptor.

Procedure

The cerebral cortex from guinea pigs is dissected and homogenized in 50 volumes ice-cold 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, in a Potter homogenizer. The homogenate is centrifuged for 15 min at 750 g. The pellet is discarded and the supernatant centrifuged at 42,000g for 15 min. The supernatant is discarded and the pellet washed superficially and then resuspended in fresh buffer. The protein concentration of the membrane suspension as determined according to Lowry et al. (1951) is about 0.3–0.4 mg/ml.

Aliquots of the membrane preparation are incubated for 60 min at 25 °C with ^{3}H(R) α-MeHA and unlabeled substances in a final volume of 1 ml. The assay is stopped by dilution with 2 × 3 ml ice-cold medium, followed by rapid filtration under vacuum over Millipore AAWP filters which are then rinsed twice with 5 ml of ice-cold medium. Radioactivity retained on the filters is measured by liquid scintillation spectroscopy.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 10 μM unlabeled α-MeHA. IC_{50} values are calculated from the percent specific binding at each drug concentration. The Ki value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

Modification of the Method

Jansen et al. (1992) described [^{125}I] iodophenpropit as a radiolabeled histamine H_3 receptor antagonist.

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Anticonvulsant Activity

Pentylenetetrazole (Metrazol)-Induced Convulsions

Purpose and Rationale

This assay has been used primarily to evaluate antiepileptic drugs. However, it has been shown that most anxiolytic agents are also able to prevent or antagonize Metrazol-induced convulsions.

Procedure

Mice of either sex with a body weight between 18 and 22 g are used. The test compound or the reference drug is injected sc. or i.p. or given orally to groups of 10 mice. Another group of 10 mice serves as control. Either min after s.c. injection, 30 min after i.p. injection, or 60 min after oral administration 60 mg/kg MTZ (Metrazol) are injected subcutaneously. Each animal is placed into an individual plastic cage for observation lasting 1 h. Seizures and tonic-clonic convulsions are recorded. At least 80 % of the animals in the control group have to show convulsions.

Evaluation

The number of protected animals in the treated groups is calculated as percentage of affected animals in the control group. ED_{50} values can be calculated. Furthermore, the time interval between MTZ injection and occurrence of seizures can be measured. The delay of onset is calculated in comparison with the control group.

Critical Assessment of the Method

The method is widely accepted as a screening procedure and has been modified by many investigators. Chlordiazepoxide (20 mg/kg i.p.), diphenylhydantoin (10 mg/kg i.p.), and phenobarbitone sodium (20 mg/kg i.p.) were found to be effective. Predominantly, the muscle relaxant and anticonvulsant effects of benzodiazepines are measured by this test. Stimulant, antidepressant, neuroleptic, and some antiepileptic drugs do not show MTZ antagonism at tolerable doses (Lippa et al. 1979). Nevertheless, the antagonism of MTZ-induced seizures appears to be a suitable procedure for detecting compounds with potential anxiolytic activity. Among a battery of tests, the MTZ antagonism has been proposed to study centrally acting skeletal muscle relaxants (Bastian et al. 1959; Domino 1964).

Modification of the Method

Different routes of administration (i.p., i.v., s.c.) have been used by various investigators. Moreover, the dose of MTZ which causes seizures in 80–90 % of the animals varies with the strain being used.

Bastian et al. (1959) published a modification, whereby mice are infused with a MTZ solution through a small-diameter polyethylene tubing into the tail vein. In the same animal three end points are registered: (1) onset of persistent clonic convulsions, (2) beginning of the tonic flexor phase, and (3) time to death. The three end points are affected differently by various drugs providing the basis for the determination of drug specificity.

Löscher et al. (1991) tested eight clinically established antiepileptic drugs in three pentylentetrazole seizure models: (1) the threshold for different types of pentylentetrazole seizures, i.e., initial myoclonus twitch, generalized clonus with loss of righting reflexes, and tonic backward extension of forelimbs (forelimb tonus) in mice; (2) the traditional pentylentetrazole seizure test with s.c. injection of the CD97 for generalized clonic seizures in mice; and (3) the s.c. pentylentetrazole seizure test in rats. Various factors may cause misleading predictions from pentylentetrazole seizure models.

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Strychnine-Induced Convulsions

Purpose and Rationale

The convulsing action of strychnine is due to interference with postsynaptic inhibition mediated by glycine. Glycine is an important inhibitory transmitter to motoneurons and interneurons in the spinal cord, and strychnine acts as a selective, competitive antagonist to block the inhibitory effects of glycine at all glycine receptors. Strychnine-sensitive postsynaptic inhibition in higher centers of the CNS is also mediated by glycine. Compounds which reverse the action of strychnine have been shown to have anxiolytic properties.

Procedure

Groups of 10 mice of either sex with a weight between 18 and 22 g are used. They are treated orally with the test compound or the standard (e.g., diazepam 5 mg/kg). One hour later the mice are injected with 2 mg/kg strychnine nitrate i.p. The time until occurrence of tonic extensor convulsions and death is noted during α 1 h period. With this dose of strychnine convulsions are observed in 80 % of the controls.

Evaluation

ED_{50} values are calculated using various doses taking the percentage of the controls as 100 %. For time response curves the interval between treatment and strychnine injection varies from 30 to 120 min.

Critical Assessment of the Method

The method has been proven to be useful in a battery of tests to characterize CNS-active drugs. (Costa et al. 1975).

Modification of the Method

McAllister (1992) induced spinal seizures in mice by rotating them along the body axis clockwise and anticlockwise alternatively three times following pretreatment with a subconvulsive dose of strychnine.

Lambert et al. (1994) tested the antagonism of a glycine derivative against seizures induced by 3-mercaptopropionic acid (3-MPA).

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Picrotoxin-Induced Convulsions

Purpose and Rationale

Picrotoxin-induced convulsions are used to further evaluate CNS-active compounds. Picrotoxin is regarded as a $GABA_A$ antagonist modifying the function of the chloride ion channel of the $GABA_A$ receptor complex.

Procedure

Groups of 10 mice of either sex with a weight between 18 and 22 g are treated either orally or i.p. with the test compound or the standard (e.g., 10 mg/kg diazepam i.p.). Either 30 min after i.p.- treatment or 60 min after oral administration the animals are injected with 3.5 mg/kg

s.c. picrotoxin and are observed for the following symptoms during the next 30 min: clonic seizures, tonic seizures, death. Times of onset of seizures and time to death are recorded.

Evaluation

For time-response curves the animals receive the drug 30, 60, or 120 min prior to picrotoxin. Protection is expressed as percent inhibition relative to vehicle control. The time period with the greatest percent inhibition is said to be the peak time of drug activity. ED_{50} values are calculated taking the percentage of seizures in the control group as 100 %.

Critical Assessment of the Method

The method has been proven to be of value among a battery of tests for CNS activity.

Modification of the Method

Buckett (1981) describes an intravenous bicuculline test in mice (see chapter "► Anti-Epileptic Activity"). The compound bicuculline antagonizes the action of GABA by competition on postsynaptic receptors. In the whole animal bicuculline reproducibly induces myoclonic seizures. An intravenous dose of 0.55 mg/kg was found to induce myoclonic seizures in 90–100 % of mice with less than 10 % mortality. GABAergic compounds such as benzodiazepines at relatively low doses antagonize the bicuculline-induced myoclonic seizures.

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Isoniazid-Induced Convulsions

Purpose and Rationale

Isoniazid can precipitate convulsions in patients with seizure disorders. The compound is regarded as a GABA synthesis inhibitor (Costa et al. 1975). Clonic-tonic seizures are elicited in mice which are antagonized by anxiolytic drugs.

Procedure

Ten mice of either sex with a weight of 18–22 g are treated with the test compound or the standard (e.g., diazepam 10 mg/kg i.p.) by oral or intraperitoneal administration. Controls receive the vehicle only. Either 30 min after i.p. or 60 min after p.o. treatment the animals are injected with a subcutaneous dose of 300 mg/kg isoniazid (isonicotinic acid hydrazide). During the next 120 min the occurrence of clonic seizures, tonic seizures, and death is recorded.

Evaluation

The percentage of seizures or death occurring in the control group is taken as 100 %. The suppression of these effects in the treated groups is calculated as percentage of controls. ED_{50} values are calculated.

Critical Assessment of the Method

The method has been proven to be of value among a battery of tests for CNS activity.

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Yohimbine-Induced Convulsions

Purpose and Rationale

Antagonism against yohimbine-induced seizures in mice is considered to be a model predictive of potential anxiolytic and GABA-mimetic agents (Dunn and Fielding 1987).

Procedure

Male Swiss-Webster mice (20–30 g) are individually placed in clear plastic cylinders and test compounds administered i.p. 30 min prior to 45 mg/kg s.c. of yohimbine HCl. The animals are observed for the onset and number of clonic seizures for 60 min.

Evaluation

ED_{50} values with 95 % confidence limits are calculated for the antagonism of yohimbine-induced clonic seizures by means of the Lichtfield-Wilcoxon procedure.

Critical Assessment of the Method

The antagonism against yohimbine-induced seizures can be regarded as a useful test among a battery of tests for anxiolytic activity.

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Antiaggressive Activity

Foot-Shock-Induced Aggression

Purpose and Rationale

Since the discovery of the taming effects of benzodiazepines in vicious monkeys (Heise and Boff 1961; Randall et al. 1961) tests for agents with antiaggressive activity have been developed for various animal species. These tests include foot-shock-induced aggression in mice and rats, fighting behavior of isolated mice, and aggressiveness of rats which become extremely vicious after lesions in the septal area of the brain (Brady and Nauta 1953).

Foot-shock-induced aggression is used for further characterization of centrally active drugs. Irwin et al. (1971) have attempted to compare drug classes with this method.

Procedure

Male mice (NMRI, Ivanovas) with a weight between 20 and 30 g are used. Two mice are placed in a box with a grid floor consisting of steel rods with a distance of 6 mm. A constant current of 0.6 or 0.8 mA is supplied to the grid floor by a LVE constant-current shocker with an associated scrambler. A 60-Hz current is delivered for 5 s followed by 5 s intermission for 3 min. Each pair of mice is dosed and tested without previous exposure. The total number of fights is recorded for each pair during the 3-min period. The fighting behavior consists of vocalization, leaping, running, rearing, and facing each other with some attempt to attack by hitting, biting, or boxing. The test compound or the standard are applied either 30 min before the test i.p. or 60 min before the test orally. For a time response, the drug is given 30, 60, and 120 min prior to testing. Six pairs of drug-treated and two pairs of vehicle-treated animals are utilized for each time period. A dose range is tested at the peak of drug activity. A minimum of 3 doses (10 pairs of mice/dose) is administered for a range of doses. Control animals receive the vehicle.

Evaluation

The percent inhibition of aggression is calculated from the vehicle control. ED_{50} values are calculated.

Critical Assessment of the Method

Not only anxiolytics but also other classes of drugs were found to be active in this test such as sedatives like meprobamate and phenobarbital, neuroleptics such as perphenazine, analgesics such as methadone, and ethyl alcohol.

Modification of the Method

A survey of aggressive behavior in the **rat** has been given by Blanchard and Blanchard (1977).

Induction of aggressive behavior by electrical stimulation in the hypothalamus of male rats was described by Kruk et al. (1979).

Mos and Olivier (1991) reviewed the concepts in animal models for pathological aggressive behavior in humans.

Play fighting, a normal behavior in several species, especially in young male rats (Pellis and McKenna 1995; Pellis et al. 1997; Pellis and Pellis 1998; Pellis 2002), has been used for evaluation of drugs by Siviy et al. (1995) and Hotchkiss et al. (2003). Schneider and Koch (2005) studied the effects of chronic pubertal treatment with cannabinoids on play behavior (pins, attacks, and defense) in rats.

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Isolation-Induced Aggression

Purpose and Rationale

Male mice, submitted to prolonged isolation, develop aggressive behavior against animals of the same sex. Compounds can be tested for their ability to suppress this isolation-induced aggression.

Procedure

Male mice of NMRI strain with an initial weight of 12 g are kept isolated in small Makrolon cages for a period of 6 weeks. Prior to the administration of the test drug, the aggressive behavior of the animals is tested. A male mouse being accustomed to live together with other animals is placed into the cage of an isolated mouse for 5 min. Immediately, the isolated mouse will start to attack the "intruder." The aggressive behavior of the isolated mouse is characterized by hitting the tail on the bottom of the cage, screaming, and biting. The reaction time until the first attack is less than 10 s for most of the isolated mice and relatively constant for one individual. After these initial tests, the isolated mice receive the test compound, the standard drug, or the vehicle either orally or subcutaneously. The aggressive behavior is evaluated 60, 120, and 240 min after oral and 30, 60, and 120 min after s.c. treatment. After treatment with certain centrally acting compounds the aggressive behavior of the isolated mice is changed. The reaction time until the first attack can be prolonged or shortened. The fighting reaction can be attenuated. Then, additional mechanical stimuli can be used to elicit the fighting behavior. With highly effective drugs the aggressive behavior is completely suppressed.

Evaluation

The number of animals with complete suppression of the fighting behavior is calculated. In animals with diminished aggressiveness the reaction time is registered. A gradual scale of inhibition of aggressiveness is established.

Critical Assessment of the Method

The fighting behavior of isolated mice is not only altered by sedative and anxiolytic compounds but also by neuroleptics and antidepressants. For example, active doses are

Lorazepam	2.5 mg/kg p.o.
Clonazepam	2.5 mg/kg sc.
Haloperidol	1.0 mg/kg p.o.
Chlorpromazine	10.0 mg/kg s.c.
Imipramine	25.0 mg/kg s.c.

In this way, antiaggressive activity of several classes can be detected.

Modification of the Method

Krsiak (1974, 1979) described the effects of various drugs on the behavior of aggressive **mice**.

Olivier and van Dalen (1982) discussed the social behavior in rats and mice as an ethologically based model for differentiating psychoactive drugs.

McMillen et al. (1987) tested the effects of drugs on aggressive behavior and brain monoaminergic neurotransmission.

Krsiak (1975) showed that about 45 % of single-housed male mice showed timidity instead of aggression on interactions with group-housed male mice. Several drugs such as benzodiazepines, chlorpromazine, and barbitone inhibited the

isolation-induced timidity without reducing other motor activities in the timid mice.

Andrade et al. (1988) tested the effect of insulin-induced hypoglycemia on the aggressive behavior of isolated mice against intruders made anosmic by application of 25 µl of 4 % zinc sulfate solution to the nasal tract 3 and 1 days before the encounters.

White et al. (1991) tested the effects of serotoninergic agents and other psychotropic compounds on isolation-induced aggression in mice at doses below those which produced debilitation in the rotarod performance. Guidotti et al. (2001) used the socially isolated mouse as a model to study the putative role of allopregnenolone and 5α-dihydroxy progesterone in psychiatric disorders.

Francès (1988), Francès et al. (1990), and Francès and Monier (1991) described another phenomenon of isolation in rodents which can be used for evaluation of psychotropic drugs: the **isolation-induced behavioral deficit**. Male Swiss NMRI mice were either housed in groups of six in home cages or isolated at the age of 4–5 weeks for 7 days. Mice were tested in pairs (one isolated and one grouped mouse) under a transparent beaker. The number of escapes was counted for the first 2 min of observation. An attempt to escape was defined as one of the following: (1) the two forepaws were placed against the wall of the beaker, (2) the mouse was sniffing, its nose at the spout of the beaker, or (3) the mouse was scratching at the glass floor. Behavioral observations were taped by an observer, blind to the treatment.

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Resident-Intruder Aggression Test

Purpose and Rationale

Sijbesma et al. (1991), Mos et al. (1992), and Muehlenkamp et al. (1995) studied the effects of drugs in a test for offensive aggression, the isolation-induced resident-intruder aggression model (Flannelly and Lore 1975; Brain et al. 1979), in the rat.

Procedure

Sprague–Dawley rats weighing 250–450 g are housed in a light–dark (12 L:12D)-, temperature (ca. 22 °C)-, and humidity (ca. 55 %)-controlled room.

Resident male rats (about 450 g) are tested in their home cages for aggression against a smaller (250 g) male intruder. They are treated by intraperitoneal injection of test drug or saline 15 min before the test. The resident female is removed from the cage 30 min prior to the start of the test period. After placing the intruder rat in the territorial cage, the behavior of the resident male is observed. The time until the first attack (in seconds), number of attacks, and duration of each attack (in seconds) are recorded for the next 15 min by a blind observer. Furthermore, a total of 49 different behavioral elements are scored and grouped into 7 behavioral categories: offensive, exploration, social interest, inactivity, avoidance, body care, and defense.

Evaluation

Paired and unpaired *t*-tests are used for comparisons of means of absolute values.

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Water Competition Test

Purpose and Rationale

The competitive water consumption test in rats (Baenninger 1970; Syme 1974) can be used to study the influence of drugs on defensive aggression and dominance (Muehlenkamp et al. 1995).

Procedure

Male Sprague–Dawley rats weighing 250–400 g are kept in a light–dark (12 L:12D)-, temperature (ca. 22 °C)-, and humidity (ca. 55 %)-controlled room.

Animals of equal weight are paired and housed in one cage. After 6 days, the animals are deprived of water for 23 h. Then, one water bottle is introduced with a shielded spout so that only one animal of a pair can drink at a time. Time (in seconds) and frequency of spout possession and water consumption are recorded in numbers with a special computer program for 5 min. Animals are then allowed another 55 min for water consumption. This test is repeated on 3 subsequent days. Saline or test substances are injected intraperitoneally 15 min before tests 3 and 4. The animal with the longest duration of water consumption and frequency of spout possession is considered to be the more aggressive and/or dominant animal.

Evaluation

Paired and unpaired t-tests are used for comparisons of means of absolute values, as well as means of differences of the absolute value of the two rats.

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Maternal Aggression in Rats

Purpose and Rationale

The model of "maternal aggression" in rats was described by Olivier et al. (1985), Olivier and Mos (1986, 1992), Mos et al. (1987, 1989). Introduction of an intruder (male or female) in the cage of a parturient female rat induces high levels of aggression against such intruders. Maternal aggression is characterized by short-latency attacks of high intensity mostly directed toward the head or neck of the intruder and is particularly pronounced during the first part of the lactating period. The behavior can be suppressed by several drugs, e.g., 5-HT_1 agonists (Mos et al. 1990; Olivier and Mos 1992).

Procedure

Female rats weighing 250–350 g are placed with a breeding male in their home cages. On the bottom of each cage an iron gauze is placed which enables the collection of ejaculation plugs. After an

ejaculation plug is detected the male is left for another week with the female after which she is placed in the observation cage provided with nesting material where she stays for the rest of the experiment. These cages are situated in an observation room under a reversed day-night rhythm. The day of birth is marked as day 0. Every parturient female is tested each day against a naive male intruder which has about 25 g less body weight than the female. Tests are performed during the first part of the dark period under red light conditions. One male intruder is placed in the female's home cage for 5 min. The ongoing behavior is videotaped and analyzed later. Each intruder is used only once and sacrificed immediately afterward with an i.p. overdose of pentobarbital, followed by shaving and describing the wounds on wound charts (Mos et al. 1984).

The aggressive behavior of the female is scored for

- Bite attack on the head (fierce biting on head and snout often causing severe wounds)
- Bite attack on the body (mostly directed to the back)
- Lateral threat (the animal kicks with a hind leg at the opponent)
- Upright posture (accompanied by boxing)
- Nipping (short and low-intensity bite on the head of the opponent)
- Pulling (the opponent is held by the teeth and drawn through the cage)
- Lunge (very rapid movement toward the opponent, mostly followed by bite attacks)
- On top (the female holds down the opponent which lies on its back)

Besides the frequency and the duration of the elements, also the latency of the first attack and the number of attacks are recorded.

Drug experiments are performed on postpartum days 3, 5, 7, and 9 using a Latin-square design of dosage. Preceding days (day 1 and 2), intervening days (4, 6, and 8), and following days (10, 11, 12, and 13) are used to establish an aggression baseline and as washout days. Drugs are administered orally 60 min before testing.

Evaluation

Analysis of variance is employed to detect overall significance, followed by Wilcoxon matched-pairs comparison between dosages. Kruskal-Wallis analysis is used to test the differences in the bite target areas after drug treatment.

Modification of the Method

Postpartum aggression in rats did not influence threshold currents for electrical brain stimulation–induced aggression in rats (Mos et al. 1987).

Maternal aggression can be observed not only in rats but also in **mice** and **hamsters** (Mos et al. 1990).

Palanza et al. (1996) examined the effects of chlordiazepoxide on the differential response pattern in aggressive-naive and aggressive-experienced lactating female mice confronting intruders of either sex in a 10 min test.

Olivier et al. (1990) highlighted ethopharmacology as a creative approach to identification and characterization of novel psychotropics.

Olivier et al. (1995) gave a review on serotonin receptors and animal models of aggressive behavior.

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Rage Reaction in Cats

Purpose and Rationale

An emotional aggressive behavior (rage reaction) can be elicited in unrestrained cats by high-frequency electrical stimulation of the hypothalamus. Benzodiazepines have been reported (Malick 1970; Murasaki et al. 1976) to elevate the stimulus threshold for eliciting this rage reaction.

Procedure

Emotional aggressive behavior is evoked by stimulation of the perifornical area of the lateral hypothalamus through chronically implanted stainless-steel bipolar concentric electrodes by using threshold impulses of 1.0–2.6 mA, delivered at 50 Hz, to evoke a control attack response. Stimulation is discontinued immediately after the slowly rising current has reached threshold strength and is performed once before drug administration as well as every 30 min thereafter. Drugs are injected by intraperitoneal route.

Evaluation

Postdrug values are expressed as percentage of the predrug control value. Student's paired *t*-test is used for each time interval.

Critical Assessment of the Test

The method has been used for evaluation of new drugs. Most studies were devoted to the role of neurotransmitters regulating feline aggression (Siegel and Schubert 1995; Siegel et al. 1998) and the neural bases of aggression and rage in the cat (Siegel et al. 1997, 1999).

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Effects on Behavior

Antianxiety Test (Light–Dark Model)

Purpose and Rationale

Crawley and Goodwin (1980) Crawley (1981) described a simple behavior model in mice to detect compounds with anxiolytic effects. Mice and rats tend to explore a novel environment but to retreat from the aversive properties of a brightly lit open field. In a two-chambered system, where the animals can freely move between a brightly lit open field and a dark corner, they show more crossings between the two chambers and more locomotor activity after treatment with anxiolytics. The numbers of crossings between the light and dark sites are recorded.

Procedure

The testing apparatus consists of a light and a dark chamber divided by a photocell-equipped zone. A polypropylene animal cage, 44 × 21 × 21 cm, is darkened with black spray over one-third of its surface. A partition containing a 13 cm long × 5 cm high opening separates the dark one-third from the bright two-thirds of the cage. The cage rests on an Animex activity monitor which counts total locomotor activity. An electronic system using four sets of photocells across the partition automatically counts movements through the partition and clocks the time spent in the light and dark compartments. Naive male mice or rats are placed into the cage. The animals are treated 30 min before the experiment with the test drugs or the vehicle intraperitoneally and are then observed for 10 min. Groups of 6–8 animals are used for each dose.

Evaluation

Dose–response curves are obtained and the number of crossings through the partition between the light and the dark chamber compared with total activity counts during the 10 min.

Critical Assessment of the Method

It has been shown that a variety of anxiolytics including diazepam, pentobarbital, and meprobamate produce a dose-dependent increase in crossings, whereas nonanxiolytic agents do not have this facilitatory effect. Furthermore, the relative potency of anxiolytics in increasing exploratory behavior in the two-compartment chamber agrees well with the potency found in clinical trials.

The test has the advantage of being relatively simple with no painful stimuli to the animals. The specificity of the method remains open.

Modification of the Method

Using a similar method, called black-and-white test box, Costall et al. (1987, 1988, 1989) studied the effects of anxiolytic agents and reported an anxiolytic effect of dopamine receptor antagonists, such as sulpiride and buspirone.

Sanchez (1995) presented a fully automated version of the black-and-white two-compartment box for mice.

Barnes et al. (1992) used this model to study the interaction of optical isomers modifying rodent aversive behavior.

Kilfoil et al. (1989) used a similar apparatus to test compounds for anxiogenic and anxiolytic activity.

Animal models of anxiety and their relation to serotonin-interacting drugs have been reviewed by Broekkamp et al. (1989) and by Griebel (1995).

Laboratory rats prefer to dwell on a solid floor rather than a grid one, particularly when resting. Manser et al. (1996) described an operant test in rats to determine the strength of preference for flooring. The apparatus consisted of a grid-floored cage and a solid-floored cage, joined via a central box containing a barrier whose weight was adjustable. The rats had to lift the barrier in order to explore the whole apparatus or were confined on the grid floor and then had to lift the barrier in order to reach the solid floor.

Hascoët and Bourin (1998) tested anxiolytic and psychostimulant drugs in a fully automated and computer-integrated two-compartment light/dark box.

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Anticipatory Anxiety in Mice

Purpose and Rationale

When group-housed mice are removed one by one from their home cage, the last mice removed have always higher rectal temperatures than those removed first (Borsini et al. 1989; Lecci et al. 1990). This phenomenon is interpreted as being caused by anticipatory fear for an aversive event (handling causes stress-induced hyperthermia). Consequently, this test is thought to be a model of anticipatory anxiety. The anticipatory increase in temperature was prevented by prior treatment with diazepam and buspirone, whereas several other drugs did not affect this phenomenon (Lecci et al. 1990). The usefulness of this model for obsessive-compulsive disorder in man is discussed.

Procedure

Groups of 18 male albino Swiss mice weighing 25–30 g are housed at constant room temperature and relative humidity for at least 7 days in Makrolon cages to adapt to the environment. Test drugs or standard (diazepam) or solvent are administered orally in various doses to groups of 18 mice prior to the test. Thirty minutes later, the first three mice are removed from the cage and the rectal temperature registered by inserting a silicone lubricated thermistor probe (2 mm diameter) 2.5 cm into the rectum. The average temperature of these three mice is taken as basal value. Mice number 4 through 15 are simply removed and again returned to the cage, and thereafter body temperature is determined in the remaining three animals. The difference of the mean value of these mice and the basal values is calculated as increase. Vehicle-treated test groups display increases of 1.1–1.3 °C.

Evaluation

The mean increase values of treated groups ± SEM are compared by ANOVA statistics with the controls.

Modification of the Method

Van der Heyden et al. (1997) adapted the group-housed stress-induced hyperthermia paradigm to single-housed animals in order to drastically reduce the number of animals used. Repeated, but not single, disturbance of animals resulted in a strong hyperthermia (Δt) within 10 min. The final test paradigm chosen involved repeated temperature measurements at 10 min intervals, thus providing both information on basal temperature and Δt in each animal within a short time frame.

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Social Interaction in Rats

Purpose and Rationale

In an unfamiliar and brightly lit environment, the normal social interaction of rats (e.g., sniffing, nipping, grooming) is suppressed. Anxiolytics counteract this suppression.

Procedure

Male Sprague–Dawley rats (225–275 g body weight) are housed in groups of five animals The apparatus used for the detection of changes in social behavior and exploratory behavior consists of a Perspex open-topped box (51 × 51 cm and 20 cm high) with 17 × 17 cm marked areas on the floor. One hour prior to the test, two naive rats from separate housing cages are treated with the test compound orally. They are placed into the box (with 60 W bright illumination 17 cm above) and their behavior observed over a 10 min period by remote video recording. Two types of behavior can be noted:

- Social interaction between the animals is determined by timing the sniffing of partner, crawling under or climbing over the partner, genital investigation of partner, and following partner.
- Exploratory motion is measured as the number of crossings of the lines marked on the floor of the test box.

Six pairs are used for each dose.

Evaluation

The values of treated partners are compared with the data from six pairs of untreated animals using single factor analysis of variance followed by Dunnett's *t*-test.

Critical Assessment of the Method

In spite of the fact that there may be some analogy between "social anxiety" in humans and the behavior of rats in the social interaction test, there appear some potential complications with this test, such as an increase of social interaction after anxiolytics independent of the environment, dependence on external variables such as time of the day, and the complicated nature of social interaction.

Modifications of the Test

Sams-Dodd (1995) described the automation of the social interaction test in rats by a commercially available video-tracking system.

Gheusi et al. (1994) studied the effects of tetrahydroaminoacridine (THA) on social recognition in rats indicating a dissociation of cognitive versus noncognitive processes.

Doses of 1.0–4.0 mg/kg i.p. phencyclidine (PCP) reduce the social interaction time in rats in a dose-dependent fashion. Reversal of the PCP-induced social withdrawal has been used as an animal model for neuroleptic-resistant schizophrenia (Carlsson and Carlsson 1990; Corbett et al. 1995).

Olfactory investigation of ovarectomized females by adult male mice decreases during repeated confrontations with the same female intruder, whereas aggressive behavior gradually increases (Winslow and Camacho 1995). Administration of scopolamine blocked decrements in olfactory investigation in repeated confrontations and significantly reduced aggression. Acetylcholinesterase inhibitors enhanced the rate of decrement of olfactory investigation but had differential effects on aggression.

Wongwitdecha and Marsden (1996) investigated the effects of isolation rearing on anxiety using the social interaction paradigm and compared the effects of diazepam on social interactive behaviors in isolation and socially reared rats.

Sams-Dodd (1997) studied the effect of novel antipsychotic drugs on phencyclidine-induced stereotyped behavior and social isolation in the rat social interaction test.

The rat social interaction model has been used by various authors to characterize the potential

anxiolytic effects of serotonin receptor antagonists, such as 5-HT_{1C} receptor antagonists (Kennett et al. 1989; Kennett 1992), 5-HT_2 receptor antagonists (Kennett et al. 1994, 1995, 1996a, b, 1997; Costall and Naylor 1995), 5-HT_3 receptor antagonists (Costall et al. 1990; Costall and Naylor 1992; Blackburn et al. 1993), 5-HT4 receptor antagonists Kennett et al. (1997), cholestystokinin receptor antagonists (Hughes et al. 1990; Costall et al. 1991; Singh et al. 1991), and nitric oxide synthase inhibitors (Volke et al. 1997).

File and Johnston (1989) reported a lack of effects of 5-HT_3 receptor antagonists in the social interaction test in the rat.

Woodall et al. (1996) described a **competition procedure** in rats. In the first week of experimentation groups of three rats (triads) are familiarized with the test box and sweetened milk from a drinking spout located on the end wall. The drinking spout is surrounded by a Perspex tube (4.5 cm diameter) which ensures that only one animal is able to drink at a time. All animals are deprived of water overnight and on the following day placed into the testing box and given access to the sweetened milk for 15 min. In the second week of testing, the rats are not longer water deprived and are given access to the testing box and sweetened milk for 5 min. During the testing period, the rats are observed every 5 s, and a note is made which animal is drinking. This procedure is carried out twice a week for a period of 5 weeks. Drugs are administered to either the dominant or subordinate rat in each triad 15 min prior to testing. Following the drug study, all triads are tested for two trials to ensure that their rank orders return to baseline levels. The access of the subordinate member to sweetened milk is increased after administration of an anxiolytic drug.

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Elevated Plus Maze Test

Purpose and Rationale

Out of many possibilities to modify maze tests (e.g., water maze (Danks et al. 1991), Y-maze, radial maze (Di Cicco et al. 1991)) the **elevated plus maze** (Montgomery 1958; Pellow et al. 1985; Corbett et al. 1991) has found acceptance in many laboratories (Liebisch et al. 1998; Landgraf et al. 1999; Schwarzberg et al. 1999; Keck et al. 2001; Brakebusch et al. 2002). The test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time; anxiogenic compounds have the opposite effect.

Procedure

The plus maze consists of two open arms, 43 × 15 cm (L × W), and two enclosed arms, 43 × 1523 cm (L × W × H), open to the top, arranged so that the two open arms are opposite to each other. The maze is elevated to a height of 70 cm (TSE Systems, Bad Homburg, Germany). The rats (200–250 g body weight) are housed in pairs for 10 days prior to testing in the apparatus. During this time the rats are handled by the investigator on alternate days to reduce stress. Groups consist of six rats for each dose. Thirty minutes after i.p. administration of the test drug or the standard, the rat is placed in the center of the maze, facing one of the enclosed arms. During a 5 min test period the following measures are taken: the number of entries into and time spent in the open and enclosed arms and the total number of arm entries. The procedure is conducted preferably in a sound attenuated room, with observations made from an adjacent room via a remote-controlled TV camera.

Evaluation

Motor activity and open arm exploratory time are registered. The values of treated groups are expressed as percentage of controls. Benzodiazepines and valproate decrease motor activity and increase open arm exploratory time.

Critical Assessment of the Method

The method is rather time consuming but can be regarded as a reliable measure of anxiolytic activity. Computerized automatic elevated plus maze systems may help to overcome these difficulties (TSE Systems, Bad Homburg, Germany).

Modification of the Method

Latency to enter a mirrored chamber by mice has been described as a behavioral assay for anxiolytic agents (Toubas et al. 1990).

Handley and McBlane (1993) provided an assessment of the elevated X maze for studying anxiety and anxiety-modulating drugs.

Lapin (1995) studied the effect of handling, sham injection, and intraperitoneal injection of saline on the behavior of mice in an elevated plus maze. These procedures produce behavior considered to be typical for anxiety-inducing drugs. Saline-treated groups taken as controls possess the behavioral profile of stressed and anxious animals.

Pokk et al. (1996) described a method of small platform-induced stress whereby mice were individually placed for 24 h on a small platform (3 cm high, 3.5 cm in diameter) which was fixed at the center of a plastic chamber (20 cm diameter, 40 cm high) and was surrounded by water (1 cm deep) at 22 °C.

Shepherd et al. (1994) described the **elevated** "**zero maze**" as a modification of the elevated plus maze model of anxiety in rats which incorporates both traditional and novel ethological measures in the analysis of drug effects. The design comprises an elevated annular platform with two opposite enclosed quadrants and two open, removing any ambiguity in interpretation of time spent in the central square of the traditional design and allowing uninterrupted exploration. A similar equipment, built for mice (TSE Systems, Bad Homburg, Germany), was used by Cryan et al. (2004) and Korsgaard et al. (2005).

Jardim et al. (1999) evaluated the elevated T-maze as an animal model of anxiety in the mouse and found important differences between mice and rats.

Based on their plus-maze behavior, that is, the time spent in the open arms, Ho et al. (2002) divided male Wistar rats into two subgroups with either "low" or "high" anxiety.

Silva and Frussa-Filho (2000) recommended the plus-maze discriminative avoidance task as a model to study memory-anxiety interactions. Mice are conditioned to choose between two enclosed arms (in one of which light and noise are presented as aversive stimuli) while avoiding the two open arms of the apparatus. The test has the advantage of measuring, at the same time and in the same animals, learning/memory (by the percentage of time spent in aversive closed arm) and anxiety (by the percentage of time spent in the open arms).

Montag-Sallaz and Montag (2003) tested cognitive and motor coordination deficits in Tenascin-R-deficient mice on an elevated plus maze. For 5 min, the behavior was recorded on videotape and the numbers of entries into the central part, the closed, or the open arms were counted, and the time spent in these departments was determined using the VideoMot 2 system (TSE Systems, Bad Homburg, Germany).

The same system was used by Karl et al. (2003) to study behavioral effects of neuropeptide Y in F344 rat substrains with reduced dipeptidyl-peptidase IV activity.

Korte and De Boer (2003) recommended fear-potentiated behavior in the elevated plus maze in rats as a robust animal model of state anxiety.

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Water Maze Test

Purpose and Rationale

Spatial learning of rats can be tested in a water maze as described by Morris (1984) and McNaughton and Morris (1987).

Procedure

The water maze consists of a circular tank with 100 cm diameter and a wall 20 cm above the water level. A circular platform (9 cm diameter, covered with white linen material for grip) is hidden 2 cm below the water level. The water is made opaque using titanium dioxide suspension and is kept at about 23 °C during the experiment. Training takes place on 3 consecutive days, with the rats receiving four consecutive trials per day with an intertrial interval of 6–10 min. Each trial is started from one of four assigned polar positions with a different sequence each day. The latency to find the platform is measured as the time of placement of the rat in the water to the time it finds the platform. If the animal fails to find the platform in any trial within 3 min it is placed on it for 10 s.

Evaluation

On day 4 a probe test is performed. The platform is removed and the time spent in the target quadrant (the quadrant in the center of which the platform has been located) and the number of annulus crossings (across the actual location where the platform has been located) in the first 60 s of exposure measured. The time to the first annulus crossing is also taken as a measure of performance on the 13th (i.e., probe) trial.

Buspirone (Rowan et al. 1990) as well as benzodiazepines (McNaughton and Morris 1987) increase the latency to find the platform in the training period and impair the number and the time of annulus crossings.

Critical Assessment of the Method

The water maze test measures learning and memory rather than the anxiolytic activity. If the test is used for memory, rats which have learned quickly and consistently to find the platform are kept in their cages for 2 weeks and then retested. Rats which solve the escape immediately are considered to have retained memory.

Modification of the Method

Bane et al. (1996) used the Morris water maze to study the adverse effects of the noncompetitive NMDA receptor antagonist dextromorphan on the spatial learning of rats.

Van der Staay (2000) studied the effects of the size of the Morris water tank on spatial discrimination learning in the CFW1 mouse. Mice can best be tested in a small pool, because the time and distance swum to find and escape onto the

platform are decreased and the probability of success is increased.

Schmitt and Hiemke (2002) reported that a γ-aminobutyric acid transport inhibitor impairs spatial learning in the Morris water maze.

Winter et al. (2004) determined long-term functional outcome after mild focal cerebral ischemia by tracking swimming performance in a Morris water maze with the computer-based VideoMot2 system (TSE Systems, Bad Homburg, Germany).

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Staircase Test

Purpose and Rationale

The staircase test for evaluating anxiolytic activity was originally described for rats by Thiebot et al. (1973). When introduced into a novel environment, rodents experience a conflict between anxiety and exploratory behavior manifested by increased vigilance and behavioral activity. In the staircase paradigm, step-climbing is purported to reflect exploratory or locomotor activity, while rearing behavior is an index of anxiety state. The number of rearings and steps climbed are recorded in a 5 min period. The dissociation of these parameters is considered to be characteristic for anxiolytic drugs. The test was modified for rapid screening of anxiolytic activity in mice (Simiand et al. 1984).

Procedure

For experiments with mice the staircase is composed of five identical steps 2.5 cm high, 10 cm wide, and 7.5 cm deep. The internal height of the walls is constant along the whole length of the staircase. Naive male mice (Charles River strain) with a weight between 18 and 24 g are used. Each animal is used only once. The drug or the standard is administered orally 1 h or 30 min subcutaneously before the test. The animal is placed on the floor of the box with its back to the staircase. The number of steps climbed and the number of rears are counted over a 3-min period. A step is considered to be climbed only if the mouse has placed all four paws on the step. In order to simplify the observation, the number of steps descended is not taken into account. After each test, the box has to be cleaned in order to eliminate any olfactory cues which might modify the behavior of the next animal.

Evaluation

Twelve mice are used for the untreated control group, each drug group, and the group receiving the standard. The average number of steps and rearings of the control group is taken as 100 %. The values of treated animals are expressed as percentage of the controls.

Critical Assessment of the Method

The staircase test has been proven as a simple and reliable method for screening of anxiolytics in several laboratories. Many applications and modifications have been described in the literature (Houri 1985; Steru et al. 1987; Keane et al. 1988; Emmanouil and Quock 1990; Simiand et al. 1993).

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Cork Gnawing Test in the Rat

Purpose and Rationale

Cork gnawing behavior in the rat has been proposed as a screening method for buspirone-like anxiolytics by Pollard and Howard (1991).

Procedure

Adult male Evans rats serve as subjects. They are housed four per cage on a regular light/dark cycle with free access to food and water except for the period between injection and the end of a test session. For the test session one animal is placed in a stainless steel cage with wire mesh bottom. A session consists of placing the subject in the test cage with a cork stopper weighing between 2 and 3 g for 30 min. Initially, the amount gnawed is relatively high and variable within and between subjects. After 30 training sessions, the amount is low and stabilized. The test compounds are injected 30 min before the test and food is withdrawn.

Evaluation

Each cork is weighed to the nearest 0.01 g before and after the session. The average cork loss during the previous control days is taken as baseline and the amount after drug treatment is expressed as percentage of baseline. Buspirone-related compounds as well as benzodiazepines and meprobamate show a dose-dependent increase of cork gnawing, but amphetamine, chlorpromazine, imipramine, and morphine do not.

Critical Assessment of the Method

The test is worthwhile to be mentioned, since buspirone – whose anxiolytic action was discovered during clinical trials to assess possible

antipsychotic action and not by use of animal tests for anxiolysis – is active in this test but not in most other classical tests for anxiolytic activity.

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Distress Vocalization in Rat Pups

Purpose and Rationale

Measurement of ultrasonic vocalization induced by tail-holding in rat pups was proposed as a simple screening method for anxiolytic drugs by Gardner (1985).

Procedure

Wistar rat pups are bred on site and left undisturbed with their mother, except for cage-bedding replacement, until the day of testing. The pups are tested at 9–12 days of age. On the day of testing the pups are separated from their mother and taken in their home cage to the quiet experimental room. In the morning all pups are subjected to handling stress and the magnitude of their ultrasound emission observed. The stress consists of holding the pup by the base of the tail, between forefinger and thumb of the experimenter, and thus suspending it 5 cm above the bench for 30 s. A prior control recording (30 s) is taken when the pup is held gently in the experimenter's hand, whereby the pups emit only a few ultrasounds. Responses when held by the tail are more than 10 times higher. This entire hand-holding–tail-holding procedure is immediately repeated. Ultrasounds are recorded with suitable detectors with 42 kHz as the center of a 10 kHz recording range. The output of the detectors is fed into pen recorders. The total number of ultrasonic cries in the two sessions of hand holding and the two sessions of tail holding are calculated and used as the control activity of each pup. Any pup producing a total of less than 50 ultrasounds when held by the tail is excluded from the drug study. The pups are kept in the home cage in the test laboratory until the afternoon. Three to four hours after the first test the pups are randomly allocated to several equally seized groups, weighed, marked, and dosed intraperitoneally either with the vehicle or drug and placed back in the home cage. Thirty minutes after dosing, each pup is subjected to the same handling stress as that used in the morning session, and the total number of sounds produced is calculated in the same way.

Evaluation

The afternoon response to tail holding is expressed as a factor of the morning response. The mean factor for the saline-treated animals is taken to be 100 % in calculations of percentage changes in ultrasound emission by drugs.

Critical Assessment of the Method

Anxiolytic benzodiazepines dose-dependently inhibit vocalization. Amitriptyline and haloperidol have no effect. Chlorpromazine, muscimol, and prazosin reduce sound at doses which also induce overt sedation. Therefore, the method can be regarded as relatively specific for anxiolytic activity.

Modification of the Method

Kehne et al. (2000) used the ULTRAVOX system (Noldus Information Technology, Wageningen, the Netherlands) to detect and quantify separation-induced ultrasonic vocalization in rat pups.

Using this equipment, Siemiatkowski et al. (2001) reported opposite effects of olanzapine and haloperidol in the rat ultrasonic vocalization test.

Molewijk et al. (1996), Griebel et al. (2002), and Steinberg et al. (2002) used maternal separation-induced distress vocalization in **guinea pig** pups to test anxiolytic and antidepressant drugs.

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Schedule-Induced Polydipsia in Rats

Purpose and Rationale

Food-deprived rats exposed to a procedure in which food is delivered intermittently will drink large amounts of water if given the opportunity to do so. This behavioral phenomenon is termed schedule-induced polydipsia and is an example of a more general class of behaviors termed adjunctive behaviors (Falk 1971; Pellon and Blackman 1992). Adjunctive behaviors have been cited as potential animal models of human obsessive-compulsive disorders (Pitman 1989).

Procedure

Male Wistar rats weighing 180–250 g are individually housed at a 12 h/12 h light/dark cycle for α 1 week acclimation period with free access to food

and water. Then they are placed on a restricted diet which maintains 80 % of their free-feeding body weight. To induce polydipsia, rats are placed in test chambers housed in sound-attenuated boxes where a pellet dispenser automatically dispenses two 45 mg pellets on a fixed-time 60-s (FT-60s) feeding schedule over a 150 min test session. Water is available at all times in the test chambers. After 4 weeks exposure to the FT-60s feeding schedule, approximately 80 % of the rats meet the predetermined criterion for water consumption (greater than 60 ml water per session) and are considered to have polydipsic behavior.

Rats receive the test compounds in various doses daily or the vehicle intraperitoneally 60 min prior to testing. They are tested once a week to assess schedule-induced polydipsia. Water bottles are weighed before and after the 150-min test sessions.

Evaluation

The experimental data comparing the effects of chronic administration of compounds on schedule-induced polydipsia are analyzed with the Mann Whitney *U*-test.

Modification of the Method

Yadin et al. (1991) proposed spontaneous alternation behavior in rats as an animal model for obsessive-compulsive disorder. Food-deprived rats were run on a T-maze in which both a white and a black goal box were equally baited with a small amount of chocolate milk. Each rat was given seven trials every other day during which it was placed in the start box and allowed to make a choice. The mean number of choices until an alternation occurred was recorded. After a baseline of spontaneous alternation was achieved the rats were treated with the nonselective serotonin agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (1.25 mg/kg i.p.) or the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT) (2 mg/kg i.p.), which both disrupted the spontaneous alternation. A course of chronic treatment (2 × 5 mg/kg for 21 days) with the selective 5-HT uptake-blocking agent fluoxetine had a protective effect on the 5-MeODMT-induced disruption of spontaneous alternation behavior. The authors speculated that serotonergic manipulations of spontaneous alternation may be a simple animal model for the perseverative symptoms or indecisiveness seen in patients with obsessive-compulsive disorder.

Bös et al. (1997) and Martin et al. (1998) tested agonists of 5-HT_{2C} receptors in the schedule-induced polydipsia task in rats and proposed them as improved therapeutics for obsessive compulsive disorder.

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Four-Plate Test in Mice

Purpose and Rationale

The four-plate test in mice has been described by Aron et al. (1971)and Boissier et al. (1968) as a method for the rapid screening of minor tranquilizers.

Procedure

The test box has the shape of a rectangle (25 × 18 × 16 cm). The floor is covered with four identical rectangular metal plates (8 × 11 cm) separated from one another by a gap of 4 mm. The plates are connected to a source of continuous current which applies to two adjacent plates a mild electrical shock of 0.35 mA for 0.5 s. This evokes a clear flight reaction of the animals.

Adult male Swiss albino mice, weighing 17–23 g, are randomly divided into different groups. Thirty minutes before the test the animals are injected intraperitoneally with the test drug or the vehicle.

At the beginning of the test, the mouse is gently dropped onto a plate and is allowed to explore the enclosure for 15 s. After this, every time the animal crosses from one plate to another, the experimenter electrifies the whole floor for 0.5 s, which evokes a clear flight reaction of the mouse which often crosses two or three plates. If it continues running, no new shock is delivered during the following 3 min.

Evaluation

The number of times the apparatus is electrified is counted each minute for 10 min. The delivery of shocks decreases dramatically the motor activity. The number of shocks received during the first min is taken as parameter. This number is increased by minor tranquilizers, such as benzodiazepines, but not by neuroleptics and psychoanaleptics.

Critical Assessment of the Method

The test is of value to differentiate minor tranquilizers, such as benzodiazepine anxiolytics, from neuroleptics. However, some stimulants (e.g., amphetamine) produce an increase in punished plate crossings and some anxiolytics do not.

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Foot-shock-Induced Freezing Behavior in Rats

Purpose and Rationale

Footshock-induced freezing behavior in rats has been proposed as a model for anxiolytics by Conti et al. (1990).

Procedure

Male Sprague–Dawley rats with a weight between 200 and 350 g are used. The animals receive a single i.p. injection of the test compound or the vehicle 30 min prior to being placed in a standard conditioning chamber (e.g., Coulborn Instruments) for a 6.5 min session. A scrambled foot-shock (0.5 mA, 0.5 s) is delivered through the grid floor of the chamber 2 and 2.5 min after the start of the session. Using an assembly of push buttons interfaced with a computer, an observer monitors the amount of time each animal spends engaged in the following mutually exclusive behaviors:

- Freezing: immobility with rigid body posture.
- Sedated posture: sitting or sleeping.
- Small exploratory movements: movements involving the torso or front paws only, vertical movements of the head, or sniffing.
- Locomotion: activity involving hind paws, grooming or rearing.
- Frequency of rearing is also counted. All behaviors are monitored for the entire 6.5 min session.

Evaluation

Duration of foot-shock-induced freezing after the second shock is taken as the critical parameter. Time spent in freezing posture after administration of test compounds is compared with the controls. Anxiolytics like diazepam and buspirone show dose-dependent effects but not haloperidol.

Critical Assessment of the Method

The method seems to discriminate anxiolytics including buspirone from other centrally acting drugs.

Modification of the Method

Foot-shock-induced ultrasonic vocalization has been suggested as another model of anxiety (Tonoue et al. 1986; Kaltwasser 1990; Miczek et al. 1991; De Vry et al. 1993; Nielsen and Sánchez 1995; Schreiber et al. 1998). Test cages ($22 \times 22 \times 22$ cm) made of grey Perspex are equipped with a metal grid floor (distance between the bars $=$ 1 cm). Electric foot-shocks are delivered from a 12 bit programmable shock source and ultrasounds picked up by a microphone (range 18–26 Hz) placed in the center of the cage lid. The total vocalization time is measured. The rats are placed individually in the test cages and immediately receive a series of 0.5 mA inescapable foot-shocks each 1 s duration with a shock interval of 5 s. The vocalization is measured for a 10 min period starting 1 min after the last shock. Drugs or saline are given subcutaneously 30 min before the test.

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Experimental Anxiety in Mice

Purpose and Rationale

Ogawa and Kuwahara (1966), Ogawa et al. 1990, 1993) designed a communication box to induce experimental anxiety in mice by employing intraspecies emotional communication. The inside of the communication box was divided into foot-shock and non-foot-shock compartments by transparent plastic boards. The animals, which were individually placed into each compartment, were unable to make physical contact with one another but were able to receive other cues such as visual, auditory, and olfactory sensations. During the foot-shock period, the animals placed in the non-foot-shock compartments were exposed to the emotional cues from foot-shocked animals, such as shrieks, smell of feces or urine, and jumping response.

Procedure

The floor of the communication box is equipped with grids for electric shock. The inside is divided into small compartments (10 × 10 cm), consisting of foot-shock compartments with a grid floor and non-foot-shock compartments with a grid floor covered by transparent plastic boards. The foot-shock compartments are arranged such as to surround the non-foot-shock compartments.

The experimental groups consist of the following three groups: sender group, responder group, and food-yoked group to responder. Sender animals receive a foot-shock of 10-s duration at intervals of 50 s for 3 h. The electric current for the shock is increased stepwise from 1.6 to 2.0 mA at a rate of 0.2 mA per 1 h. Responders are exposed daily to the emotional responses of sender animals, 3 h per day for 3 days. Sender animals are changed daily to naive mice to prevent a reduced emotional response to foot-shock based on adaptation or learned helplessness due to repeated exposure. Both sender and responder animals are placed individually in each compartment of the communication box 15 min before beginning of the shock period. On day 1, responder animals are returned to their home cages after the 3-h foot-shock period. On day 2, after completing the foot-shock period, they are transferred to metal cages and are housed in the cages with four animals per cage under food deprivation condition. Food-yoked control animals are maintained to the metal cage during the foot-shock period under the aggregated housing condition (five animals each) and then returned to the home cages after the foot-shock period. From beginning of the day-2 experiment to completion of the day-3 experiment, they are maintained in the metal cages under aggregating housing. On day 3, just after completing the foot-shock period, the responders are sacrificed by chloroform, and their stomachs are removed. The stomachs are visually inspected for lesions.

Drugs are administered orally at different doses either with a single dose on day 3 or daily 30 min before the shock period.

Evaluation

Data are reported as the incidence of mice with gastric lesions characterized by slight erosions or bleeding. Active anxiolytics reduce the incidence of gastric ulcers found in food-deprived animals. The incidence in food-yoked animals is much lower. The data are analyzed by Fisher's exact probability test.

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mCPP-Induced Anxiety in Rats

Purpose and Rationale

The metabolite of the antidepressant drug trazodone 1(3-chlorphenyl)piperazine (= mCPP), classified as 5-HT_{1C} agonist (Rocha et al. 1993; Gibson et al. 1996) or 5-$HT_{1B/2C}$ agonist (Dryden et al. 1996), has been shown to be anxiogenic both in man and in rats (Curzon et al. 1991). The compound induces hypophagia (Samanin et al. 1979; Dryden et al. 1996; Yamada et al. 1996) and hypolocomotion (Kennett et al. 1996, 1997a), inhibits social interaction in rats, diminishes exploratory activity of rats in the open field test (Czyrak et al. 1994; Meert et al. 1997) and in the light/dark box test (Bilkei-Gorzo et al. 1998), induces hyperthermia (Aulakh et al. 1995; Kennett et al. 1997b), and reduces ultrasound-induced defensive behavior in the rat (Beckett et al. 1996). Antagonism against these symptoms has been proposed as a screening model for anxiolytic drugs (Bilkei-Gorzo et al. 1996, 1998; Wallis and Lal 1998).

Procedure

Male Sprague Dawley rats (220–250 g) are housed in groups of six under a 12 h light/dark cycle with free access to food and water.

mCPP-Induced Locomotion

Rats are placed in a room adjacent to the experimental room on the day of the procedure. They are dosed either orally 1 h or i.p. 30 min before the locomotion test with test compound or vehicle and injected 20 min before the test with 7 mg/kg mCPP i.p. or saline in groups of four. Rats are returned to their home cages after dosing. At 0 h they are each placed in automated locomotor activity cages made of black Perspex with a clear Perspex lid and sawdust-covered floor under red light for 10 min. During this time, locomotion is recorded by means of alternately breaking two photocell beams traversing opposite ends of the box 3.9 cm above floor level.

mCPP-Induced Hypophagia

Rats are individually housed on day 1, and on day 3 they are deprived of food. Twenty-three hours later, they are orally treated with the test drug or vehicle and returned to their home cages. Forty minutes later, they are given 5 mg/kg mCPP or saline i.p. and again returned to their home cages. After a further 20 min, weighted amounts of their normal food pellets are placed in their food hoppers and the amount remaining after 1 h measured.

Evaluation

The effect of the test compound on mCPP-induced hypolocomotion is determined by one-way ANOVA and Newman-Keuls test. The dose producing 50 % disinhibition of mCPP is also estimated. Feeding test data are subjected to one-way ANOVA and Dunnett's test.

Modification of the Method

Griebel et al. (1991) described neophobic and anxious behavior in mice induced by m-CPP.

Czyrak et al. (1994) measured the antagonism of antipsychotics against the mCPP-induced hypothermia in mice.

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Acoustic Startle Response in Rats

Purpose and Rationale

The acoustic startle reflex is a relatively simple behavior that occurs naturally in mammals and is affected by a variety of treatments. It consists of a series of rapid movements beginning at the head and moving caudally involving contraction and

extension of major muscle groups in response to auditory stimuli with a rapid onset, or rise time. Responses are graded in amplitude in relation to stimulus intensity and may show habituation and sensitization. Startle response can be used to determine sites and mechanisms of drug action (Davis 1982).

Procedure

Male Wistar rats weighing about 200 g are used. Acoustic startle reflexes are measured in a specially built apparatus, e.g., Coulborn Instruments Acoustic Response Test System or TSE Systems, Bad Homburg, Germany. The animals are individually placed in 8 × 8 × 16 cm open air cages that restrict locomotion but do not immobilize the animal and are placed on one of four platforms within a sound-attenuating acoustic chamber. A ventilating fan provides an ambient noise level. Acoustic stimuli consist of white noise bursts lasting 20 ms at 98 dB and 124 dB SPL. Simultaneously with the rapid onset of each stimulus, the animal's physical movement within the cage on the platform is measured for 200 ms as an electrical voltage change via a strain gauge which is converted to grams of weight change following analog-to-digital conversion. Data are recorded automatically by an interfaced microcomputer.

Pretests are performed with all animals to obtain control values. The animals are treated 2 h prior the experiment with test drugs or vehicle given orally or subcutaneously.

Evaluation

The results are given as percentage of the change, related to the values obtained in the pretest and assessed by a one-way ANOVA, followed by Dunnett's test when appropriate.

Modification of the Method

The test has been modified in various ways, e.g., inhibition by a prepulse (Keith et al. 1991; Rigdon and Viik 1991; Taylor et al. 1995) or fear-induced potentiation (Davis 1986, 1992).

Schulz et al. (1996) performed acoustic startle experiments in rats with a potent and selective nonpeptide antagonist of the corticotropin-releasing factor receptors.

Walker and Davis (1997) found that the amplitude of acoustic startle response in rats was increased by high illumination levels.

Devices to register the intensity of fear-potentiated startle response in rats were described by Hijzen et al. (1995). Yilmazer-Hanke et al. (2002) studied fear-potentiated startle and exploration-related anxiety in inbred Roman high- and low-avoidance rats.

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Unconditioned Conflict Procedure (Vogel Test)

Purpose and Rationale

Vogel et al. (1971) described a simple and reliable conflict procedure for testing antianxiety agents. Thirsty, naive rats were administered shocks while licking water.

Procedure

The apparatus is a clear Plexiglas box (38 × 38 cm) with a black Plexiglas compartment (10 × 10.5 cm) attached to one wall and an opening from the large box to the small compartment. The entire apparatus has a stainless-steel grid floor. A water bottle with a metal drinking tube is fitted to the outside of the small compartment, so that the tube extends into the box at a height 3 cm above the grid. Rats lick in bursts with a relatively constant rate of seven licks per sec.

A drinkometer circuit is connected between the drinking tube and the grid floor of the apparatus, so that the rat completes the circuit whenever it licks the tube. Shock is administered to the feet of the animal by switching the connections to the drinking tube and grids from the drinkometer to a shocker which applies an unscrambled shock between the drinking tube and the grid floor.

Naive adult male rats are used. Thirty minutes after intraperitoneal injection, the rat is placed in the apparatus and allowed to find the drinking tube and to complete 20 licks before shock (available at the tube for 2 s) is administered. The rat controls shock duration by withdrawing from the tube. A 3-min timer is automatically started after the termination of the first shock. During the 3-min period, shocks are delivered following each 20th lick. The number of shocks delivered during the 3-min session is recorded for each animal.

Evaluation

The number of shocks received after treatment is compared with untreated animals. Benzodiazepines increase dose-dependently the number of shocks. Barbiturates in low doses and meprobamate, but not damphetamine or scopolamine, are active in this test.

Critical Assessment of the Method

The method is far more simple and less time consuming than the methods using conflict behavior after intensive training. The specificity may be less than that of the Geller paradigm.

Modification of the Method

The method and the apparatus have been modified by Patel and Malick (1982), Patel et al. (1983), Sanger et al. (1985), Langen et al. (2005), and Mathiasen and Mirza (2005).

Miklya and Knoll (1988) showed an increase of sensitivity of the method using rats deprived of food but supplied with tap water ad libitum for 96 h, then fed with dry pellets and punished for drinking during feeding. The punished drinking test has not only been used for identifying and studying anxiolytic agents but also as a method for measuring anxiogenic activity (Uyeno et al. 1990).

La Marca and Dunn (1994) studied α_2-antagonists after intravenous administration in the Vogel lick-shock conflict paradigm.

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Novelty-Suppressed Feeding

Purpose and Rationale

Placing a hungry rat into an unfamiliar environment with access to food results in a suppression of feeding behavior relative to the condition when the test environment is familiar. This effect has been termed hyponeophagia (Shephard and Broadhurst 1982) and occurs because of the novelty of the test environment. The avoidance of novel foods is termed food neophobia. Both hyponeophagia and food neophobia have been assumed to measure emotionality or anxiety by eliciting a conflict situation arising from a fear of the novel setting and foods and the drive to eat (Porschel 1971). A number of investigators have adopted these paradigms to explore the behavioral effects of anxiolytics (Soubrie et al. 1975; Cooper and Crummy 1978; Borsini et al. 1993).

Procedure

The testing apparatus consists of individual Plexiglas open fields, 76 × 76 × 46 cm. Thirty Purina lab chow pellets are placed in a pile directly in the center of the open field.

Animals are handled for 3 weeks prior to the behavioral testing. Forty-eight hours prior to testing, all food is removed from the home cage, although water is still available ad lib. One hour prior to testing, animals receive an intraperitoneal injection of test drugs or vehicle. At the time of testing, the animals are placed into individual open fields containing the food, and the latency to begin eating is measured. If the animal has not eaten within 720 s, the test is terminated and the animal assigned a latency score of 720 s.

Evaluation

The data are analyzed by a one-way analysis of variance followed by Fisher least significant difference post hoc tests. An anxiolytic effect is defined as a significant decrease in mean latency to begin eating compared with vehicle controls.

Critical Assessment of the Method

The test has the advantage of simplicity for screening procedures.

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Shock Probe Conflict Procedure

Purpose and Rationale

The shock probe conflict procedure, an assay responsive to benzodiazepines, barbiturates, and related compounds, was described by Meert and Colpaert (1986). Rats being placed in a novel test environment containing a probe explore the environment and also the probe. The exploration of the probe, quantified as the number of times that the animal makes physical contact with it, is reduced when the probe is electrified. Rats treated with anxiolytics continue to touch the electrified probe.

Procedure

Apparatus: The test environment consists of a Plexiglas chamber measuring 40 × 40 × 40 cm and having a metal grid floor. A Teflon probe (Ø 1 cm) with two uninsulated wires (Ø 0.5 mm), each independently wrapped 25 times around it, is inserted from the front panel protruding for a length of 6.5 cm into the test box, 3 cm above the floor of the chamber. The wires are connected to a shocker. Whenever the animal touches both wires simultaneously with some part of its body, a DC current flows through the animal. At the same time, a counter is triggered. Normally, a shock intensity of 0.9 mA is used.

Sixty min after treatment with saline or test substance, the animal is placed in a back corner of the test box facing away from the probe. The test session starts from the moment it makes the first contact and receives the first shock. The number of responses the animal makes during the subsequent 5-min episode is counted.

Evaluation

Dose–response curves can be established for various drugs at different shock intensities. The Mann–Whitney *U*-test is used to evaluate differences between experimental conditions. To control whether a drug treatment increases responding above the saline control level, a one-tail *t*-test is used; a two-tail test is used in other cases.

Critical Assessment of the Method

The procedure requires neither behavioral training nor expensive equipment and overcomes some of the limitations that are typical for other conflict procedures. However, the procedure still uses electric shock as inhibitory stimulus.

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Ultrasound-Induced Defensive Behavior in Rats

Purpose and Rationale

Rats exposed to aversive stimuli display specific defence behavior as a part of their natural survival strategy. One component of this behavior is the production of ultrasonic calls in the 20–27 kHz range, which are thought to serve a communication role. Artificially generated ultrasound produces intensity-related locomotion characteristic of defensive behavior (Beckett et al. 1996).

Procedure

The apparatus (Beckett and Marsden 1995) consists of a circular open field arena, 75 cm in diameter, 46 cm high walls, with a video camera suspended above. Locomotor behaviors are recorded and analyzed using a computer-automated tracking system capable of following rapid movements (VideoTrack, CPL Systems, Cambridge, UK). This allows the ultrasound-induced change in locomotor behavior to be quantified in maximum speed, average speed, and distance traveled by the animals. Data are expressed as 15 sequential 20-s bins over the duration of the experiment.

Ultrasound (continuous tone, square wave, 20 kHz) is produced using a multifunction signal generator at sound pressure intensities of 65, 72, and 75 dB, as measured from the arena, 20 cm horizontally from the speaker. Sound is delivered to the testing arena via a high-frequency

piezoelectric speaker mounted at a height of 40 cm on the wall of the testing arena. The signal frequency and intensity delivered to the speaker are monitored using a digital oscilloscope. White noise is generated using a standard generator and the sound intensity measured as above.

Animals are placed in the test arena 20 min after intraperitoneal injection of drug or vehicle and locomotor activity is measured. After 2 min they are exposed to a 1-min, 20 kHz, square wave ultrasound tone (65, 72, or 75 dB sound pressure intensity, randomized) followed by a further 2 min without sound. This procedure is repeated for each intensity with a 1-min interprocedure interval. Locomotor activity values are then calculated for the maximum speed, average speed, and total distance traveled throughout the 5-min test period and expressed as a series of 15–20-s time epochs.

Evaluation

Maximum speed is analyzed using a two-way ANOVA. Significant interactions between treatment and time are followed by one-way ANOVAs for individual time points with post hoc Duncan's new multiple range test.

Modification of the Method

Moleweijk et al. (1995) evaluated ultrasound vocalizations of adult male rats in association with aversive stimulation as a screening method for anxiolytic drugs.

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Anxiety/Defense Test Battery in Rats

Purpose and Rationale

Blanchard et al. (1989, 1990, 1992) described a set of procedures designed to assess the defensive reactions of rats to a natural predator, the cat. These tests involve a brief confrontation of laboratory rats with an unconditioned threat stimulus (cat) which, to preclude physical contact, is presented behind a wire mesh barrier. The primary measures, taken both during and after cat presentation, include movement arrest and risk assessment (proxemics/activity test) and the inhibition of nondefensive behaviors (eat/drink or freezing test).

Procedure

The test apparatus for both the proxemics/activity and eat/drink procedures consists of two parallel subject chambers (53 × 20 × 25 cm). The inside walls of each chamber are constructed of opaque black Plexiglas, while outer walls and lids are clear Plexiglas to allow video recording from lateral and overhead views. The end wall of each chamber, constructed of wire mesh, adjoins a separate cat compartment. Subject movements are monitored by five photocells mounted at equal distances over the length of each chamber, and a food hopper and drinkometer are positioned 2.5 cm to each side of the central photocell. Access to the food hopper/drinkometer can be prevented by insertion of Plexiglas gates.

Each rat (Long-Evans strain, female or male, about 100 days old) receives the same injection (drug or saline) in each of the two successive paradigms. The initial study assesses the effects of cat exposure on proxemics/activity followed 7 days later by analysis of eat/drink behavior during and after cat exposure. Both procedures are carried out under dim red light.

Proxemic/activity testing. Rats are individually placed in each compartment of the test apparatus. Following a 5-min precat period, the cat is

introduced to the cat compartment for 5 min. Following removal of the cat, behavior is recorded for a further 15 min postcat period, for which measures are summed in three 5-min blocks. The test session is video recorded for analysis of lying, crouching, rearing, locomotion, and grooming. Proxemic location is measured by a digitizing system which divides the length of the subject compartment into thirds, indicating animal's location near the cat compartment, in the midsection of the box, or far from the cat compartment. Assessment of transits indicates movement from one section to another.

Eat/drink testing. Rats are individually given 2 g of finely crushed chocolate cereal on the 2 days after the proxemic/activity test, to familiarize them with this highly preferred food. In order to induce a mild water deprivation, water bottles are removed, 24 h prior to eat/drink testing. On the test day, animals are individually placed in the subject compartments for a 5-min precat period, during which the food hopper and water dispenser are concealed with Plexiglas gates. At the beginning of the 5-min cat period, these gates are removed allowing free access to the crushed chocolate cereal and water. After removal of the cat, the rats are monitored for a further 15-min postcat period. Measures of eating frequency and duration and drinking frequency are taken for the cat and postcat periods.

Evaluation

The data are analyzed by analysis of variance (ANOVA). Subsequent comparisons between treatment groups and control are carried out using Newman-Keuls procedures.

Modifications of the Test

Farook et al. (2001, 2004a, b) and Wang et al. (2003) described a **cat-freezing test apparatus**. The apparatus consisted of a completely enclosed, black, cat compartment (55 × 38 × 30 cm) with a wire mesh floor opening 42 × 19 cm and an open-top rat compartment (38 × 24 × 19 cm) made of clear Plexiglas to allow observation. The cat compartment could be placed stably on the top of the rat compartment. Male PVG hooded rats weighing 210–260 g were used. Locomotor activity was monitored using an Opto-Varimex Mini (Columbus Instruments), which measured the interruptions of optical beams that were placed 2.5 cm apart. The predator cat was a 4.3-kg male, which was selected for a total absence of aggressive behavior toward the rodents. During a 20-min test period, the duration of freezing, defined as the absence of all movements except movements related to breathing, was monitored. Freezing was expressed as the percentage of time the rat spent frozen during this 20-min session.

Blanchard et al. (1986a, b, 1989) developed a battery of tests designed to elicit a wide range of active and passive defensive activities in **wild rats**.

Griebel et al. (1997, 1998a, b, 2001, 2002) designed a mouse defense test battery in which **Swiss mice** were confronted with a natural threat (a rat) and behaviors associated with this threat were recorded.

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Repetitive Transcranial Magnetic Stimulation

Purpose and Rationale

Transcranial magnetic stimulation was introduced by Barker et al. (1985). The regional electrical activity in the brain is influenced by a pulsed magnetic field. Repetitive transcranial magnetic stimulation can cause functional changes in the cortex. Effects have been demonstrated in humans as well as in animals (Weissman et al. 1992; Fleischmann et al. 1995; Zyss et al. 1997; Ji et al. 1998; Ben-Shachar et al. 1999; Hausmann et al. 2000; Luft et al. 2001). Most studies indicated an antidepressant or anxiolytic effect (Keck et al. 2000, 2001; Tsutsumi et al. 2002; Kanno et al. 2003); however, this effect is dependent on frequency of stimulation (Sachdev et al. 2002) and duration of treatment (Hedges et al. 2003, 2005; Isogawa et al. 2003). Isogawa et al. (2005) found that 10-day repetitive transcranial magnetic stimulation induced anxiety in normal rats, as evidenced by expression of anxiety behaviors in the elevated plus maze.

Procedure

Male Wistar rats were used. All animals received repetitive transcranial magnetic stimulation for 10 days. On day 10, groups of five rats were treated with saline i.p. or anxiolytics or antidepressants.

Magnetic stimulation was delivered via a high-frequency magnetic stimulator with a round coil (4 cm in diameter) positioned over the rat's head. The stimulator delivered a biphasic cosine current with a pulse width of 200 |is in duration. The switching elements transfer up to 250 J per pulse to the coil, depending on the intensity setting. The peak magnetic flux at the center of the coil is approximately 2.2 T at maximal output. The stimulus amplitude was set at an intensity of 1.5 motor thresholds for motor-evoked potentials recorded from the gastrocnemius muscles.

To deliver the stimulation, awake animals were gently held by hand to minimize any discomfort. The coil was held tangentially in direct physical contact with the rat's head. To keep the same brain region stimulated, the center of the coil was set at

the center of the vertex of the skull. All animals received one set of training stimuli daily between 13:00 and 18:00 h for 10 days. To control for the auditory stimulation that accompanies high-frequency magnetic stimulation, sham stimulations were performed with the coil held far enough away (15 cm) from the induced magnetic field.

On the first day of testing, all rats were evaluated on the elevated plus maze. This consisted of two opposite open arms (50 × 10 cm) without side walls and two opposite enclosed arms (50 × 8 × 40 cm) and was elevated 50 cm above the floor. At the beginning of the test, rats were placed in the middle of the maze facing one of the open arms, immediately left alone in the test room, and were observed for 300 s using a video camera. The following parameters were measured: (1) time spent in the open arms, (2) total number of entries into the open arms, (3) total number of entries into the closed arms, (4) number of stretched-attend postures, and (5) number of head dips over the edge of the platform.

As a measure of exploratory activity, animals were placed in a standard apparatus. General motor activity was determined by the number of infrared photobeam breaks in a 2-h period. Rats were tested again after treatment with drugs on day 10.

Evaluation

The results are presented as the means ± SEM of individual values from each group. Overall statistical significance was determined using one-way ANOVA with a post hoc Dunnett test. For general motor activity, results were analyzed using two-way ANOVA.

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Marmoset Human Threat Test

Purpose and Rationale

The behavior of the common marmoset (*Callithrix jacchus*) as described by Stevenson and Poole (1976) can be used for evaluation of potential anxiolytic drugs (Costall et al. 1988).

Procedure

Male or female laboratory-bred common marmosets weighing 350–400 g are housed in single-sex pairs. Holding rooms are maintained at 25 °C at a humidity of 55 % on a 12 h light/dark cycle. Tests are conducted between 13:30 and 15:30 in the normal holding room. The holding cages are 75 cm high, 50 cm wide, and 60 cm deep.

A behavioral change characterized by retreat from and posturing toward a human threat is initiated by a human observer standing in close proximity in front of the holding cage. Changed behavior is recorded over a 2 min period by the observer. The behavioral measures selected are

(i) The percentage of time spent on the cage front in direct confrontation with the human threat
(ii) The number of body postures, primarily shown as raising the tail to expose the genital region with varying degrees of piloerection, anal scent marking, and slit stare with flattened ear tufts

The animals are used at 7 day intervals and are subject to a random crossover of treatments.

Drugs are administered 45 min before exposure to the human threat situation.

Evaluation

Statistical analysis is performed with one-way analysis of variance followed by Dunnett's *t*-test.

Critical Assessment of the Method

The human threat test in marmosets has the advantage of using primates instead of rodents. However, it is subject to individual scoring of the observer.

Modifications of the Test

Cilia and Piper (1997) developed a method of measuring conspecific confrontation-induced behavioral changes in common marmosets together with automated monitoring of locomotor activity as a possible model of anxiety.

Barros et al. (2000) measured fear and anxiety in the marmoset (*Callithrix penicillata*) with a novel predator confrontation model. The wild cat (*Felis tigrina*) was chosen as taxidermized predator to induce anxiety-related behaviors.

Borsini et al. (1993) used female **cynomolgus monkeys** to test aggressiveness against the observer.

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Psychosocial Stress in Tree Shrews

Purpose and Rationale

Male tree shrews (*Tupaia belangeri*) provide an animal model to study the neurobehavioral and endocrine consequences of chronic psychosocial stress (Fischer et al. 1985; Fuchs et al. 1993, 1996; Fuchs and Flügge 2002; Fuchs 2005). When living in visual and olfactory contact with a male conspecific by which it has been defeated, the subordinate tree shrew shows dramatic behavioral, physiological, and neuroendocrine changes. The pattern of these changes resembles a depression-like symptomatology.

Procedure

Animals are subjected to a 10-day period of psychosocial conflict to elicit stress-induced behavioral and endocrine alterations before the onset of drug treatment. For a treatment period of 30 days, animals are given either saline or an oral dose of test compound.

Evaluation

At the end of the test period, the following parameters are recorded: behavioral changes (marking, grooming, locomotor activity) and endocrine changes (urinary cortisol and norepinephrine excretion).

Modification of the Method

Lucassen et al. (2004) found that treatment with an antidepressant reduces apoptosis in the hippocampal dentate gyrus and temporal cortex induced by a 7-day stress period in tree shrews.

Czéh et al. (2005) examined the effects of a NK_1 receptor antagonist in the chronic psychological stress model of adult male tree shrews.

Shively et al. (2005) described social stress–associated depression in adult female **cynomolgus monkeys**.

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Aversive Brain Stimulation

Purpose and Rationale

Electrical stimulation of brain aversive areas, in particular the midbrain central gray, induces defensive reaction and/or flight behavior in several species and, therefore, may be viewed as an animal model of anxiety or of panic attack. Most studies used intracerebral microinjections of neurotransmitters, their agonists, and antagonists to elucidate the mechanisms of aversive or antiaversive effects (Schütz et al. 1985; Graeff et al. 1986, 1990, 1993, 1997; Graeff 1991; Audi et al. 1988, 1991; Brandão et al. 1991; Brandão 1993; Broekkamp et al. 1991; Nogueira and Graeff 1991, 1995; Motta and Brandão 1993; Aguiar and Brandão 1994, 1996; Melo and Brandão 1995; Motta et al. 1995; De Araujo et al. 1998). Other groups evaluated the effect of drugs, e.g., serotonin receptor antagonists, on periaqueductal gray stimulation-induced aversion after peripheral application (Bovier et al. 1982; Clarke and File 1982; Jenck et al. 1989, 1995, 1996, 1998, 1999; Beckett and Marsden 1997). Dorsal periaqueductal gray-induced aversion is considered as a model of panic anxiety.

Procedure

Surgery. A stainless-steel bipolar twisted electrode, insulated to the tip which is cut square to the shaft, is implanted to the dorsal part of the periaqueductal gray matter of male Wistar rats weighing 370–450 g under pentobarbital anesthesia. According to the atlas of Paxinos and Watson (1982), the coordinates for the electrode tip are 5.8 mm posterior to the bregma, 0.2 mm lateral to the midline, and 5.0 mm ventral to the surface of the skull. The electrode is held in place with dental cement and five screws threaded into the skull.

Behavioral Procedure. Animals are placed in a rectangular cage (20 × 36 × 20 cm high) with a grid floor and a 2 cm high barrier dividing the cage in half. They are allowed to explore freely for 10–15 min before the stimulation begins.

Brain stimulation consists of constant current square wave, 0.1 ms duration, monophasic pulses conducted from the neurostimulator (Grass S88 + stimulus isolation unit SIU8) to the electrode by way of flexible wire leads. Pulse duration, pulse frequency, and stimulation intensity are monitored by an oscilloscope. Animals are screened for stimulation-induced aversion using a fixed stimulation frequency of 50 Hz; current is raised slowly until aversive behavioral signs are observed. Aversive effects are first characterized by visible autonomic reactions (increase in respiratory rates, piloerection, eventually mydriasis) in animals which are behaviorally frozen. Increasing the intensity induces, following a freezing period,

active behavioral signs, ranging from ear dressing and head weaving to sudden running and attempts to escape out of the cage.

With this fixed stimulation intensity, aversive behavior is shaped into an operant escape response: rats are trained to stop the stimulation by escaping from one compartment to the opposite compartment of the cage. Brain stimulation is switched off when the rat crosses the middle line separating the two compartments or after a maximal cutoff time of 20 s. A trial is applied every min. Three to 10 daily sessions of 30 trials are required to obtain stable responses.

Self-Interruption Threshold Determination

Once an animal displays steady performances during 3 consecutive days on this task with fixed intensity and fixed stimulation frequency, it undergoes the next step of training aimed at determining its stimulation frequency threshold for escape reaction. This consists of testing the animals in a threshold procedure in which the frequency is varied while stimulation intensity is held constant. This procedure keeps the size of the stimulation field around the electrode tip constant.

A stimulation intensity is chosen and defined as the threshold intensity eliciting escape when the stimulation frequency is 50 Hz. With this intensity held fixed, a method of limits is employed to determine the frequency threshold for escape: the stimulation frequency is either decreased or increased depending on the response displayed by the animal on the previous trial. Starting from 50 Hz, stimulation frequency is decreased by 5 Hz steps following a trial in which the rat responds to the stimulation and is increased by 5 Hz steps, in case the animal fails to respond to the stimulation. When a response is made, the time elapsing between the onset of the stimulation and the moment the animal crosses the midline barrier is also recorded as escape latency; no response is associated with the maximum cutoff time of 20 s.

Frequency threshold is calculated as the average frequency eliciting an escape reaction during a 20 min preinjection session; an average escape latency is calculated the same way. Drugs are then injected intraperitoneally at various doses, and 35 min following administration, frequency threshold is determined again over a 25–30 min postinjection session. Drug effects can be estimated by comparing thresholds and latencies for each animal before and after injection. Doses are injected at least 4 days in a counterbalanced order. Animals serve as their own controls and can undergo several treatments.

Evaluation

Data are analyzed by means of analysis of variance, followed by paired *t*-tests. Dose–response curves are established for active drugs.

Modification of the Method

The group of Graeff et al. (1986) used an electrode-cannula, called chemitrode, for electrical stimulation and microinjection of drugs or neurotransmitters at the same place of the periaqueductal gray. This chemitrode was made of stainless-steel cannula (outside diameter 0.6 mm, length 12.5 mm) glued to a brain electrode made of stainless-steel (diameter 250 mm) enamel insulated except at the cross section of the tip, reaching 1 mm below the lower end of the cannula (Nogueira and Graeff 1995).

Schenberg et al. (2001) appraised the isomorphism of dorsal periaqueductal gray-evoked defensive behaviors and panic attacks both in rats and humans.

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Conditioned Behavioral Responses

Sidman Avoidance Paradigm

Purpose and Rationale

Sidman (1953) described an apparatus for the evaluation of two temporal parameters (shock-shock interval and response-shock interval) of the maintenance of avoidance behavior by the white rat. The procedure has been widely used to evaluate CNS depressant compounds, neuroleptics, anxiolytics, and sedatives.

Procedure

The test cage is equipped with a single lever and a light. This cage is enclosed in a sound-attenuating chamber with a fan and with a speaker emitting a white-noise auditory background. The test cage has a grid floor of steel bars which are attached to a scrambled shock source. The data are recorded in an adjacent room. Male Sprague–Dawley rats with a starting weight of 250–300 g are housed in individual cages. They are trained to avoid an unsignaled shock by repetitive lever-pressing responses. A shock (1.5 mA for 0.5 s) is delivered to the grid floor every 15 s if no responses occur (shock-shock interval of 15 s = SS-15 s). A lever press (response) will delay the oncoming shock for 30 s (response-shock interval of 30 s = RS-30 s). The responses do not accumulate for delays of shock; a shock will be delivered 30 s after the last response is made even if 10 responses are made 31 s prior. Every 30 min, the total number of shocks received and the total number of responses made are accumulated and constitute the basic data. The animals are trained until they maintain a stable response rate and receive no more than 100 shocks/5 h test session. After reaching these criteria of performance, experimental compounds are administered and their effects on the performance of this learned avoidance behavior evaluated. The experimental compounds or the standard are usually administered by i.p. injection immediately prior to testing in volumes of 1 ml/kg of body weight. Depressant drugs lower the rate of lever presses and increase the number of shocks received. Stimulant drugs increase the rate of lever pressing.

Evaluation

The effect of a drug on the performance of an animal is compared to the data generated in the previous nondrug sessions. Each animal thereby serves as its own control. The basic measures of performance during a specific time interval, responses, and shocks are used for evaluation. Responses are reported both as total and as percent of control responses. Shocks are reported as totals and as shock-avoided (SHA) as percent of control. This latter measure is computed by subtracting the number of shocks received from the total number of possible shocks if no responses had been made. In the initial screening of experimental compounds, the results are reported in terms of the total effect during a 5-h test. However, an ED_{50} is usually estimated during a representative time of peak activity.

Critical Assessment of the Method

Anxiolytics show activity in this test; however, it has been proven to be more reliable for neuroleptic activity. Apparently, the present conditioned active avoidance paradigms do not constitute a reliable method for screening anxiolytic agents, in spite of their homologies with human anxiety.

Modification of the Method

Heise and Boff (1962) and Galizio et al. (1990) extended the method by using two levers, an "avoidance lever" and an "escape lever" for calculating ratios between shock rate, escape failure rate, and avoidance rate.

Balfour (1990) described the effect of drugs on rat behavior in an unsignaled Sidman avoidance schedule.

Wadenberg et al. (1998) described and evaluated a newly designed apparatus for the assessment of conditioned avoidance response performance in rats.

Patel and Migler (1982) reported a sensitive and selective conflict test in **squirrel monkeys**.

Szewczak et al. (1995) tested antipsychotic agents using continuous avoidance behavior in adult male squirrel monkeys.

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Geller Conflict Paradigm

Purpose and Rationale

Experimentally induced conflict by punishing food-rewarded behavior has been used to differentiate between various psychoactive drugs by Geller and Seifter (1960). The basic principle has been used and modified by many authors to reveal possible antianxiety effects of experimental compounds.

Procedure

Male albino rats with a starting body weight of 300–400 g are housed individually. They are food deprived until the body weight is gradually reduced by approximately 20 % of original and it is maintained at this level by restricted food diet. Conditioning is carried out in commercially available Skinner boxes (e.g., Campden Instruments, London, UK) equipped with a house light, a single lever, cue lights, a liquid dripper, and a grid floor connected to a shocker. Sweetened condensed milk delivered by the liquid dipper serves as the positive reinforcer. The data are recorded on cumulative recorders.

The animals are trained to lever press for the milk reward in two distinct response-reward sections. In the anxiety or "conflict" segment (signaled by onset of both tone and cue lights), a dipper of milk is delivered in response to each

lever press (continuous reinforcement schedule = CRF) However, lever presses during this period are also accompanied by a 40-ms pulse of aversive foot-shock through the grid floor. This creates a conflict between milk reward and a painful foot-shock. This conflict period is 3 min in duration.

During the other segment of this paradigm, the lever presses produce a drop of milk only at variable intervals of time from 60 to 210 s with an average reward of once per 2 min (variable interval = VI-2 min). No shocks are administered during this variable interval phase of testing which is 15 min in duration.

The test procedure consists of four 15 min nonshock variable interval segments where reinforcement is available on a restricted basis. Each variable interval period is followed by a 3 min CRF conflict period phase when reinforcement is constantly available but always accompanied by an aversive foot-shock. The shock level is adjusted for each subject to reduce the CRF responding to a total of less than 10 lever presses during the entire test. The rats are tested 2–4 days a week. Drugs are administered once per week and the performance compared to the previous day's control trial. The VI responses are used to evaluate any general debilitating drug effects while the CRF responses are used to evaluate any antianxiety effects as indicated by the increased responding during the CRF conflict period.

The test compounds are administered intraperitoneally 30 min or orally 60 min before the test period.

Evaluation

The total number of lever presses during the conflict periods (CRF) and the nonconflict periods (VI) are counted. Values of treatment sessions are expressed as percentage of values of the preceding nontreatment day. An increase of lever presses in the conflict period is regarded as indication of an antianxiety effect and a decrease of lever presses in the nonconflict period as an indication for a sedative effect. In this procedure linear dose–response curves are rarely found. Therefore, minimal effective doses (MED) are calculated.

Critical Assessment of the Method

The method is suitable to distinguish anxiolytics from other centrally active drugs, such as sedatives and neuroleptics. The relative potency of various anxiolytic agents in a number of species compares favorably with their relative potency in humans. The negative aspects are the time-consuming procedure, the high expenses for the apparatus, and the difficulty to obtain ED_{50} values.

The relevance of this test and other models has been challenged by Bignami (1988).

Modification of the Method

The method has been modified by many authors.

Davidson and Cook (1969)and Cook and Davidson (1973) introduced a schedule of 10 lever presses for the delivery of one food pellet together with an electric foot-shock. In the variable interval reinforcement period, responses were reinforced by food delivery at varying intervals of time; the mean interval was 30 s. Also under these conditions differentiation of various psychotropic drugs could be achieved.

Iorio et al. (1986) and Chipkin et al. (1988) used a procedure during which a tone (5 s) preceded and then overlapped for 10 s with scrambled foot-shocks. After the start of the tone, the rat has the option to avoid (in the no-shock period) or escape (in the shock period) the shock by jumping onto a platform located 17 cm above the grid floor.

Thiébot et al. (1991) developed the method further by introducing "safety signal withdrawal," a behavioral paradigm in rats sensitive to both anxiolytic and anxiogenic drugs.

Commissaris and Fontana (1991) published a potential animal model for the study of antipanic treatments. Rats were trained to drink their daily water ration during 10-min sessions. These sessions were characterized by alterations of silence (unpunished periods) and the presence of a tone (punished periods). Tube contact during the tone periods resulted in a 0.25–0.5 mA shock delivered to the mouth of the animal for the duration of the tube contact. After 4 weeks of training, subjects received either chronic post-test treatment with an antidepressant or vehicle, twice daily, 7 days per week. Acute pretest administration of traditional anxiolytics (benzodiazepines, barbiturates)

increased dramatically the number of punished contacts made in a dose-dependent manner. Typical antidepressants were not active after pretest administration but were after chronic post-test treatment.

Beaufour et al. (1999) investigated the effects of chronic antidepressants in the conditioned suppression of operant behavior in rats. In daily 18-min sessions, three periods of nonpunished lever pressing for food alternated with two 4-min periods signaled by a light-on conditioned stimulus during which 50 % of the responses were randomly punished by electric foot-shocks. Antidepressants were administered once daily for 7–8 weeks to trained food-restricted rats.

The conflict test has also been adapted to the **mouse** (Prado de Carvalho et al. 1986).

The conflict behavior and anticonflict effect of anxiolytics has been demonstrated in a variety of species, including **pigeons** (Morse 1964; Wuttke and Kelleher 1970; McMillan 1973; Gleeson et al. 1989; Barrett et al. 1989, 1994; Barrett 1991; Schipper et al. 1991; Pollard et al. 1992; Mos et al. 1997).

Patel and Migler (1982) described a sensitive and selective conflict model in male squirrel **monkeys** in which antianxiety agents exhibit pronounced anticonflict activity.

Ervin et al. (1987), Ervin and Cooper (1988), van Heest et al. 1991, and Simiand et al. (1993) used **conditioned taste aversion** as a conflict model. Moderate taste aversions were induced by pairing the initial consumption of 0.25 % sodium saccharin with either 25 mg/kg 5-hydroxytryptophan or 30 mg/kg i.p. LiCl. Antagonism was found with benzodiazepines and nonbenzodiazepine-anxiolytic drugs.

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Progressive Ratio Procedure

Purpose and Rationale

The progressive ratio schedule was introduced as a model for studying the psychomotor stimulant activity of drugs in the rat by Poncelet et al. (1983). Male Wistar rats were trained to press a lever with food reinforcement according to a continuously reinforced schedule (CRF). Afterward, rats were subjected to three experimental sessions (30 min each) during which responding was rewarded according to a progressive ratio schedule (following an initial 2 min CRF period, the number of presses necessary for the pellet delivery was doubled every 2 min). Responding during the first half of each session, i.e., pressing for food, was maintained at a significant level, whereas it was almost suppressed during the second part of the session. Animals treated with stimulants showed an increased rate of responding.

Several authors used the progressive ratio test (Richardson and Roberts 1991; McGregor et al. 1993; Bourland and French 1995; Ferguson and Paule 1996; Pulvirenti et al. 1997, 1998; Duvauchelle et al. 1998; Grottnick et al. 2000; Mobini et al. 2000; Wilcox et al. 2000; Woolverton et al. 2002; Schneider und Koch 2002, 2003; Kozinowski et al. 2003; Solinas et al. 2004).

Procedure

Drews et al. (2005) studied the effects of a cannabinoid agonist on operant behavior and locomotor activity in rats. The progressive ratio test was conducted in an operant chamber (24 × 28 × 28 cm) (Operant Behavior System, TSE Systems, Bad Homburg, Germany). First, male Wistar rats were habituated for 1 day to the test chamber, the palatable casein pellets, and the noise of the magazine response. After shaping, rats were trained over 3 days at lever pressing in sessions of 30 min on a continuous reinforcement schedule until they reached a stable baseline. After lever pressing was completed, one progressive ratio test session (for 30 min) was conducted on the next day. The progressive ratio schedule (i.e., lever presses for one pellet) was changed every 2 min according to the following exponential progression: 1, 2, 4, 6, 9, 12, 15, etc., derived from the formula 5. $e^{02\ n}$–5, where n is the position in the sequence of ratios (Mobini et al. 2000). The so-called break point, the conventional index of performance on a progressive ratio schedule of reinforcement (Reilly 1999), was defined as the first progressive ratio sequence where lever pressing decreased $\leq$50 % relative to the previous phase without increasing $\geq$100 % in the following phase.

Evaluation

Student's *t*-test was used to evaluate operant responding in the progressive ratio test.

Modification of the Method

Barr and Phillips (1999) found that withdrawal following exposure to D-amphetamine decreases responding for a sucrose solution as measured by a progressive ratio schedule of reinforcement. The authors concluded that the progressive ratio procedure may be a useful technique for evaluating changes in motivation to natural reinforcing stimuli following withdrawal from psychostimulant drugs.

For safety studies, the response requirement increases for every infusion or after a fixed period of time until the animal ceases to lever-press for a predetermined interval, defined as the breaking point. The presumption of progressive ratio studies is that the higher the breaking point the greater the reinforcing effectiveness, although the same potential complications regarding pharmacokinetics that were noted above for a simple (FR) schedule of self-administration also apply to progressive ratio schedules.

Weed et al. (1997) studied the relationship between reinforcing effects and in vitro effects of D1 agonists in **rhesus monkeys**.

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Conditioned Defensive Burying in Rats

Purpose and Rationale

Besides the well-known defensive reactions of animals in typical laboratory settings, like freezing, fleeing, and attacking, rats tested in the presence of bedding material develop a peculiar behavior when shocked through a stationary prod by burying the shock source. This behavior has been proposed as a new paradigm for the study of anxiolytic agents (Pinel and Treit 1978, 1983; Treit et al. 1981).

Procedure

Male adult rats weighing 250–400 g are used. The testing is performed in 44 × 30 × 44 cm Plexiglas test chambers, the floor of which is covered with 5 cm of a commercial bedding material. In the center of each of the four walls, 2 cm above the level of the bedding material, is a small hole through which a 6.5 × 0.5 × 0.5 cm wire-wrapped wooden dowel (i.e., the shock prod) can be inserted. Electric current is administered through the two uninsulated wires wrapped around the prod. The behavior of each rat is monitored for 15 min from a separate room via closed-circuit television.

Before each of the experiments, the rats are placed in the Plexiglas test chamber in groups of 5 or 6 for 30-min periods on each of 4 consecutive days. In the experiments, groups of 10 animals are used for each dose and control. The rat is injected intraperitoneally with the test drug or saline before being placed into the test chamber in which the shock prod is inserted. The animal is placed into the center of the chamber so that is faces away from the prod. When the rat first touches the prod with its forepaw, it receives a brief electric shock (1 mA) which typically elicits a flinch away from the prod and withdrawal toward the back of the chamber. Afterward, the rat moves directly toward the prod, pushing and spraying a pile of bedding material ahead with rapid shoveling movements of its snout and alternating pushing movements of its forepaws. The prod is buried in a pile of bedding material. The duration of burying is recorded.

Evaluation

The mean duration of burying in treated animals is compared with controls. Anxiolytics as well as neuroleptics shorten the burying behavior dose-dependent at low shock intensity (1 mA). A second measure is the height of the bedding material. At high shock intensity (10 mA) only neuroleptics but not benzodiazepines are active.

Critical Assessment of the Method

The method of conditioned defensive behavior can be regarded as a simple and reliable method to detect anxiolytic activity of benzodiazepines and neuroleptics. However, using higher shock intensity – where only chlorpromazine was found to be active – a separation between benzodiazepines and neuroleptics has been demonstrated.

The drug class specificity of the test has been challenged by Craft et al. (1988).

Modification of the Method

Diamant et al. (1991) used telemetry to register autonomic and behavioral responses in the shock-prod burying test in rats.

Wiersma et al. (1996) reported that microinfusion of corticotropin-releasing hormone in the central amygdala of freely moving rats enhanced the active behavior responses in the conditioned defensive burying paradigm.

Fernandez-Guasti and Lopez-Rubalcava (1998) used the rat burying behavior test to study the effect of various potential anxiolytics.

Broekkamp et al. (1986), Njung'e and Handley (1991a, b, and Gacsályi et al. 1997) described burying of marbles by *mice* as harmless objects without punishment by electrical shocks as a model for detection of anxiolytics. Thirty minutes after treatment with drugs, male Swiss mice, weighing 20–24 g, were individually placed in a 23 × 17 × 14 cm cage with 25 glass marbles, 1.5 cm in diameter. The glass marbles were placed in close contact in the middle of the cage on a 5 cm layer of sawdust. The mice were left in the cage with the marbles for 30 min after which the test was terminated by removing the mice and counting the number of marbles that were more than two thirds covered with sawdust.

DeBoer and Koolhaas (2003) reviewed the ethology, neurobiology, and psychopharmacology of defensive burying in rodents.

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Taste Aversion Paradigm

Purpose and Rationale

When ingestion of a taste stimulus is paired with internal malaise the animal remembers the taste and rejects its ingestion thereafter. This phenomenon is called a conditioned taste aversion or taste aversion learning. In the classical experiment in rats, the conditioned stimulus is a 0.01 M drinking solution of saccharin paired with an intraperitoneal injection of 0.15 M LiCl solution as unconditioned stimulus. The underlying neural mechanisms (Yamamoto 1993; Agüero et al. 1993, 1996; Swank et al. 1995) as well as facilitating and inhibiting factors (Lipinski et al. 1995; Sobel et al. 1995) were investigated. Moreover, drugs by themselves can be studied. Rats are presented a fluid with palatable taste and immediately after consumption of this liquid injected with a drug, whose effect the animals have not experienced before. Subsequently, on a later occasion and under nondrug conditions, avoidance of the taste associated with the drug is measured (De Beun et al. 1996).

Procedure

Male Wistar rats weighing 220–250 g are housed in groups of four per cage under a normal 12 L:12D regime at 22–23 °C with free access to food and water. Twenty-four hours before the first conditioned taste aversion session, the animals are water deprived and fluid access from then on restricted to daily experimental sessions of 15 min which takes place individually in a test cage. After each session, the animals are returned to their home cages. Food is freely available in the home cages throughout the procedure, but not available during the sessions. For a given subject, all six sessions required to complete a conditioned taste aversion experiment take place in the same test cage. Animals designated to the same experimental group are run in parallel. During the first four sessions (day 1 through day 4), both bottles contain plain tap water. During this phase of the procedure, the animal learns to drink a reasonable amount of fluid in a short period of time. For the 5th session (day 5, conditioning session), both bottles are filled with a 0.1 % saccharin solution and immediately after completion of this session the animals injected with either the vehicle or different doses of the test drug. Per animal, only one dose (or the vehicle) of a particular drug is tested. On days 6 and 7, no sessions are conducted (washout period), and the animals have free access to tap water in the home cages from the end of day 5 until the morning of day 7, when the animals are again deprived of water, 24 h prior to the final 6th session (day 8, test session). During this last session, one bottle contains the saccharin solution, and the other bottle is filled with tap water. To control for location bias, the saccharin is presented in the left bottle for half of the animals in each group and in the right bottle for the other half. By measuring the amount of fluid consumed from both bottles separately, drug-induced conditioned taste aversion can be determined by comparison of the relative saccharin intake in the drug-treated groups and their vehicle-treated controls.

Evaluation

Data of test drugs are submitted separately to one-way analysis of variance, with the between-subjects factor DOSE. The dependent variable is the ratio of (saccharin solution/saccharin solution + tap water) intake. Fluid intake scores are calculated in grams. Post hoc analyses are used with Tukey HSD multiple comparisons. Results are considered significant when $p < 0.05$.

Modification of the Method

Besides LiCl, several other drugs were used to induce taste aversion, such as ethanol (Gauvin and Holloway 1992; June et al. 1992; Thiele et al. 1996; Bienkowski et al. 1997), morphine (Miller et al. 1990; Bardo and Valone 1994),

cocaine (Van Haaren and Hughes 1990; Glowa et al. 1994), naloxone (Mucha 1997), apomorphine (McAllister and Pratt (1998), caffeine (Brockwell et al. 1991), D-amphetamine (Davies and Wellman 1990; Lin et al. 1994), nicotine (Shoaib and Stolerman 1996), quinine (Parker 1994), cisplatin (Mele et al. 1992), benzodiazepine and nonbenzodiazepine anxiolytics (Neisewander et al. 1990), 5-hydroxytryptamine (Rudd et al. 1998), dopamine D_3 agonists (Bevins et al. 1996), cyclosporine A (Exton et al. 1998), cholecystokinin (Ervin et al. 1995; Mosher et al. 1996), and Δ9-tetrahydrocannabinol (Parker and Gillies 1995).

Turenne et al. (1996) found individual differences in reactivity to the aversive properties of drugs. Rats were assigned to high conditioned taste avoidance and low conditioned taste avoidance groups on the basis of their intake of saccharin solution previously paired with morphine, amphetamine, lithium, or fenfluramine.

Willner et al. (1992) investigated the influence of drugs on taste-potentiated odor aversion learning in rats.

Rabin and Hunt (1992) studied taste aversion learning in **ferrets**.

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Cued and Contextual Fear Conditioning

Purpose and Rationale

Fear conditioning is a form of Pavlovian conditioning where an animal is trained to associate a neural stimulus (e.g., a 10-s presentation of light) with an aversive, unconditioned stimulus (US), such as an electric foot-shock. After such pairings, the light alone predicts the occurrence of the shock and acts as a conditioned stimulus (CS), eliciting a state of fear. Tones, lights, odors, and tactile stimuli have been used as CS in fear-conditioning experiments (Anagnostaras et al. 1999; Fendt and Fanselow 1999). A startle response is elicited by a sudden acoustic, visual, or tactile stimulus and is composed of a fast, sequential muscle contraction, with the most prominent reaction around the face, neck, and shoulders.

The **fear-potentiated startle paradigm** was initially described by Brown et al. (1951). Rats are given several pairings of light CS and foot-shock. After this procedure, the mean amplitude of the acoustic startle response to a loud noise is usually 50–100 % higher in the presence of the light CS than to the noise alone. The difference between these two trial types represents the fear potentiation of the startle response and acts as a measure of fear.

In the **cued and contextual fear conditioning paradigm**, animals learn to associate a novel environment (context) and a previously neutral stimulus (conditioned stimulus, a tone) with an aversive foot-shock stimulus. Testing then occurs in the absence of the aversive stimulus. Conditioned animals, when exposed to the conditioned stimuli, tend to refrain from all but respiratory movement. Freezing responses can be triggered by exposure to either the context in which the shock was received (context test) or the conditioned stimulus.

Sullivan et al. (2003) determined the effects of doxapram for cue and contextual fear conditioning in rats.

Procedure

Fear Conditioning Apparatus

Pavlovian fear conditioning was performed using a clear polycarbonate conditioning chamber encased by a sound-attenuating outer box and fitted with metal bars as a floor that can deliver current (context 1). For fear conditioning to cue, the context was changed between the conditioning and the testing day to enhance the specificity of the fear response to the tone cue on testing. For the testing day, striped walls were placed on the outer surface of the clear chamber walls, the floor was fitted with a black panel covering the bars, the ambient light was lower, and the floor had a peppermint odor from cleaning with a scented detergent (context 2). A microcamera on the ceiling of the chamber allowed observation during the experiments and a videotape record for scoring of behavior.

Cued Fear Conditioning Behavioral Procedure

On conditioning day 0, adult male Sprague Dawley rats were habituated to the conditioning chamber for 15 min. On conditioning day 1 the rats received an intraperitoneal injection of drug and were returned to the home cages. After 10 min each rat was placed in the conditioning chamber (context 1) and was trained with two trials of tone-shock pairings. The tone (10 kHz, 72 dB) lasted 20 s and coterminated with a 0.5-s, 7 mA shock. The intertrial interval was variable (mean 120 s) and was generated by a computer program. Videotape of the conditioning allowed later scoring of the level of freezing during each tone. On test days 1, 2, and 3, rats were tested with the different contextual stimuli in the chamber (context 2). On each day they received ten presentations of the 20-s tone (no shocks) while being videotaped for later scoring of the time spent freezing during each tone. Rats were matched by weight for control and various doses of test drug.

Contextual Fear Conditioning Behavioral Procedure

On conditioning day 0, the rats were habituated to the conditioning chamber (context 1) for 20 min.

On conditioning day 1, the rats received an intraperitoneal injection of drug and were returned to their home cages. After 7 min, each rat was placed in the same chamber (context 1), and at 8 min after injection the computer program was initiated that trained with two shocks separated by a variable intertrial interval (mean 120 s). The shocks were 1.5 mA and lasted 0.5 s, and the total time in the chamber was 8 min for all subjects. On day 2, rats were tested in the same chamber (context 1) for 5 min while being videotaped for later scoring. The 30-s periods before and after shock 1, the 30-s period after shock 2, and the first 30 s of each minute during the 5 min of testing were scored for freezing. Rats were matched by weight for vehicle- and drug-treated groups.

Evaluation

Data were analyzed with a series of one-way analyses of variance with repeated measures or with Student's *t*-tests.

Modification of the Method

Several modifications of this procedure have been used for various purposes (Phillips and LeDoux 1992; Lu and Wehner 1997; Maren 1998; Laurent-Demir and Jaffard 2000; DeLorey et al. 2001; Gupta et al. 2001; Malkani and Rosen 2001; McKay et al. 2002; Riedel et al. 2002; Cambon et al. 2003; Fischer et al. 2003; Maciejak et al. 2003; Walker and Carrive 2003; Sienkiewicz-Jarosz et al. 2003; Célérier et al. 2004; Kudo et al. 2004; Mesches et al. 2004; Roberts et al. 2004; Takahashi 2004; Wehner et al. 2004).

Zhang et al. (2004) used a telemetric system for recording cardiovascular data and automated measurement of conditioned freezing behavior.

Contarino et al. (2002) described automated assessment of conditioning parameters for context and cued fear in mice.

Several authors (Radulovic et al. 1998; Stiedl et al. 1999, 2000; Kishimoto et al. 2000; Eckart et al. 2001; Maciejak et al. 2003; Tezval et al. 2004; Võikar et al. 2004; Radyushkin et al. 2005; Tovote et al. 2005) used a commercially available fear conditioning system (TSE Systems, Bad Homburg, Germany) consisting of the following components: up to four boxes with animal location sensors, shock grid, and test arena; box housing with software-controlled loud speaker, software-controlled light, and ventilator; control unit with integrated shocker/scrambler; fear conditioning software; PC interface.

Crestani et al. (2002) and Gould et al. (2004) distinguished between delay fear conditioning and trace fear conditioning. For delay fear conditioning, after 3 min exposure to the chamber mice received three successive tone-shock pairings with the shock delivered during the last 500 ms of the tone. Freezing was recorded 48 h later in a modified context (new olfactory, tactile, and visual cues) for 3 min and subsequently in the presence of the tone for 8 min. For trace fear conditioning, the procedure was similar to that for delay conditioning, except that an empty trace interval of 1 s was interposed between the tone and the foot-shock in three learning trials.

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Effects on the Endocrine System

Plasma Catecholamine Levels During and After Stress

Purpose and Rationale

A wide variety of stressors causes significant biochemical, physiological, and behavioral changes. These changes include marked increases in plasma catecholamines, heart rate, and blood pressure. Treatment with anxiolytic drugs can attenuate the stress-induced increases of norepinephrine and epinephrine in plasma (Vogel et al. 1984; Livesey et al. 1985; Taylor et al. 1989; de Boer et al. 1990; Krieman et al. 1992).

Procedure

Male Sprague–Dawley rats, 300–350 g, are individually housed and given food and water ad libitum. After 1 week of acclimatization, an aortic catheter is surgically implanted in each animal, running on top of the psoas muscle and brought out through an incision at the back of the neck. The catheters are flushed daily with heparinized saline. After a recovery period of 48 h baseline (time −15) blood samples are drawn and blood pressure recordings taken using a Grass model

7 polygraph. The animals are then given an i.p injection of the test compound or the vehicle. Fifteen minutes after the injection (time 0), blood samples, heart rate, and blood pressure measurements are taken. Animals in control and treatment groups are then stressed by immobilization for 1 h and blood samples, heart rate, and blood pressure recordings taken at time 15, 30, and 60 min during stress. Immobilization is performed by taping the legs of the animals to the laboratory bench. After the stress period of 1 h, the animals are released, returned to the home cage for recovery, and 1 h poststress samples and recordings taken. Nonstressed, compound, or vehicle-treated animals remain in the home cage for the entire test period and are sampled in the same manner as the stressed animals. Approximately 0.3 ml blood is withdrawn for each sample and an equal amount of 0.9 % saline reinfused to prevent changes in blood volume.

Evaluation

Determinations of norepinephrine and epinephrine in plasma are made using a radioenzymatic assay. Heart rate and systolic and diastolic blood pressure are determined directly from the polygraph tracings. The data are analyzed using a three-way analysis of variance (ANOVA) with repeated measures, two-way ANOVA, Students *t*-test, Student-Newman-Keuls method, and the trapezoidal rule for the area under the curve.

Critical Assessment of the Method

Reduction of catecholamine levels but no changes of the cardiovascular parameters could be found after treatment with anxiolytics. The test can be used as a method for evaluation of the influence of psychoactive drugs on the endocrine system (Krieman et al. 1992).

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Plasma Corticosterone Levels Influenced by Psychotropic Drugs

Purpose and Rationale

Corticosterone levels in the blood of rats are elevated not only after stress but also after application of selective 5-HT receptor agonists (Koenig et al. 1987). This has been used to differentiate typical and atypical neuroleptics (Nash et al. 1988).

Procedure

Male Sprague Dawley rats weighing 200–250 g are housed six per cage under temperature- and light/dark–controlled conditions with free access to food and water. On the day prior to an experiment, the animals are transferred to the experiment room. On the day of the experiment, various doses of test drugs or saline are injected intraperitoneally. After 60 min 2.5 mg/kg

6-chloro-2-(1-piperazinyl)pyrazine (MK-212) are injected intraperitoneally followed by decapitation after further 60 min. Trunk blood is collected and allowed to clot. Serum is obtained following centrifugation and stored at −20 °C for the radioimmunoassay of corticosterone.

Evaluation

Data are analyzed with a two-way analysis of variance. Differences between treatment groups are evaluated using the Student-Newman-Keuls test.

Modification of the Method

Korte et al. (1991) studied the effect of a 5-HT_{1A} agonist on behavior and plasma corticosterone levels in male Wistar rats before and after psychological stress of defeat.

Broqua et al. (1992) measured corticosterone and glucose levels in blood together with parameters of 5-HT metabolism in brain in stressed animals treated with the antidepressant tianeptine.

Rittenhouse et al. (1992) measured plasma concentrations of renin, corticosterone, ACTH, and prolactin in rats after treatment with a 5-HT_{1A} agonist in three stress paradigms: immobilization, forced swim, and conditioned fear.

Groenink et al. (1995) studied the corticosterone secretion in rats after application of 5-HT_{1A} receptor agonists and antagonists.

Aulakh et al. (1988, 1993) reported higher baseline levels of plasma corticosterone in fawn-hooded rats relative to Wistar and Sprague–Dawley strain rats. Long-term treatment (21 days) with antidepressant drugs significantly decreased plasma corticosterone in fawn-hooded rats. The authors recommended this strain as a genetic model of depression.

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Benzodiazepine Dependence

General Considerations

Benzodiazepine dependence is a hypothetical construct for the adaptive changes that occur as a result of chronic drug exposure. Two measures are usually considered to reflect dependence: the development to a drug's effects and the abstinence signs of drug withdrawal.

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Benzodiazepine Tolerance and Dependence in Rats

Purpose and Rationale

Induction of benzodiazepine tolerance and physical dependence has been reported for several animal species, such as rats, mice, dogs, and monkeys. Ryan and Boisse (1983) and Boisse et al. (1986) developed a reproducible model exemplified for chlordiazepoxide in rats.

Procedure

Male Sprague Dawley rats weighing 350–575 g are used. Chordiazepoxide hydrochloride is administered as solution 75 mg/ml by gavage after an initial loading dose of 450 mg/kg given at 7:00 a.m. and 5:00 p.m. Impairment of motor function is evaluated by a neurological screen including five different ladder and open field tests.

Neurological test	Maximum depression points
Ladder, head down	2
Ladder, head up	4
Ladder, grasp reflex	1
Motor activity	3
Walking	5
Maximum	15

The animals are rated by three independent observers. Once dependence is revealed, lower doses are used which are then increased in appropriate steps to induce an average depression rating of about five. Treatment is continued for 5 weeks during which time the dose has to be increased. The degree of increase (about fivefold in the case of chlordiazepoxide) reflects the **tolerance** of the test compound.

To test **dependence**, rats are challenged with the benzodiazepine receptor antagonist Ro 15–1788 (flumazenil; ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo -4H -imidazo (1,5a) (1,4)benzodiazepine-3-carboxylate) at a dose of 25 mg/kg i.p. Withdrawal reactions are recorded just before and 5, 15, 30, and 60 min after administration of the antagonist. Motor, autonomic, and behavioral signs are monitored by operational defined criteria, such as position of the claws, salivation, and diarrhea. The total withdrawal expression for each observation and each animal is estimated by summing the grades of all signs as recorded by three independent observers.

Evaluation

For each animal and observation time, the scores are estimated by the average of all co-observers. From these estimates group means are computed and compared by *t*-test between control and test and by paired *t*-test for self-control comparisons before and after antagonist administration.

Modification of the Method

Further studies in *rats* with benzodiazepines were performed by Vellucci and File (1979), Treit (1985), and Nath et al. (1997).

File (1985) found very different rates at which tolerance develops to the sedative, anticonvulsive, and anxiolytic actions of benzodiazepines.

Bonnafous et al. (1995) studied the increase of gastric emptying induced by benzodiazepine withdrawal in rats. Gastric emptying was measured with a test meal containing ^{51}Cr sodium chromate administered in rats, either previously receiving 15 mg/kg diazepam or DMSO i.p. for 7 days.

Benzodiazepine-like dependence potential of a putative 5-HT$_{1A}$ agonist anxiolytic was assessed in rats by Goudie et al. (1994).

Studies in *mice* on tolerance and physical dependence with benzodiazepines were performed by Patel et al. (1988), Gallaher et al. (1986), Stephens and Schneider (1985), Nutt and Costello (1988), and Piot et al. (1990).

These phenomena were studied in *dogs* by McNicolas et al. (1988) and Löscher et al. (1989).

Studies in monkeys were performed by Lukas and Griffiths (1982), Yanagita (1983), Lamb and Griffiths (1984), and France and Gerak (1997).

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Genetically Modified Animals in Psychopharmacology

General Considerations

The use of genetically altered animals in biological research has also affected psychopharmacology. Genetically engineered strains of mice modified by transgenesis or gene targeting ("knockout") have been generated and are used as research tools for deciphering the genetic basis of behavior. These animals are designed to evaluate the efficacy of new pharmacological and gene therapy treatments in human hereditary diseases (Crnic 1996; Mayford et al. 1997; Costentin 1998; Picciotto and Wickmanm 1998; Anagnostopoulos et al. 2001).

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Special Reports on Genetically Altered Animals Useful for Evaluation of Drugs Against Anxiety

Wilson et al. (1996) reported that the **transgenic (mREN2) rat**, basically a model of genetically engineered hypertension, showed an anxiogenic profile in several tests, e.g., open field or elevated X-maze.

Saudou et al. (1994) found enhanced aggressive behavior in **mice lacking the 5-HT_{1B}receptor**. When confronted with an intruder, mutant mice attacked the intruder faster and more violently than did wild-type mice.

Trillat et al. (1998) studied the antidepressant effect of selective serotonin reuptake inhibitors (SSRIs) in homozygote mice deficient in the serotonin **5-HT_{1B}receptor**. The findings suggested that 5-HT_{1B} autoreceptors limit the effect of SSRIs particularly in the hippocampus while postsynaptic 5-HT_{1B} receptors are required for the antidepressant activity of SSRIs.

Parks et al. (1998) described increased anxiety in **mice lacking the serotonin 1A receptor**. Mice with an inactivated gene encoding the 5-HT_{1A} receptor have an increased tendency to avoid a novel and fearful environment and to escape a stressful situation.

Likewise, Heisler et al. (1998) found elevated anxiety and antidepressant-like responses in serotonin **5-HT_{1A} mutant mice**.

Ramboz et al. (1999) described **serotonin receptor 1A knockout mice** as an animal model of anxiety-related disorder. The authors demonstrated that mice without 5-HT_{1A} receptors display decreased exploratory activity and increased fear of aversive environments (open or elevated spaces). 5-HT_{1A} knockout mice also exhibited a decreased immobility in the forced swim test, an effect commonly associated with antidepressant treatment. These results showed that 5-HT_{1A} receptors are involved in the modulation of exploratory and fear-related behaviors and suggested that reductions in 5-HT_{1A} receptor density due to genetic defects or environmental stressors might result in heightened anxiety.

Gross et al. (2000) used genetically altered mice to determine the influence of different neurotransmitter receptors on fear and anxiety. **Mice with a genetic deletion of the 5-HT_{1A} receptor** were more fearful in a number of behavioral conflict tests, confirming the importance of this receptor in modulating anxiety.

Knapp et al. (2000) bred rats selectively for high or low hypothermic responses to the specific 5-HT_{1A} receptor agonist 8-hydroxy-2-di-n-propylamino tetralin (8-OH-DPAT). These rats differed in responses related to anxiety and depression.

Holmes (2001) reviewed targeted gene mutation approaches to the study of anxiety-like behavior in mice.

Schramm et al. (2001) found that the $\alpha 2_A$ adrenergic receptor plays a protective role in mice behavioral models of depression and anxiety. The genetic **knockout of the α_{2A}-adrenergic receptor** makes mice less active in a modified Porsolt's swim test and insensitive to the antidepressant effect of imipramine in this paradigm. Furthermore, α_{2A}-adrenergic receptor knockout mice appear more anxious than wild-type C57 Bl/6 mice in rearing and light/dark models of anxiety.

Quinlan et al. (2000) reported that **mice lacking the long splice variant of the gamma 2 subunit of the $GABA_A$ receptor** are more sensitive to benzodiazepines. Lack of the gamma 2 L subunits may shift the $GABA_A$ receptor from an inverse-agonist-preferring toward an agonist-preferring configuration.

Yamada et al. (2000) described neurobehavioral alterations in mice with a targeted **deletion of the tumor necrosis factor alpha gene** and the implications for emotional behavior.

Miyakawa et al. (2001) studied the behavior of **mice lacking the m_1 muscarinic acetylcholine receptor**. The animals exhibited a pronounced increase in locomotor activity in various tests, including open field, elevated plus maze, and light/dark transition tests. However, hippocampus-dependent learning was intact.

Picciotto et al. (2001) studied physiological and behavioral phenotypes of **neuronal nicotine acetylcholine receptor subunit knockout mice** and discussed possible implications for drug development.

Rupniak et al. (2001) used assays for antidepressant and anxiolytic drugs, such as resident-intruder or the forced swim test, to compare the phenotype of **NK1R−/− mice** with pharmacological blockade of the substance P (NK1) receptor.

Montag-Sallaz and Montag (2003) found severe cognitive and motor coordination deficits in **Tenascin-R-deficient mice**. The animals were tested for grip strength, open field behavior, elevated plus maze, light/dark avoidance, and hole board examination in the Morris water maze and in two-way active avoidance learning using several systems from TSE Systems, Bad Homburg, Germany.

Bilkei-Gorzo et al. (2004) studied behavioral and drug effects in **pre-proenkephalin-deficient mice** in the elevated zero maze (using the VideoMot 2 system), the light–dark test using the animal activity monitor Actimot, and the startle response test (all systems provided by TSE Systems, Bad Homburg, Germany)

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Anti-Epileptic Activity

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General Considerations

Epilepsy is a disease of high prevalence, being well known since thousands of years as "morbus sacer." In spite of intensive investigations, the pathophysiology of epilepsy is still poorly understood. Studies with various animal models have provided ample evidence for heterogeneity in the mechanisms of epileptogenesis. New evidence derives from investigations of kindling, which involves the delivery of brief, initially subliminal, electrical, or chemical stimuli to various areas of the brain. After 10–15 days of once-daily stimulation, the duration and intensity of afterdischarges reach a stable maximum, and a characteristic seizure is produced. Subsequent stimulation then regularly elicits seizures.

Surveys of methods being used to test compounds with anticonvulsant properties have been provided by Toman and Everett (1964), Woodbury (1972), Hout et al. (1973), Swinyard (1973), Koella (1985), Meldrum (1986), Rump and Kowalczyk (1987), Löscher and Schmidt (1988), Fisher (1989), Rogawski and Porter (1990), and Porter and Rogawski (1992).

Epilepsy becomes drug resistant in 20–30 % of patients. Out of the animal models, the amygdala-kindled rat seems to be a suitable approach (Löscher 1997, 1998, 2002a, b). Furthermore, the rat cortical dysplasia model is recommended (Smyth et al. 2002).

Several biochemical hypotheses have been advanced, involving the inhibitory GABAergic system and the system of the excitatory amino acids glutamate and aspartate. Excitatory receptors have been divided into subtypes according to the actions of specific agonists or antagonists. Agents which reduce $GABA_A$ synaptic function provoke convulsions. A convulsive state is induced by the direct blockade of $GABA_A$ receptors (e.g., to the action of bicuculline) or a reduction in the GABA-mediated opening of the chloride ion channel (e.g., by picrotoxin). One major factor in epileptogenesis seems to be a decreased function of $GABA_A$ synapses.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids, in particular, glutamate. Of the three receptors of glutamate, the NMDA (*N*-methyl-D-aspartate) receptor is considered one of the most interested in epilepsy, and competitive NMDA receptor antagonists are proposed as potential antiepileptic drugs. Excessive excitatory amino acid neurotransmission is thought to be associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders. Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA), and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987).

Fabene and Sbarbati (2004) underlined the value of in vivo MRI in different models of experimental epilepsy.

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In Vitro Methods

[^{3}H]-GABA Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

$GABA_A$ Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

$GABA_B$ Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

The in vitro assays for GABAergic compounds described in the chapter "► Tests for Anxiolytic Activity" (anxiolytics) are similarly used for evaluation of antiepileptic compounds.

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[^{3}H]-GABA Uptake in Rat Cerebral Cortex Synaptosomes

Purpose and Rationale

Roberts (1974) and others have proposed that the inhibitory action of the amino acid γ-aminobutyric acid (GABA) is the fine tuning control for pacemaker neurons. Disruption of this interplay due to inadequacies of the GABA

system results in various disorders, in particular, convulsive seizures (Roberts 1974; Korgsgaard-Larsen 1985). The nonspecific action of GABA-mimetics makes inhibition of the uptake mechanism, which terminates the neurotransmitter action, the ideal choice for increasing GABA's concentration at specific sites (Roberts 1974; Tapia 1975; Meldrum et al. 1982; Brehm et al. 1979). Demonstration of the high-affinity mechanism that best reflects the in vivo condition utilizes GABA-depleted cerebral cortex synaptosomes (Ryan and Roskoski 1977; Iversen and Bloom 1972; Roskoski 1978). Although the physiological role of GABA transport systems is still unclear, uptake inhibitors such as THPO [4,5,6,7-tetrahydroisoxazolo-(4,5-C)pyridine-3-ol], nipecotic acid, cis-4-hydroxynipecotic acid, and guvacine exhibit anticonvulsant effects (Meldrum et al. 1982; Brehm et al. 1979). Furthermore, a number of neuroleptics have been shown to inhibit GABA uptake (Fjalland 1978). In particular, fluspirilene was found to be equivalent to the most potent uptake inhibitors known.

The assay is used as a biochemical screen for potential anticonvulsants or GABA (γ-aminobutyric acid) mimetic compounds that act by inhibiting GABA uptake.

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.4.
2. Ringer's solution + 10 mM Tris buffer, pH 7.4 containing.
   - Glucose 10.0 mM,
   - NaCl 150.0 mM
   - KCl 1.0 mM
   - $MgSO_4$ 1.2 mM
   - Na_2HPO_4 1.2 mM
3. Depolarizing Ringer's solution, pH 7.4 reagent 2 containing:
   - KCl 56 mM
   - $CaCl_2$1 mM
4. 0.32 M sucrose.
5. [^{3}H]-GABA is diluted to 2.5×10^{-4} M with distilled water. Forty microliters of this solution in 1 ml of reaction mixture will yield a final concentration of 10^{-5} M.
6. Test compounds.

   A 10 mM stock solution is made up in distilled water, ethanol, or DMSO and serially diluted, such that the final concentration in the assay ranges from 10^{-3} to 10^{-8} M. Total and nonspecific controls should use solvent of test compound.

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Cerebral cortex is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter–Elvehjem homogenizer. The homogenate is centrifuged at 1000 g for 10 min. The supernatant (S_1) is decanted and recentrifuged at 1000 g for 10 min. The pellet (P_2) is resuspended in 9 volumes of 0.32 M sucrose and centrifuged at 24,000 g for 10 min. The washed pellet is resuspended in 15 volumes of depolarizing Ringer's solution, incubated at 25 °C for 10 min and centrifuged at 3000 g for 10 min. The resulting pellet is resuspended in 15 volumes of Ringer's solution and is ready for use.

Assay

60 µl Ringer's solution
100 µl vehicle or appropriate drug concentration
800 µl tissue suspension

Microcentrifuge tubes are set up in triplicate. Nonspecific controls are incubated at 0 °C and total at 25 °C for 10 min. 40 µl of [^{3}H]-GABA are added and the tubes are reincubated for 10 min. All tubes are centrifuged at 13,000 g for 1 min. The supernatant is aspirated and 1 ml of solubilizer (Triton X-100 + 50 % EtOH, 1:4, v/v) is added and mixed to dissolve pellets. Tubes are incubated at 90 °C for 3 min, then centrifuged at 13,000 g for 15 min. 40 µl of supernatant is counted in 10 ml Liquiscint scintillation cocktail.

Evaluation

Active uptake is the difference between cpm at 25 °C and 0 °C. The percent inhibition at each drug concentration is the mean of three

determinations. IC_{50} values are derived from log–probit analysis.

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GABA Uptake and Release in Rat Hippocampal Slices

Purpose and Rationale

The GABA transporter, the subsynaptic $GABA_A$ receptor, and the $GABA_B$ autoreceptor are therapeutically the most relevant targets for drug actions influencing GABAergic synaptic transmission. Uptake inhibitors are potential anticonvulsants.

Procedure

For measurement of GABA uptake, rat hippocampal slices are cut with a McIlwain tissue slicer (100-μm-thick prisms) and dispersed in ice-cold Krebs–Ringer solution with HEPES buffer (pH 7.4). Following two washes, slices (15 mg) are incubated at 37 °C for 15 min in the presence or absence of test compound. [^{3}H]-GABA is added, and samples are incubated for an additional 5 min before filtration through Whatman GF/F filters. Samples are then washed twice with 5 ml ice-chilled 0.9 % saline. Distilled water is added, and samples are allowed to sit at least 60 min before measured for radioactivity by liquid scintillation spectroscopy. Blanks are treated in an identical manner but are left on ice throughout the incubation.

For measurement of GABA release, rat hippocampal slices are prepared and dispersed in ice-cold HEPES-buffered (pH 7.2) Krebs–Ringer solution and incubated with 0.05 μM [^{3}H]-GABA for 15 min at 37 °C. Following two washes, the slices are incubated for an additional 15 min and finally resuspended in medium. Tissue (10 mg) is incubated at 37 °C for a 15 min release period in the presence or absence of test compound. At the end of the release period, the medium is separated from tissue by centrifugation at 500 g for approximately 1 min and poured into 0.5 ml of perchloric acid (0.4 N). The tissue is homogenized in 0.13 N perchloric acid. Radioactivity in the samples is measured by using liquid scintillation spectroscopy.

Evaluation

For GABA uptake, IC_{50} values (μM) are determined.

In GABA release experiments, results are expressed as the amount of radioactivity released as a percent of the total radioactivity.

Modifications of the Method

Roskoski (1978) studied the net uptake of GABA by high-affinity synaptosomal transport systems.

Nilsson et al. (1990, 1992) tested GABA uptake in astroglial primary cultures.

The **isolated nerve-bouton preparation** was used to study GABA release (Jang et al. 2001; Kishimoto et al. 2001; Akaike et al. 2002; Akaike and Moorhouse 2003). The technique was developed by Drewe et al. (1988), Vorobjev (1991), Haage et al. (1998), Rhee et al. (1999), and Koyama et al. (1999).

The method is based on the local application of mechanical vibration directly to the chosen site of a brain slice and does not require the enzymatic pretreatment of the tissue. The mechanical vibration is applied via a glass rod (0.5 mm in diameter) mounted on a piezoelectric bimorph crystal at the site of the chosen brain tissue. The dissociated cells are allowed to settle at the bottom of a Petri dish for 20 min. The cell bodies are usually 10–15 pm at their longest axis, rounded or elongated in shape. Some cells had remaining neurites up to 100 pm long. The majority of cells had neurites less than 15 μm long.

In other studies (Koyama et al. 1999; Kishimoto et al. 2001), a custom-built vibrating stylus was placed in the appropriate region for mechanical dissociation. The glass capillary (1.5 mm o.d.) was pulled to a fine tip and fire polished. The tip was placed within the appropriate region by a manipulator. The vibrating stylus was driven by an electronic relay, and the tip was horizontally moved (excursions of 2–3 mm at 0.5–2 Hz) for 2 min.

Neurons with adherent functional synaptic terminals were investigated by tight-seal whole-cell recordings from the postsynaptic cells.

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Glutamate Receptors: [^{3}H]CPP Binding

Purpose and Rationale

The ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. The family comprises three pharmacologically defined classes that were originally named after reasonably selective ligands: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (Cotman and Iversen 1987; Watkins and Olverman 1987; Collingridge and Lester 1989; Monaghan et al. 1989; Carlsson and Carlsson 1990; Young and Fagg 1990; Nakanishi 1992; Cunningham et al. 1994; Herrling 1994; Iversen and Kemp 1994; Mayer et al. 1994; Meldrum and Chapman 1994; Monaghan and Buller 1994; Watkins 1994; Bettler and Mulle 1995; Fletcher and Lodge 1995; Becker et al. 1998; Danysz and Parsons 1998; Meldrum 1998; Chittajallu et al. 1999; Dingledine et al. 1999; Hatt 1999; Gallo and Ghiani 2000; Lees 2000; Meldrum 2000). It turned out that NMDA, AMPA, and kainate receptor subunits are encoded by at least six gene families as defined by sequence homology: a single family of AMPA receptors, two for kainate, and three for NMDA (Dingledine et al. 1999; Mayer and Armstrong 2004).

The NMDA subtype is a hetero-oligomer consisting of an NR1 subunit combined with one or more NR2 subunits and a third subunit, NR3 (Loftis and Janowsky 2003). The receptor has two amino acid recognition sites, one for glutamate and one for glycine, both of which must be occupied to promote channel opening. A variety of drugs have been identified which block the channel selectively (Bräuner-Osboren et al. 2000; Kemp and McKernan 2002).

The AMPA subtype is a hetero-oligomer formed from combinations of iGluR1–4. Selective agonists and competitive antagonists acting at the glutamate recognition site have been useful for defining the physiological and pathophysiological roles played by the receptor. AMPA receptor modulators have been discussed as cognitive enhancers (Lynch 2004).

The kainate subtype consists of hetero-oligomers, comprising five subunits (Hollmann and Heinemann 1994; Huettner 2003).

Excessive excitatory amino acid neurotransmission has been associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and

Olverman 1987; Parsons et al. 1998). Antagonism of NMDA receptor function appears to be the mechanism of action of some anticonvulsant and neuroprotective agents (Löscher 1998; Tauboll and Gjerstad 1998). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized (Thomsen 1997). NMDA receptors are believed to be coupled to a cation channel which converts to an open state with NMDA receptor activation (Kemp et al. 1987; Mukhin et al. 1997). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+}, and Zn^{2+}. Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and novel anticonvulsants, such as MK-801, block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as CPP and the phosphono analogues of L-glutamate, AP7, and AP5 (2-amino-5-phosphonopentanoic acid), are inhibitors at the excitatory amino acid binding site (Olverman et al. 1986; Davies et al. 1986; Harris et al. 1986; Murphy et al. 1987; Lehmann et al. 1987).

The following assay is used to assess the affinity of compounds for the excitatory amino acid binding site of the NMDA receptor complex. [^{3}H]CPP 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid is a structurally rigid analogue of the selective NMDA receptor antagonist 2-AP7 (2-amino-7-phosphonoheptanoic acid).

Procedure

Reagents

1. Buffer A: 0.5 M Tris HCl, pH 7.6
   60.0 g Tris HCl
   13.9 g Tris base
   q.s. to 1 l with distilled water
2. Buffer B: 50 mM Tris HCl, pH 7.6
   Dilute buffer A 1:10 with distilled water
3. L-Glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg of L-glutamic acid (Sigma G1251) with 10.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^4 M.
4. [^{3}H]CPP is obtained from New England Nuclear, specific activity 25–30 Ci/mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 10 mM.
5. Test compounds. A stock solution of mM is made with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.
6. Triton X-100,10 % (v/v) (National Diagnostics, EC-606). A stock solution of Triton X-100, 10 %, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton X-100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of Triton X-100, 10 %, to give a final concentration of 0.05 % (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 60, 15 s) and spin at 7600 g (SS34, 8000 rpm, 4 °C) for 20 min. Save the supernatant, swirl off the upper buffy layer of the pellet and add to the supernatant. Centrifuge the supernatant at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Resuspend the pellet with 15 volumes of cold distilled water and centrifuge. Discard the supernatant and store the pellet at −70 °C.

On the day of the assay, resuspend the pellet in 15 volumes ice-cold 50 mM Tris buffer, pH 7.6. Preincubate the homogenate with Triton X-100 in a final concentration 0.05 % (v/v) for 15 min at 37 °C with agitation. Centrifuge the homogenate at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Wash the pellet an additional three times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 20 times the original wet weight.

Assay

1. Prepare assay tubes in triplicate.
   380 µl distilled water
   50 µl buffer A, 0.5 M Tris HCI, pH 7.6
   20 µl L-glutamic acid, 10^{-4} M, or distilled water, or appropriate concentration of inhibitor
   50 µl [^{3}H]CPP
   500 µl tissue homogenate
2. Following the addition of the tissue, the tubes are incubated for 20 min at 25 °C with agitation. Place the tubes in an ice bath at the end of the incubation. Terminate the binding by centrifugation (HS4, 7000 rpm, 4 °C) for 15 min. Return the tubes to ice. Aspirate and then discard the supernatant. Carefully rinse the pellet three times with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml, and add an additional 4 ml scintillation fluid.

Evaluation

Specific binding is determined from the difference of binding in the absence of presence of 10^{-4} M L-glutamic acid and is typically 60–70 % of total binding. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Modifications of the Assay

Glutamate (Non Selective)

The assay measures the binding of glutamate, which binds non selectively to ionotropic glutamate receptors including the NMDA, AMPA, and kainate subtypes (Foster and Fagg 1987). In addition, glutamate binds to a family of metabotropic glutamate receptors.

Whole brains (except cerebellum) are obtained from male Wistar rats. A membrane fraction is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1.6 nM [^{3}H]L-glutamate for 10 min at 37 °C. Non-specific binding is estimated in the presence of 50 µM L-glutamate. Membranes are filtered and washed three times to separate bound from free ligand, and filters are counted to determine [^{3}H]L-glutamate bound.

Convulsions induced in mice by intravenous injections of 2.0 mmol/kg L-glutamic acid can be inhibited by glutamate antagonists (Piotrovsky et al. 1991).

Glutamate AMPA

The assay measures the binding of [^{3}H]AMPA (*a*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), a selective agonist which binds to the AMPA receptor subtype of glutamate-gated ion channels (Honore et al. 1982; Olsen et al. 1987; Fletcher and Lodge 1995).

Membranes are prepared from male rat brain cortices by standard techniques. Fifteen mg of membrane preparation is incubated with 5 nM [^{3}H]AMPA for 90 min at 4 °C. Nonspecific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed three times and the filters are counted to determine [^{3}H]AMPA bound.

Mutel et al. (1998) recommended [^{3}H]Ro 48–8587 as specific for the AMPA receptor.

Fleck et al. (1996) described AMPA receptor heterogeneity in rat hippocampal neurons. AMPA receptor antagonists were described by Kohara et al. (1998), Wahl et al. (1998), Kodama et al. (1999), and Nielsen et al. (1999) and reviewed by Chimirri et al. (1999).

Glutamate Kainate

The assay measures the binding of [^{3}H]kainate, a selective agonist that binds to the kainate subtype of the ionotropic glutamate receptors in rat brain (London and Coyle 1979; Clarke et al. 1997).

Whole brains (except cerebellum) are obtained from male Wistar rats. Fifteen mg of a membrane fraction prepared by standard techniques is incubated with 5.0 nM [^{3}H]kainate for 1 h at 4 °C. Nonspecific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed three times to separate free from bound ligand, and filters are counted to determine [^{3}H]kainate bound.

Toms et al. (1997) and Zhou et al. (1997) recommended [^{3}H]-(2S,4R)-4-methylglutamate as kainate receptor selective ligand.

Irreversible inhibition of high-affinity [^{3}H]kainate binding by a photoactivatable analogue was reported by Willis et al. (1997).

Worms et al. (1981) described the behavioral effects of systemically administered kainic acid.

Hu et al. (1998) described neuronal stress and seizure-induced injury in C57/BL mice after systemic kainate administration.

Glutamate NMDA Agonist Site

The assay measures the binding of CGS 19755, a selective antagonist, to the agonist site of the NMDA receptor (Lehmann et al. 1988; Murphy et al. 1988; Jones et al. 1989).

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NMDA Receptor Complex: [^{3}H]TCP Binding

Purpose and Rationale

The purpose of this assay is to determine the binding affinity of potential noncompetitive NMDA antagonists at the phencyclidine (PCP) binding site which is believed to be within or near the NMDA-regulated ion channel. TCP, 1-[1-(2-thienyl)cyclohexyl]-piperidine, is a thienyl derivative of PCP.

Excessive activity of excitatory amino acid neurotransmitters has been associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987). Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA), and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized. NMDA receptors are believed to be coupled to a cation channel which converts to an open state following activation (Kemp et al. 1987). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+}, Zn^{2+}, and polyamines (Loo et al. 1986; Snell et al. 1987, 1988; Reynolds et al. 1988; Thomson 1989;

Sacaan and Johnson 1989; Thedinga et al. 1989; Williams et al. 1989). Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and the neuroprotective agent MK-801 block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid (CPP), and the phosphono analogues of L-glutamate, 2-amino-7-phosphonoheptanoic acid (2-AP7), and 2-amino-5-phosphonopentanoic acid (2-AP5) are inhibitors at the excitatory amino acid recognition site.

Molecular cloning and functional expression of rat and mouse NMDA receptors (Moriyoshi et al. 1991; Meguro et al. 1992), a family of AMPA-selective glutamate receptors (Keinänen et al. 1990), and the metabotropic glutamate receptors mGluR1–mGluR6 (Schoepp et al. 1990; Masu et al. 1991; Abe et al. 1992; Bashir et al. 1993; Nakajima et al. 1993; Tanabe et al. 1993) have been reported.

Procedure I

Reagents

1. Buffer A: 0.1 M HEPES, pH 7.5
   Weigh 23.83 g HEPES.
   Add approximately 900 ml distilled water.
   Adjust pH to 7.5 with 10 N NaOH.
   q.s. to 1 l with distilled water.
2. Buffer B: 10 mM HEPES, pH 7.5
   Dilute buffer A 1:10 with distilled water and adjust pH to 7.5.
3. L-glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg with 10.0 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
4. Glycine, 5×10^{-4} M
   Dissolve 3.75 mg with 10.0 ml distilled water.
   Dilute 1:10 with distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-5} M.
5. Phencyclidine HCl (PCP) is used for nonspecific binding.
   Dissolve 0.7 mg in 0.5 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
6. [^{3}H]TCP is obtained from New England Nuclear, specific activity 42–60 Ci/mmol. For IC_{50} determinations, a 50 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 2.5 nM.
7. Test compounds. A stock solution of 5 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Cerebral cortex of male Wistar rats, 7–10 weeks of age, is dissected over ice and homogenized in ice-cold 0.32 M sucrose, 30 volumes of original tissue weight, for 60 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g for 20 min (SS34, 12,000 rpm, 4 °C). The pellet is resuspended with cold distilled water, to 50 volumes of original tissue weight, using the Tissumizer, 60 s at setting of 70. The homogenate is incubated at 37 °C for 30 min, transferred to centrifuge tubes, and centrifuged at 36,000 g for 20 min (SS34, 16,500 rpm, 4 °C). The pellet is again resuspended in 50 volumes distilled water, incubated and centrifuged. All resuspensions with the Tissumizer are for 60 s at a setting of 70. The resulting pellet is resuspended in 30 volumes of ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and washed once again (resuspension and centrifugation) with buffer. Following resuspension in 30 volumes of buffer, the homogenate is frozen in the centrifuge tube and stored at −70 °C until the day of the assay.

On the day of the assay, the homogenate is thawed and centrifuged at 36,000 g for 20 min (SS34, 16,500 rpm, 4 °C). The pellet is washed three times by resuspension with ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and finally resuspended in 30 volumes of buffer. Aliquots of 500 µl are used for each assay tube, final volume 1000 µl, and correspond to approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate. For each test compound, inhibition of [^{3}H]TCP binding is measured both in the absence (basal) and presence (stimulated) of 100 μM L-glutamic acid and 10 mM glycine.

Basal	Stimulated	
380 μl	340 μl	Distilled water
50 μl	50 μl	Buffer A, 0.1 M HEPES, pH 7.5
20 μl	20 μl	PCP (reagent A5) or distilled water, or appropriate concentration of inhibitor
0 μl	20 μl	L-glutamic acid (reagent A3)
0 μl	20 μl	Glycine (reagent A4)
50 μ	50 μl	[^{3}H]TCP (reagent A6)
500 μl	500 μ	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by separating the bound from nonbound radioligand by rapid filtration with reduced pressure over Whatman GF/B filters, presoaked in 0.05 % polyethyleneimine, using the Brandel cell harvesters. The filters are rinsed once with buffer before filtering the tubes and rinsed two times after filtration. The filters are counted with 10 ml Liquiscint.

Evaluation

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M PCP. Specific binding is typically 50 % of total binding in basal conditions and 90 % of total binding when stimulated by L-glutamic acid and glycine. L-glutamic acid and glycine typically increase specific binding to 300 % and 200 % of basal binding, respectively. The combination of L-glutamic acid and glycine typically produce a greater than additive effect, increasing specific binding to 700 % of basal binding. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Protocol Modification for Crude Membrane Homogenates

This modified procedure for the preparation of membrane homogenates does not use extensive lysing and washing of the tissue to remove endogenous L-glutamate, glycine, and other endogenous compounds which enhance [^{3}H]TCP binding. This procedure may be used for rapid screening of compounds for inhibition of [^{3}H]TCP binding site without specifically defining an interaction at the ion channel or modulatory sites of the NMDA receptor complex.

Procedure II

Reagents

1. Buffers A and B are prepared as described above.
2. Phencyclidine HCl is used for nonspecific binding and is prepared as described above.
3. [^{3}H]TCP is prepared as described above.
4. Test compounds are prepared as described above.

Tissue Preparation

Cortical tissue is dissected and homogenized in 30 volumes of 0.32 M sucrose, and a crude P_2 pellet is prepared as described above. The pellet is resuspended in 30 volumes of 10 mM HEPES, pH 7.5, centrifuged at 36,000 g (SS34, 16,500 rpm, 4 °C) for 20 min, and again resuspended in 100 volumes of buffer. This homogenate is used directly in the assay in aliquots of 500 μl.

Assay

1. Prepare assay tubes in triplicate.

Volume	Solution
380 μl	Distilled water
50 μl	Buffer A, 0.1 M HEPES, pH 7.5
20 μl	PCP (reagent IA5) or distilled water, appropriate concentration of inhibitor
50 μl	[^{3}H]TCP (reagent IA6)
500 μl	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by rapid filtration as described above. The filters are rinsed and counted for bound radioactivity as above.

Evaluation

Specific binding is determined from the difference of binding in the presence or absence of 10^{-4} M PCP. Specific binding is typically 90 % of total

binding. IC_{50} values for the competing drug are calculated by log–probit analysis.

Modifications of the Method

Instead of [^{3}H]TCP, radiolabeled [^{3}H]MK-801 has been used as ligand (Wong et al. 1988; Javitt and Zukin 1989; Williams et al. 1989).

Sills et al. (1991) described [^{3}H]CGP 39653 as a *N*-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat brain.

Nowak et al. (1995) reported that swim stress increases the potency of glycine to displace 5,7-[^{3}H]dichlorokynurenic acid from the strychnine-insensitive glycine recognition site of the *N*-methyl-D-aspartate receptor complex.

NMDA receptor cloning studies have shown that NMDA receptors contain at least one of seven different NMDAR1 subunits (NR1A–NR1G) (Sugihara et al. 1992) and at least one of four NMDAR2 subunits (NR2A–NR2D) (Kutsuwada et al. 1992; Ishii et al. 1993). While the NR1 subunits are generated by alternative splicing of a single gene, the NR2 subunits are products of four highly homologous genes. Thus, there are thousands of potential subunit combinations yielding complexes of four or five subunits.

Grimwood et al. (1996) reported generation and expression of stable cell lines expressing recombinant human NMDA receptor subtypes, two cell lines expressing NR1A/NR2A receptors, and one cell line expressing NR1A/NR2B receptors.

NR2B selective NMDA antagonists were described by Fischer et al. (1997), Kew et al. (1998), Reyes et al. (1998), and Chenard and Menniti (1999).

For discovery of novel NMDA receptor antagonists, Bednar et al. (2004) developed a high-throughput functional assay based on fluorescence detection of intracellular calcium concentrations. Mouse fibroblasts L(tk-) cells expressing human NR1A/NR2B NMDA receptors were plated in 96-well plates and loaded with fluorescence calcium indicator fluo-3 AM. NR2B antagonists were added after stimulation of NMDA receptors with 10 μM glutamate and 10 μM glycine. Changes in fluorescence after addition of the antagonists were fitted with a single exponential equation providing kobs.

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Metabotropic Glutamate Receptors

Purpose and Rationale

In addition to ionotropic (AMPA, kainate, and NMDA) receptors, glutamate interacts with a second family of receptors, metabotropic or mGlu receptors (Tanabe et al. 1992, 1993; Schoepp

and Conn 1993; Hollmann and Heinemann 1994; Nakanishi and Masu 1994; Okamoto et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995, 1996; Pin and Duvoisin 1995; Conn and Pin 1997; Alexander et al. 2001; Skerry and Genever 2001; DeBlasi et al. 2001; Pin and Acher 2002; Conn 2003). Three groups of native receptors are distinguishable on the basis of similarities of agonist pharmacology, primary sequence, and G protein-effector coupling: Group I ($mGlu_1$ and $mGlu_5$ and splice variants) are coupled via $G_{q/11}$ to phosphoinositide hydrolysis. Group II ($mGlu_2$ and $mGlu_3$) are negatively coupled via Gi/Go to adenylyl cyclase and inhibit the formation of cAMP following exposure of cells to forskolin or activation of an intrinsic G protein-coupled receptor (e.g., adenosine A_2 receptor). Group III receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) also inhibit forskolin-stimulated adenylyl cyclase.

Various agonists and antagonists for metabotropic glutamate receptors were described (Ishida et al. 1990, 1994; Porter et al. 1992; Jane et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995; Annoura et al. 1996; Bedingfield et al. 1996; Thomsen et al. 1996; Acher et al. 1997; Doherty et al. 1997; Brauner-Osborne et al. 1998; Kingston et al. 1998; Monn et al. 1999; Jane and Doherty 2000). Schoepp et al. (1999) reviewed pharmacological agents acting at subtypes of metabotropic glutamate receptors. Gasparini et al. (2002) described allosteric modulators of group I metabotropic glutamate receptors as novel subtype-selective ligands and their therapeutic perspectives.

Several radioligands for metabotropic glutamate receptors were described:

- For subtype mGluR4a receptor by Eriksen and Thomsen (1995),
- For group II mGlu receptors by Cartmell et al. (1998), by Ornstein et al. (1998), and by Schaffhauser et al. (1998).

Riedel and Reymann (1996) discussed the role of metabotropic glutamate receptors in hippocampal long-term potentiation and long-term depression and their importance for learning and memory. Furthermore, possible roles in the treatment of neurodegenerative disorders (Nicoletti et al. 1996; Bruno et al. 1998) and of Parkinson's disease (Konieczny et al. 1998) were discussed. Anticonvulsive properties (Atwell et al. 1998; Thomsen and Dalby 1998; Gasparini et al. 1999) as well as anxiolytic properties (Helton et al. 1998) of metabotropic glutamate receptor ligands were reported. Christoffersen et al. (1999) found a positive effect on short-term memory and a negative effect on long-term memory of the class I metabotropic glutamate receptor antagonist, AIDA, in rats.

Procedure

Cultured cells are prepared from the cerebral cortex of 17-day-old embryos of Wistar rats. Prior to the experiments, the culture is maintained for 8–12 days with minimum essential medium (MEM) containing 5 % fetal calf serum and 5 % horse serum.

For **cyclic AMP assays**, the cultured cellsx are preincubated with HEPES-buffered Krebs–Ringer solution containing 5.5 mM glucose (HKR) for 1–1.5 h, then exposed to various agonists for 15 min in the absence or presence of 10 μM forskolin. The content of cyclic AMP is measured using a radioimmunoassay kit after homogenization with 0.1 M HCl.

For **phosphoinositide turnover assays**, the cultured cells are prelabeled with myo-1,2-[^{3}H] inositol in MEM for 8–10 h. The cells are washed twice with HKR containing 10 mM LiCl and then exposed to various agonists in HKR containing 10 mM LiCl for 30 min. The reaction is terminated with 2 % trichloroacetic acid, and the homogenized samples are analyzed for inositol constituents by anion exchange chromatography (Berridge et al. 1982). The extracts are applied to columns containing 1 ml of Dowex 1 in the formate form. The phosphate esters are then eluted by the stepwise addition of solutions containing increasing concentrations of formate. Glycerophosphoinositol and inositol 1:2-cyclic phosphate are eluted with 5 mM sodium tetraborate plus 150 mM sodium formate. The penultimate solution contains 0.1 M formic acid plus 0.3 M ammonium formate, followed by 0.1 M formic acid plus 0.75 M ammonium

formate, each of which removes more polar inositol phosphates. The 1 ml fractions eluted from the columns are counted for radioactivity after addition of 10 ml of Biofluor.

The percentage of radioactivity of inositol phosphates to the total applied to the column is calculated.

Evaluation

Dose–response curves for inhibition of forskolin-stimulated cAMP formation and for percentage of phosphoinositide hydrolysis are established for each test compound.

Modifications of the Method

Thomsen et al. (1993, 1994) used baby hamster kidney (BHK) cells stably expressing $mGluR_{1\alpha}$, $mGluR_2$, or $mGluR_4$ for measurements of phosphoinositol hydrolysis or cAMP formation.

Varney and Suto (2000) recommended functional high throughput screening assay for the discovery of subtype-selective metabotropic glutamate receptor ligands.

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Excitatory Amino Acid Transporters

Purpose and Rationale

Glutamate is not only the predominant excitatory neurotransmitter in the brain but also a potent neurotoxin. Following release of glutamate from presynaptic vesicles into the synapse and activation of a variety of ionotropic and metabotropic glutamate receptors, glutamate is removed from the synapse. This is achieved through active uptake of glutamate by transporters located presynaptically but also postsynaptically, or glutamate can diffuse out of the synapse and be taken up by transporters located on the cell surface of glial cells. The excitatory amino acid transporters form a gene family out of which at least five subtypes were identified (Robinson et al. 1993; Seal and Amara 1999). A role for glutamate transporters has been postulated for acute conditions

such as stroke, CNS ischemia, and seizure, as well as in chronic neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis. Glutamate transport is coupled to sodium, potassium, and pH gradients across the cell membrane creating an electrogenic process. This allows transport to be measured using electrophysiological techniques (Vandenberg et al. 1997).

Procedure

Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, are subcloned into pOTV for expression in *X. laevis oocytes* (Arriza et al. 1994; Vandenberg et al. 1995). The plasmids are linearized with BamHI, and cRNA is transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5',7-methyl guanosine using the mMESSAGE mMACHINE (Ambion, Austin, TX). cRNA (50 ng) encoding either EAAT1 or EAAT2 is injected into defolliculated Stage 5 *X. laevis oocytes*. Two to 7 days later, transport is measured by two-electrode voltage-clamp recording using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab 2e recorder (ADInstruments, Sydney, Australia) and controlled using a pCLAMP 6.01 interfaced to a Digidata 1200 (Axon Instruments). Oocytes are voltage-clamped at – 60 mV and continuously superfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). For transport measurement, this buffer is changed to one containing the indicated concentration of substrate and/or blocker. The voltage dependence of block of glutamate transport is measured by clamping the membrane potential at −30 mV and then applying a series of 100 ms voltage pulses from −100 to 0 mV and measuring the steady-state current at each membrane potential. This protocol is applied both before and during the application of the compound in question and then the baseline current at each membrane potential is subtracted from the current in the presence of the compounds to get a measure for the transport-specific current at the various membrane potentials.

Evaluation

Current (I) as a function of substrate concentration ([S]) is fitted by least squares to

$$I = I_{\max}[\mathrm{S}]/(K_{\mathrm{m}} + [\mathrm{S}])$$

where $I_{\max}$ is the maximal current and K_{m} is the Michaelis transport constant. The $I_{\max}$ values for the various substrates are expressed relative to the current generated by a maximal dose of L-glutamate in the same cell. $I_{\max}$ and K_{m} values are expressed as mean ± standard error and are determined by fitting data from individual oocytes. The potent competitive blockers are characterized by Schild analysis (Arunlakshana and Schild 1959) and the K_{b} estimated from the regression plot. The less potent blockers are assumed to be competitive, and K_{i} values calculated from IC_{50} values using the equation

$$K_{\mathrm{i}} = IC_{50}/(1 + [\mathrm{glutamate}]/K\mathrm{m})$$

where K_{i} is the inhibition constant, IC_{50} is the concentration giving half maximum inhibition, K_{m} is the transport constant, and [glutamate] is 30 μM. The fraction of the membrane electric field sensed by transport blockers when bound to the transporters is estimated using the Woodhull equation (Woodhull 1973),

$$K_{\mathrm{i}} = K_{\mathrm{i}}^{0}\exp(-\zeta\delta FE/RT)$$

where K_{i} is the inhibition constant, K_{i}^{0} is the inhibition constant at 0 mV, ζ is the charge on the blocker, δ is the fraction of the membrane field, F is Faraday's constant, E is the membrane potential, R is the gas constant, and T is temperature in K.

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[^{35}S]TBPS Binding in Rat Cortical Homogenates and Sections

Purpose and Rationale

To screen potential anticonvulsant agents which interact at the convulsant binding site of the benzodiazepine/GABA/chloride ionophore complex by measuring the inhibition of binding of [^{35}S]TBPS to rat cortical membranes.

TBPS, t-butylbicyclophosphorothionate, is a potent convulsant which blocks GABAergic neurotransmission by interacting with the convulsant (or picrotoxin) site of the GABA/benzodiazepine/chloride ionophore receptor complex (Casida et al. 1985; Gee et al. 1986; Olsen et al. 1986; Squires et al. 1983; Supavilai and Karabath 1984). Picrotoxin, pentylenetetrazol, and the so-called cage convulsants are believed to change the state of the chloride channel to a closed conformation and thereby block GABA-induced increases in chloride permeability. Anticonvulsants, such as the barbiturates and the pyrazolopyridines, cartazolate, etazolate, and tracazolate, appear to interact at depressant sites allosterically coupled to the convulsant sites and facilitate the effects of GABA on chloride permeability, by converting the ionophore to the open conformation. Benzodiazepines interact at a separate recognition site to modulate the actions of GABA. Convulsant compounds and some anticonvulsants can inhibit [^{35}S]TBPS binding. These two classes can be differentiated by their effects on dissociation kinetics (Macksay and Ticku 1985; Trifiletti et al. 1984, 1985). [^{35}S]TBPS dissociates slowly, half-life approximately 70 min, in a monophasic manner in the presence of convulsant compounds; anticonvulsants produce a biphasic dissociation, with rapid and slow-phase components. It has been postulated that the rapid and slow phases of [^{35}S]TBPS dissociation may correspond to the open and closed conformation of the chloride ionophore.

Procedure

Reagents

1. Buffer A: 0.05 M Tris with 2 M KCl, pH 7.4
   6.61 g Tris HCl
   0.97 g Tris base
   149.1 g KCl
   q.s. to 1 l with distilled water
2. Buffer B
   A 1:10 dilution of buffer A in distilled water (5 mM Tris, 200 mM KCl, pH 7.4)
3. [^{35}S]TBPS is obtained from New England Nuclear with a high initial specific activity, 90–110 Ci/mmol. For an inhibition assay with a 2 nM final concentration of TBPS, a specific activity of 20–25 Ci/mmol will provide sufficient counts due to a high counting efficiency (87 %) for ^{35}S. The specific activity of [^{35}S]TBPS can be reduced with the addition of 3–5 volumes (accurate measurement with a Hamilton syringe) of an equimolar ethanolic solution of non-radiolabeled TBPS (7.9×10^{-6} M). The new specific activity (Ci/mmol)

is calculated by dividing the number of curies by the number of mmols TBPS. Since [^{35}S] TBPS has a relatively short half-life, 87.1 days, the specific activity is calculated for each assay, based on the exponential rate of decay:

A_0 = initial specific activity

A = specific activity at time t

t = days from date of initial calibration of specific activity

$t_{1/2}$ = half-life of [^{35S}] in days (87.1)

For IC_{50} determinations, a 40 nM stock solution is made with distilled water and 25 µl is added to each tube to yield a final concentration of 2 nM in the assay.

4. Unlabeled TBPS is available from New England Nuclear. A stock dilution of 7.923×10^{-6} M is prepared in ethanol.
5. Picrotoxin is obtained from Aldrich Chemical Company. A solution of 5×10^{-4} M is prepared with distilled water, with sonication if necessary. Aliquots of 10 µl are added to assay tubes to give a final concentration of 10^{-5} M.
6. Test compounds. A stock solution of 1 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

The whole cerebral cortex of male Wistar rats is dissected over ice and homogenized with a Tekmar Tissumizer, 20 s at setting 40, in 20 volumes of 0.32 M sucrose, ice-cold. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm, 4 °C). The resulting pellet is resuspended in 20 volumes of ice-cold distilled water with two 6-s bursts of the Tissumizer, setting 40. The homogenate is centrifuged at 40,000 g for 30 min. The pellet is washed (resuspended and centrifuged) once with 20 volumes ice-cold buffer (Tris HCl 5 mM, KCl 200 mM, pH 7.4). The resulting pellet is resuspended with 20 volumes buffer and frozen at −70 °C overnight. The following day, the tissue homogenate is thawed in a beaker of warm water, approximately 15 min, and then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm, 4 °C). The pellet is washed twice with 20 volumes of ice-cold buffer, and then resuspended and frozen at −70 °C for future use. On the day of the assay, the homogenate is thawed and centrifuged at 40,000 g for 30 min. The resulting pellet is washed once with 20 volumes ice-cold buffer and finally resuspended in 30 volumes buffer. Aliquots of 250 µl are used for each assay tube, final volume 500 µl, and correspond to 8.35 mg original wet weight tissue per tube, approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate:
   190 µl distilled water
   25 µl Tris 0.05 M, KCl 2 M, pH 7.4
   10 µl picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor 25 µl [^{35S}] TBPS, final concentration 2 nM
   250 µl tissue preparation, 1:30 homogenate
2. Following the addition of the tissue, the tubes are incubated at 25 °C for 150 min with agitation. The assay is terminated by rapid filtration over Whatman GF/B filter circles, presoaked in buffer, with 5 × 4 ml rinses of ice-cold buffer. Vacuum filtration is performed with the 45-well filtration units to avoid contamination of the Brandel harvesters with [^{35}S]. The filters are counted with 10 ml Liquiscint.

Evaluation

Specific binding is determined from the difference between binding in the absence or presence of 10 mM picrotoxin and is typically 85–90 % of total binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Modifications for Dissociation Experiments

1. Prepare assay tubes as follows:
   185 µl distilled water
   25 µl Tris 50 mM, KCl 2 M, pH 7.4
   10 µl test compound or vehicle
2. Add 250 ml tissue homogenate to tube s. Vortex. Preincubate 30 min at 25 °C.

3. Add 25 ml [^{35}S]TBPS. Vortex. Incubate 180 min at 25 °C.
4. Add 5 ml picrotoxin (10^{-3} M) to give a final concentration of 10^{-5} M. Vortex.
5. At various times after the addition of picrotoxin (0–120 min), tubes are filtered and rinsed as described above.

Modification for [^{35}S]TBPS Autoradiography

1. Sections of rat brain, 20 mm thickness, are collected onto gel-chrome alum-subbed slides, freeze-dried for approximately 1 h, and stored at −70 °C until used.
2. After thawing and drying at room temperature, the sections are preincubated for 30 min in buffer B.
3. Preparation of slide mailers for incubation:
   (a) For scintillation counting:
   2.47 ml distilled water
   0.325 ml buffer A
   3.25 ml buffer B 0.13 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
   0.325 ml [^{35}S]TBPS, final concentration 2 nM
   6.50 ml final volume
   (b) For autoradiography:
   4.56 ml distilled water
   0.60 ml buffer A
   6.00 ml buffer B
   0.24 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
   0.60 ml [^{35}S]TBPS, final concentration 2 nM
   12.0 ml final volume
4. Sections are incubated in slide mailers at room temperature with [^{35}S]TBPS in the absence or presence of appropriate inhibitors for 90 min.
5. Slides are transferred to vertical slide holders and rinsed in ice-cold solutions as follows: dip in buffer B, two 5 min rinses in buffer A and a dip in distilled water.
6. Slides are dried under a stream of cool air and desiccated overnight at room temperature.
7. Slides are mounted onto boards with appropriate [^{35}S] brain mash standards.
8. In the dark room under safelight illumination (GBX filter), slides are opposed to Kodak X-OMAT AR film and stored in cassettes for 7–10 days.
9. Develop films as described in "X-OMAT AR Film Processing."

	[^{35}S]TBPS binding parameters	
	Slide-mounted sections	Cortical homogenates
Assay conditions		
Tissue	20 p sections, rat freeze-dried, 1 h	Whole cortex, rat 1:30 homogenate prepared with five washes and two freeze-thaw cycles
	30 min	No preincubation
	Preincubation	
Buffer	5 mM Tris, 200 mM KCl, pH 7.4	5 mM Tris, 200 mM KCl, pH 7.4
Incubation time	90 min, 21–22 °C	150 min, 25 °C
Nonspecific	10^{-5} M picrotoxin	10^{-5} M picrotoxin
Tissue linearity	2.5–25 mg tissue per 0.5 ml assay tube	
Equilibrium constants		
KD (nM)	32.8	25.2
Bmax	1615	2020
(fmol/mg prot)		
Binding kinetics		
Association kobs (min^{-1})	0.0496	0.0138
k + 1 (nM^{-min-1})	0.0164	0.0021
Dissociation k–1 (min^{-1})	0.017	0.001
Dissociation constant	1.03	4.73
k + 1/k – 1 (nM) IC_{50}M		
Picrotoxin	2.8×10^{-7}	3.4×10^{-7}
TBPS	8.7×10^{-8}	8.1×10^{-8}
GABA	1.7×10^{-6}	2.1×10^{-6}
Pentobarbital	1.2×10^{-4}	6.0×10^{-4}
Phenobarbital	None at 10^{-3}	None at 10^{-3}
Clonazepam	None at 10^{-6}	None at 10^{-6}

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[^{3}H]glycine Binding in Rat Cerebral Cortex

Purpose and Rationale

The amino acid glycine is a major inhibitory transmitter in the vertebrate system. Glycinergic synapses are particularly abundant in spinal cord and brain stem, but are also found in higher regions, including the hippocampus. The inhibitory actions of glycine are potently blocked by strychnine. Glycine modulates and may activate the excitatory amino acid receptors of the NMDA subtype (Thomson 1989; Laube et al. 2002).

The strychnine-sensitive, postsynaptic glycine receptor is a ligand-gated chloride channel protein that belongs to the nicotinic acetylcholine receptor family. It is a pentameric transmembrane protein composed of α and β subunits (Lynch 2004).

Glycine has been shown in vitro to potentiate the effects of L-glutamate or NMDA on the stimulation of [^{3}H]TCP binding (Snell et al. 1987, 1988; Bonhaus et al. 1989) and [^{3}H]norepinephrine release (Ransom and Deschenes 1988) and in vivo to act as a positive modulator of the glutamate-activated cGMP response in the cerebellum (Danysz et al. 1989; Rao et al. 1990). The activation of NMDA receptors requiring the presence of glycine is necessary for the induction of long-term potentiation (LTP), a type of synaptic plasticity which may be fundamental to learning processes (Oliver et al. 1990). A [^{3}H]glycine binding site in the brain has been identified and characterized as a strychnine-insensitive site associated with the NMDA receptor complex (Kessler et al. 1989; Monahan et al. 1989; Cotman et al. 1987). Autoradiographic studies have shown a similar distribution of [^{3}H]glycine and [^{3}H]TCP (NMDA ion channel radioligand) binding sites (Jansen et al. 1989). Compounds which interact with the glycine site offer a novel mechanism of action for intervention with NMDA receptor function.

Schmieden and Betz (1995) reviewed the pharmacology of the inhibitory glycine receptor, the agonist and antagonist actions of amino acids, and piperidine carboxylic compounds.

Hyperekplexia is a hereditary neurological disorder in humans characterized by an excessive

startle response which can be caused by mutations in the α1 subunit of the heteropentameric inhibitory glycine receptor (Rees et al. 2002). Becker et al. (2002) generated transgenic mice resembling this disease.

The following assay is used to assess the affinity of compounds for the glycine binding site associated with the *N*-methyl-D-aspartate (NMDA) receptor complex using [^{3}H]glycine as the radioligand.

Procedure

Reagents

1. Buffer A: 0.5 M Tris maleate, pH 7.4 59.3 g Tris maleate
   q.s. to 0.5 l
   Adjust pH to 7.4 with 0.5 M Tris base.
2. Buffer B: 50 mM Tris maleate, pH 7.4
   Dilute buffer A 1:10 with distilled water; adjust pH with 50 mM Tris maleate (acid) or 50 mM Tris base.
3. Glycine, 5×10^{-2} M
   Dissolve 3.755 mg of glycine (Sigma G7126) with 1.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-3} M.
4. [^{3}H]Glycine is obtained from New England Nuclear, specific activity 45–50 Ci mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 µl are added to yield a final assay concentration of 10 nM.
5. Test compounds. A stock solution of 5 mM is prepared with a suitable solvent and serially diluted, such that the final concentrations in the assay ranges from 10^{-4} to 10^{-7} M. Higher or lower concentrations may be used, depending on the potency of the compound.
6. Triton X-100,10 % (v/v) (National Diagnostics, EC606). A stock solution of Triton X-100, 10 %, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton X-100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of the 10 % solution to give a final concentration of 0.04 % (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. Three cortices are pooled for one preparation. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 70, 15 s) and spin at 7600 g (SS34, 8000 rpm 4 °C) for 20 min. The pellet is resuspended with 15 volumes of cold distilled water and centrifuged. Discard the supernatant and store the pellet at −70 °C.

On the day of the assay, the pellet is resuspended in 15 volumes ice-cold 50 mM Tris maleate, pH 7.4. Preincubate the homogenate with Triton X-100 in a final concentration of 0.04 % (v/v) for 30 min at 37 °C with agitation. Centrifuge the suspension at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Wash the pellet an additional three times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 25 times the original wet weight.

Assay

1. Prepare assay tubes in quadruplicate.
   380 µl distilled water
   50 µl buffer A, 0.5 M Tris maleate, pH 7.4
   20 µl glycine, 10^{-3} M final concentration, or distilled water or appropriate concentration of inhibitor
   50 µl [^{3}H] glycine, final concentration 10 nM
   500 µl tissue homogenate.
   1000 µl final volume
2. Following the addition of the tissue, the tubes are incubated for 20 min in an ice bath at 0–4 °C. The binding is terminated by centrifugation (HS4, 7000 rpm, 4 °C) for 20 min. Aspirate and discard the supernatant. Carefully rinse the pellet twice with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml, and add an additional 4 ml scintillation fluid.

Evaluation

Specific binding is determined from the difference of binding in the absence or in the presence of 10^{-4} M glycine and is typically 60–70 % of total binding. IC_{50} values for the competing compound are calculated by log–probit analysis of the data.

Modifications of the Method

Baron et al. (1996), Hofner and Wanner (1997), Chazot et al. (1998) described [^{3}H]MDL 105,519 as a high-affinity ligand for the NMDA associated glycine recognition site.

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[^{3}H]Strychnine-Sensitive Glycine Receptor

Purpose and Rationale

The strychnine-sensitive glycine receptor is a member of the family of ligand-gated ion channel receptors. Within this family, the glycine receptor is most closely related to the GABA receptor. Like the $GABA_A$ receptor, the glycine receptor has an inhibitory role, mediating an increase in chloride conductance. However, in contrast to the $GABA_A$ receptor, the glycine receptor is located mainly in the spinal cord and lower brainstem, where glycine appears to be the major inhibitory neurotransmitter. Purification and molecular cloning has shown that the glycine receptor is an oligomeric transmembrane protein complex composed of three α and two β subunits. The inhibitory actions of glycine are potently blocked by strychnine. In addition to strychnine, the steroid derivative RU5135 (Simmonds and Turner 1985), phenylbenzene-α-phosphono-a-amino acid (Saitoh et al. 1996), and 5,7-dichloro-4hydroxyquinoline-3-carboxylic acid (Schmieden et al. 1996) antagonize glycine responses in cultured neurons or cells expressing recombinant glycine receptors.

A glycine receptor agonist may be a potential antispastic agent.

Procedure

Male Wistar rats weighing about 200 g are sacrificed. About 220 mg of frozen pons and medulla are homogenized in 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1, by an Ultra-Turrax homogenizer. The homogenate is centrifuged for 10 min at 30,000 g at 0–4 °C in a refrigerated centrifuge. The pellet is rehomogenized in another 2 × 10 ml portion of

the same buffer and recentrifuged as before. This washing procedure is repeated a total of four times. The final pellet is resuspended in 200 vol/g original tissue in ice-cold 50 mM potassium phosphate buffer, pH 7.1, with or without 1000 mM NaCl, and used directly for binding assays.

Binding assays consist of 1 ml tissue homogenate, 50 µl test solution (water or 5 % v/v ethanol/water is used for serial dilutions), 50 µl water, 5 % ethanol/water or glycine solution (40 mM final concentration), and 25 µl [^{3}H]strychnine working solution, final concentration 2 nM. The samples are mixed well and incubated for 20 min in an ice bath. Free and bound radioactivity are separated by filtration through Whatman GF/C glass fiber filters followed by washing with 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1. Tritium on the filters is monitored by conventional scintillation counting in 3 ml Hydroluma. Nonspecific binding is binding in the presence of 40 mM glycine and is always subtracted from total binding to give specific binding.

Evaluation

K_i values are calculated as

$$K_i = (IC_{50}/1 + [K_D]/[L])$$

whereby IC_{50} are the concentrations that inhibit by 50 % the specific binding of [^{3}H]strychnine determined in two independent experiments using at least three concentrations of the agent in duplicate assays, [L] is the concentration of the radioligand, and K_D is the affinity constant in the absence or the presence of 1000 mM NaCl.

NaCl shift used for differentiating glycine agonists from glycine antagonists is the ratio K_i 1000 mM NaCl versus K_i 0 mM NaCl.

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Electrical Recordings from Hippocampal Slices In Vitro

Purpose and Rationale

The transverse hippocampal slice has been described as a well-defined cortical structure maintained in vitro (Skrede and Westgard 1971). The hippocampus slice has the advantage that each slice may contain all hippocampal structures: The chain of neurons goes from the perforant path to granule cells of the dentate gyrus, through mossy fibers to CA3 pyramidal cells and then through Schaffer collaterals to CA1 cells with their axons leaving the hippocampus through the alveus. The pyramidal cells lie close together and can be easily seen and penetrated with fine microelectrodes.

Procedure

Male guinea pigs weighing 300–400 g are anesthetized with ether, the brains removed, and the hippocampi dissected. Transverse slices of the hippocampus (300–400 pm thick) are cut in parallel to the alvear fibers. After preparation, the slices are submerged in 28 °C warm saline which is equilibrated with 95 % O_2 and 5 % CO_2. After a preincubation period of 2 h, slices are transferred in a Perspex chamber (1.5 × 4 cm) and attached to the bottom consisting of optically plain glass. The chamber is mounted on an inverted microscope allowing detailed inspection of the excised tissue. The slices are superfused by an approximately 3-mm-thick layer of 32 °C warm saline. Intracellular recordings are achieved by means of micropipettes with tip diameters of less than 0.5 pm which are filled with 3 mol/l potassium chloride. Under microscopic control, the tips of the micropipettes are placed within the stratum pyramidale and moved by means of a step motor-driven hydraulic microdrive. For intracellular injections of drugs, e.g., pentylenetetrazol, via the recording microelectrode, a passive bridge is used. Alternatively, drugs are added to the incubation bath.

Evaluation

The resting membrane potential and paroxysmal depolarizations are recorded before and after application of drugs.

Critical Assessment of the Method

The hippocampal slice has been one of the most useful models for the study of basic mechanisms underlying the epilepsies. The model has also been recommended for screening of putative anticonvulsant drugs.

Modifications of the Method

Harrison and Simmonds (1985) performed quantitative studies on some antagonists of *N*-methyl-D-aspartate in slices of rat cerebral cortex consisting of cerebral cortex and corpus callosum.

Tissue culture models of epileptiform activity were described by Crain (1972).

Oh and Dichter (1994) studied the effect of a GABA uptake inhibitor on spontaneous postsynaptic currents in cultured rat hippocampal neurons by the whole-cell patch-clamp method.

Blanton et al. (1989) described whole-cell recordings from neurons in slices of reptilian and mammalian cerebral cortex. Synaptic currents and membrane properties could be studied in voltage and current clamps in cells maintained within their endogenous synaptic currents.

Gähwiler (1988) and Stoppini et al. (1991) described methods for organotypic cultures of nervous tissue. Hippocampal slices from 2 to 23-day old rats were maintained in culture at the interface between air and the culture medium. They were placed on a sterile, transparent, and porous membrane and kept in Petri dishes in an incubator. This yielded thin slices which remained

one to four layers thick and were characterized by a well-preserved organotypic organization. Excitatory and inhibitory synaptic potentials could be analyzed using extra- or intracellular recording techniques. After a few days in culture, long-term potentiation of synaptic responses could reproducibly be induced.

Using this method, Liu et al. (1995) studied dopaminergic regulation of transcription factor expression in organotypic cultures of developing striatum of newborn rats.

Stuart et al. (1993) reported the implementation of infrared differential interference contrast video microscopy to an upright compound microscope and a procedure for making patch pipette recordings from visually identified neuronal somata and dendrites in brain slices.

Bernard and Wheal (1995) described an ex vivo model of chronic epilepsy using slices of rat hippocampus previously lesioned by stereotactic injections of kainic acid. Extracellular population spikes were recorded from the stratum pyramidale of CA1 after stimulation by bipolar twisted wire electrodes placed in the stratum radiatum of CA1 area proximally to stratum pyramidale near the recording electrode.

Using hippocampal slices prepared from brain tissue of patients undergoing neurosurgery for epilepsy, Schlicker et al. (1996) showed that the serotoninergic neurons of the human hippocampus are endowed with presynaptic inhibitory autoreceptors.

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Electrical Recordings from Isolated Nerve Cells

Purpose and Rationale

The use of the cell-attached patch-clamp configuration to record action potential currents has shown to have utility in the testing for drug actions on ion channels in excitable cell membranes (Kay and Wong 1986; McLarnon and Curry 1990; McLarnon 1991).

Procedure

Preparation of Cultured Cells

The cultured cells are obtained from the hippocampus or the hypothalamus of rat brain. The isolation of the hippocampal CA1 neurons is performed according to the procedure of Banker and Cowan (1977). The dissociated hypothalamic neurons are prepared according to Jirikowski et al. (1981). The hippocampal and hypothalamic neurons that are selected for electrophysiological recording are bipolar in shape with the long axis dimension between 10μm and 15 μm. The neurons are studied over a period of 5–10 days after isolation.

Electrophysiology

The cell-attached patch-clamp configuration is used to record spontaneous action potentials in the cultured neurons. The bath solution contains 140 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.3. The composition of the patch pipette solution is the same as the bath solution. The drugs used in the experiments are added to the bath solution.

The patch pipettes (Corning 7052 glass) fabricated using a specific patch pipette puller (PP-83; Narishige, Tokyo) are fire-polished and filled immediately prior to use. The resistance of the pipettes is in the range 4–8 MΩ and the tip diameters are between 1 and 2 pm. An axopatch amplifier (Axon Instruments, Foster City, CA), with low-pass filter set at 5 kHz, is used to record the capacitative currents. After recording, at a sampling frequency of 5 kHz, the data are stored on hard disk or video tape for subsequent analysis. All data are obtained at room temperature (21–24 °C).

Evaluation

The capacitative component of current recorded by the patch pipettes is proportional to the rate of change of membrane potential and can be expressed as $I_C = CdV/dt$, where C is the specific membrane capacitance. Assuming a value of C of 1 μF/cm^2 and a tip diameter of the patch pipette of 2 μm, the membrane area isolated by the patch pipette is about 3×10^{-8} cm^2. Using a value of d V/d t of 100 mV/ms gives an approximate expected magnitude of I_C near 3 pA. When a class III antiarrhythmic drug that blocks a delayed rectifier K^+ channel is added to the bath, the portion of I_C corresponding to the after-hyperpolarization component of the action potential is completely abolished. The Na^+ spike is not altered by the drug. The cell-attached recordings of I_C can also be used to determine effects on the Na^+ spike when tetrodotoxin is included in the bath solution. Thus, the spontaneous action potential can be used for evaluation of drug effects on both K^+ and Na^+ channels in excitable membrane.

Modifications of the Method

Chen et al. (1990) measured current responses mediated by $GABA_A$ receptors in pyramidal cells acutely dissociated from the hippocampus of mature guinea pigs according to the procedure of Kay and Wong (1986) using whole-cell voltage-clamp recordings.

Caulfield and Brown (1992) studied inhibition of calcium current in NG108–15 neuroblastoma cells by cannabinoid receptor agonists using whole-cell voltage-clamp recordings.

Gola et al. (1992, 1993) performed voltage recordings on non-dissociated sympathetic neurons from rabbit coeliac ganglia using the whole-cell configuration of the patch-clamp technique (Neher and Sakmann 1976; Sakmann and Neher 1983).

Stolc (1994) used the voltage-clamp technique in internally dialyzed single neurons isolated from young rat sensory ganglia to study the effects of pyridoindole stobadine on inward sodium and calcium currents and on slow non-inactivating components of potassium outward current.

McGivern et al. (1995) examined the actions of a neuroprotective agent on voltage dependent Na^+ currents in the neuroblastoma cell line, NIE-115, using the whole cell variant of the patch-clamp technique.

Smith (1995) reviewed the use of patch and voltage-clamp procedures to study neurotransmitter transduction mechanisms.

Using whole-cell and perforated-patch recordings, Delmas et al. (1998) examined the part played by endogenous G protein $\beta\gamma$ subunits in neurotransmitter-mediated inhibition of N-type Ca^{2+} channel current in dissociated rat superior cervical sympathetic neurons.

Gonzales et al. (1985) registered membrane potentials with intracellular electrodes in cultured olfactory chemoreceptor cells.

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Isolated Neonatal Rat Spinal Cord

Purpose and Rationale

The spinal cord of the neonatal rat is a useful in vitro preparation, originally proposed by Otsuka and Konishi (1974). In this preparation, ventral root potentials of ten seconds of duration can be recorded after supramaximal electrical stimulation of the lumbar dorsal root. Variously implicated in the generation of these slow ventral root potentials are tachykinins, such as substance P and neurokinin B (Yanagisawa et al. 1982; Akagi et al. 1985; Otsuka and Yanagisawa 1988; Guo et al. 1998) and agonists at the glutamate receptor sites (Evans et al. 1982; Ohno and Warnick 1988, 1990; Shinozaki et al. 1989; Ishida et al. 1990, 1991, 1993; Woodley and Kendig 1991; Bleakman et al. 1992; King et al. 1992; Thompson et al. 1992; Zeman and Lodge 1992; Pook et al. 1993; Jane et al. 1994; Boxall et al. 1996). These long-lasting reflexes are thought to reflect a nociceptive reflex for several reasons: the threshold of activation corresponds to that of C fiber primary afferents (Akagi et al. 1985); they can be depressed by opioids (Yanagisawa et al. 1985; Nussbaumer et al. 1989; Faber et al. 1997) and α_2-adrenoceptor agonists (Kendig et al. 1991); and a similar response can be evoked by peripheral noxious stimulation (Yanagisawa et al. 1995).

Procedure

Preparation of Spinal Cord

Male Wistar rats aged 6–9 days are used. Under ether anesthesia, the spinal column is quickly removed from the animal and placed in a Petri dish, filled with oxygenated physiological

solution. A laminectomy is performed on the dorsal surface of the spinal column at room temperature. The spinal cord of the mid-thoracic to mid-sacral level is then carefully removed from the column and hemisected in the longitudinal plane under a dissecting microscope. After removal of the dura mater, the hemisected cord is completely submerged in the recording chamber (total volume: approximately 0.5 ml), which is perfused with physiological solution (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 15 mM $NaHCO_3$, 11 mM glucose) at a flow rate of 1.5–2.5 ml/min. The perfusion medium is continuously bubbled with a gas mixture of 95 % O_2 and 5 % CO_2, and the temperature is kept at 25 ± 0.5 °C. The cut ends of the corresponding dorsal and ventral roots in an $L_{3–5}$ segment are fixed to a pair of suction electrodes for stimulating and recording. The preparation is stabilized in the recording chamber for at least 90 min to allow recovery from the dissection and the sealing of the roots to suction electrodes.

Recording of Monosynaptic Reflexes

Test stimulations, composed of square wave pulses of 0.05–0.2 ms duration and 5–30 V, are applied to the dorsal root every 10 s. The discharges of the corresponding ventral root are recorded with a suction electrode, amplified and monitored on an oscilloscope and stored on an analogue data recorder or computer disks for later analysis. The mean values for the waveform of the monosynaptic reflex (amplitude, area, and latency) are obtained from 6 to 18 successive responses in each experiment before and during application of drugs.

Recording of Single Motoneuron Activity

Test pulses (0.01–0.1 ms duration and 5–15 V) are applied to the dorsal or ventral root every 2 s. The activity of single motoneurons is recorded extracellularly using glass microelectrodes (electrical resistance approximately 10–30 MΩ) filled with 3 M sodium chloride or 2 M sodium acetate. The microelectrode is inserted into the ventral part of the cord through the hemisected surface while monitoring the field potential. The motoneurons in the ventral horn are identified by the short and consistent latency of antidromic spikes (1.66 ± 0.46 ms, $n = 5$), following the stimulation of the ventral root. The motoneurons also produce transsynaptic spikes with orthodromic stimulation of the dorsal root, of which the latency is 10.26 ± 1.05 ms upon supramaximal stimulation. The spike generation of motoneurons is displayed on an oscilloscope and stored on magnetic tapes. The spontaneous firing of the motoneuron is also monitored on an oscilloscope and recorded through a window discriminator and spike counter. The mean number and latency of spikes and latency of the dorsal root-elicited spikes are obtained from 20 to 40 successive responses in each experiment. Comparisons are made before and 3–5 min after application of drugs.

Evaluation

All data are expressed as the mean ± SEM. Statistical significance of the data is determined by repeated measures analysis of variance (ANOVA) and, when appropriate, Student's *t*-test. A *P* value of less than 0.05 is considered statistically significant.

Modifications of the Method

Smith and Feldman (1987) and Wong et al. (1996) described an in vitro neonatal rat brainstem/spinal cord preparation. The brainstem and cervical spinal cord were isolated from 0 to 4 days old ether-anesthetized Sprague–Dawley rats. The en bloc neuraxis was pinned down with ventral surface upward in a recording chamber and superfused continuously with artificial cerebrospinal fluid. Respiratory activity was recorded with suction electrodes from the C_4 ventral root.

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Cell Culture of Neurons

Purpose and Rationale

Cell culture of neurons, especially of hippocampal neurons, has become a widely used tool in pharmacological studies (Banker and Cowan 1977; Skaper et al. 1990, 1993, 2001; Araujo and Cotman 1993; Brewer 1997, 1999; Brewer et al. 1998; Li et al. 1998; Mitoma et al. 1998; Semkowa et al. 1998, 1999; Chaudieu and Privat 1999; May et al. 1999; Hampson et al. 2000; Novitskaya et al. 2000; Pickard et al. 2000; Vergun et al. 2001).

The basic information on methodology of cell culture of rat hippocampal neurons was given by Banker and Cowan (1977). One modification used by Skaper et al. (1990, 2001) studying the role of mast cells on potentiation by histamine of synaptically mediated excitotoxicity in cultured hippocampal neurons is described below.

Procedure

Preparation of Hippocampus

Timed pregnancies are obtained in female Sprague–Dawley rats by daily checking vaginal washings for sperm, the day on which sperm is found being regarded as day 0. At the appropriate stage of gestation, the pregnant rats are anesthetized and the uterus removed to a sterile dish. The remainder of the cell preparations is performed in a sterile hood.

The brains are removed from the fetuses with a pair of fine scissors, and the cerebral hemispheres separated from the brain stem. When the hemisphere of an 18–19-day-old fetus is viewed in a dissecting microscope, the hippocampus can be clearly seen on its medial surface. The hippocampal fissure, usually marked by a conspicuous group of blood vessels, indicates the approximate junction between the hippocampus and the adjoining subicular and entorhinal cortex. The developing fimbria is seen as a white translucent band along the free margin of the hippocampus. Before separating the hippocampus from the hemisphere, the meninges and adherent chorioid plexus are carefully pulled off with fine forceps. At this stage, the full depth of the hippocampal fissure can be seen. Then with iridectomy scissors, the hippocampus is separated from the adjoining cortex by a cut parallel to the hippocampal fissure and by transverse cuts at its rostral and caudal ends.

Cell Culture

Hippocampi isolated from embryonic rats (gestational age 17.5 days) are incubated with 0.08 % trypsin and dissociated in neurobasal medium containing 10 % heat-inactivated calf serum. Cells are pelleted by centrifugation (200 g, 5 min) and resuspended in neurobasal medium containing B27 (Life Technologies, Inc.) supplements (with antioxidants), 25 μM glutamate, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cell suspension is plated onto poly-D-lysine (10 μg/ml) coated 48-well culture plates at a density of 4.5×10^4 cells per cm^2. Cultures are maintained at 37 °C in a humidified atmosphere

of 5 % CO_2–95 % air. After 5 days, one-half of the medium is replaced with an equal volume of maintenance medium (plating medium but containing B27 supplements without antioxidants and lacking glutamate). Additional medium exchanges (0.5 volume) are performed every 3–4 days thereafter. Cells are used between 14 and 16 days in culture. During this period, neurons develop extensive neuritic networks and form functional synapses.

Mast cells are collected from the peritoneal lavage of male Sprague–Dawley rats and isolated over a bovine serum albumin gradient to >90 % purity, as judged by toluidine blue and safranin staining.

Neurotoxicity Assays

Cultures are washed once with Locke's solution (pH 7.0–7.4) with or without 1 mM $MgCl_2$. Drug treatments are carried out for 15–30 min (25 °C) in a final volume of 0.5 ml. In the case of mast cell neuron co-cultures, transwell inserts (3-μm pore size, 9 mm diameter) are seeded with 5×10^4 mast cells in RPMI-1640 medium and placed in 24-well plates overnight. Inserts with mast cells are then placed into wells with hippocampal cells. Mast cell activation is achieved using an antigenic stimulus (0.3 μg /ml anti-DNP IgE/0.1 μg /ml DNP albumin). The mast cell-containing inserts are removed at the end of the Mg^{2+} – free incubation. After this time, all cell monolayers are washed with complete Locke's solution and returned to their original culture medium for 24 h. Cytotoxicity is evident during 24 h after the insult. Viable neurons have phase-bright somata of round-to-oval shape, with smooth, intact neurites. Neurons are considered nonviable when they exhibit neurite fragmentation and somatic swelling and vacuolation. Cell survival is quantified 24 h after the insult by a colorimetric reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).

Evaluation

Data are analyzed by one-way ANOVA with Student-Newman-Keuls post hoc test for differences between groups.

Modifications of the Method

Brewer (1997) reported the isolation and culture of adult rat hippocampal neurons. Using different proteases and special separation techniques, about 90,000 viable neurons could be isolated from each hypothalamus at any age rat from birth to 24–36 months. Neurons were cultured for more than 3 weeks.

Flavin and Ho (1999) found that propentofylline protects hippocampal neurons in culture from death triggered by macrophage or microglia secretory products.

To study neurite outgrowth in cultured hippocampal cells from Wistar rat embryos, 5000-well cells were seeded in 8-well LabTec tissue culture slides with a grown surface of permanox plastic and grown in neurobasal medium supplemented with B27 (Life Technologies, Inc.), 20 mM HEPES, 0.4 % bovine serum albumin, penicillin (100 IU/ml), and streptomycin (100 pg/ml) (Novitskaya et al. 2000). For image analysis, cells were fixed in 4 % paraformaldehyde and stained for 20 min with Coomassie Blue R250. Cover slides were observed in an inverted microscope using phase contrast optics. To measure neurite outgrowth from hippocampal neurons, an unbiased counting frame containing a grid with a number of test lines was superimposed on the images of cells. The number of intersections of cellular processes with the test lines was counted and related to the number of cell bodies, thereby allowing quantification of neurite length per cell.

Cell culture experiments were also performed with **neuronal cells from other areas of the brain** besides the hippocampus.

Brain tissue samples of rat embryos containing either septum plus preoptic area or retrochiasmatic hypothalamus were dissociated and cultured for 14 and 21 days by Jirikowski et al. (1981). By means of immunofluorescence, LHRH, α-MSH, vasopressin, and neurophysin-containing hormones could be identified.

Sinor et al. (2000) studied NMDA and glutamate-evoked excitotoxicity at distinct cellular locations in rat cortical neurons in vitro.

Canals et al. (2001) examined neurotrophic and neurotoxic effects of nitric oxide on

neuronal-enriched fetal midbrain cultures from embryonic Sprague–Dawley rats.

López et al. (2001) investigated the release of amino acid neurotransmitters in cultured cortical neurons obtained from gestation day 19 rats by nicotine stimulation.

Ehret et al. (2001) studied the modulation of electrically evoked acetylcholine release in cultured septal neurons from embryonic Wistar rats.

Tang et al. (2001) found a lack of replicative senescence in cultured rat oligodendrocyte precursor cells.

Yamagishi et al. (2001) used cultured rat cerebellar granule neurons as a model system for studying neuronal apoptosis.

Noh and Koh (2000) prepared mixed **mouse** cortical cultures containing both neurons and astrocytes and pure astrocyte cultures, from fetal (15 days of gestation) and neonatal (1–3 postnatal days) mice.

Saluja et al. (2001) found that PPAR δ agonists stimulate oligodendrocyte differentiation in glial cell culture of mouse cerebra.

Uchida et al. (2000) succeeded to directly isolate clonogenic **human** central nervous system stem cells from fresh human brain tissue, using antibodies to cell surface markers and fluorescence-activated cell sorting.

For further studies with brain cell cultures.

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In Vivo Methods

Electroshock in Mice

Purpose and Rationale

The electroshock assay in mice is used primarily as an indication for compounds which are effective in grand mal epilepsy. Tonic hind limb extensions are evoked by electric stimuli which are suppressed by antiepileptics but also by other centrally active drugs.

Procedure

Groups of 6–10 male NMRI mice (18–30 g) are used. The test is started 30 min after i.p. injection or 60 min after oral treatment with the test compound or the vehicle. An apparatus with corneal or ear electrodes (Woodbury and Davenport 1952) is used to deliver the stimuli. The intensity of stimulus is dependent on the apparatus, e.g., 12 mA, 50 Hz for 0.2 s have been used. Under these

conditions, all vehicle-treated mice show the characteristic extensor tonus.

Evaluation

The animals are observed closely for 2 min. Disappearance of the hind leg extensor tonic convulsion is used as positive criterion. Percent of inhibition of seizures relative to controls is calculated. Using various doses, ED_{50} values and 95 % confidence interval are calculated by probit analysis.

ED_{50} values after oral administration are:

- Diazepam 3.0 mg/kg
- Diphenylhydantoin 20.0 mg/kg

Critical Assessment of the Method

The electroshock test in mice has been proven to be a useful tool to detect compounds with anticonvulsant activity.

Modifications of the Method

Cashin and Jackson (1962) described a simple apparatus for assessing anticonvulsant drugs by the electroshock seizure test in mice.

Kitano et al. (1996) developed the increasing-current electroshock seizure test, a new method for assessment of anti- and proconvulsant activities of drugs in mice. A single train of pulses (square wave, 5 ms, 20 Hz) of linearly increasing intensity from 5 mA to 30 mA was applied via ear electrodes. The current at which tonic hind limb extension occurred was recorded as the seizure threshold. The method allows the determination of seizure threshold current for individual animals.

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Pentylenetetrazol Test in Mice and Rats

See chapter "► Tests for Anxiolytic Activity".

Strychnine-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity."

Picrotoxin-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity".

Isoniazid-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity".

These tests, already described for evaluation of the anticonvulsive activity of anxiolytics, can be used and show activity for antiepileptics.

Many other agents induce seizures in animals and have been used to test the anticonvulsant activity of drugs (Stone 1972), e.g., glutarimides (Hahn and Oberdorf 1960), pilocarpine (Tursky et al. 1987), methionine sulfoximine (Toussi et al. 1987), *N*-methyl-D-aspartic acid (Leander et al. (1988), γ-hydroxybutyrate (Snead 1988).

Shouse et al. (1989) described mechanisms of seizure suppression during rapid eye movement (REM) sleep in cats. Spike–wave paroxysms in the EEG accompanied by bilateral myoclonus of the head and the neck were induced by i.m. injection of 300,000–400,000 IU/kg sodium penicillin G.

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Bicuculline Test in Rats

Purpose and Rationale

Seizures can be induced by the $GAGA_A$ antagonist bicuculline and are antagonized by known antiepileptics.

Procedure

Female Sprague–Dawley rats are injected i.v. with 1 mg/kg bicuculline. At this dose, a tonic convulsion appears in all treated rats within 30 s after injection. Test compounds are administered orally 1 or 2 h before bicuculline injection. Dose–response curves can be obtained.

Evaluation

Percentage of protected animals is evaluated. ED_{50} values and 95 % confidence limits are calculated by probit analysis.

Critical Assessment of the Method

Like the electroshock test, the bicuculline test is considered to be relatively specific for antiepileptic activity.

Modifications of the Method

Czuczwar et al. (1985) studied the antagonism of *N*-methyl-D,L-aspartic acid-induced convulsions by antiepileptic drugs and other agents.

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4-Aminopyridine-Induced Seizures in Mice

Purpose and Rationale

The K^+ channel antagonist 4-aminopyridine is a powerful convulsant in animals and in man. The drug readily penetrates the blood–brain barrier and is believed to induce seizure activity by enhancing spontaneous and evoked neurotransmitter release. Although both excitatory and inhibitory synaptic transmission are facilitated by 4-aminopyridine, the epileptiform activity induced by the drug is predominantly mediated by non-NMDA-type excitatory amino acid receptors. In mice, parenterally administered 4-aminopyridine induces clonic–tonic convulsions and lethality.

Procedure

Male NIH Swiss mice weighing 25–30 g are allowed to acclimatize with free access to food and water for a 24 h period before testing. Test drugs are administered in various doses intraperitoneally 15 min prior to s.c. injection of 4-aminopyridine at a dose of 13.3 mg/kg which was found to be the LD_{97} in this strain of mice. Controls treated with 4-aminopyridine only exhibit characteristic behavioral signs, such as hyperreactivity, trembling, intermitted forelimb/hind limb clonus followed by hind limb extension, tonic seizures, opisthotonus, and death. The mean latency to death at the LD_{97} is about 10 min. Groups of eight mice are used for each dose.

Evaluation

The percentage of protected animals at each dose is used to calculate ED_{50} values. Phenytoin-like anticonvulsants such as carbamazepine and broad-spectrum anticonvulsants such as phenobarbital and valproate are effective whereas GABA enhancers such as diazepam, several NMDA antagonists, and $C\varsigma^{2+}$ channel antagonists such as nimodipine are not.

Critical Assessment of the Method

The profile of drugs effective in this seizure model is distinct from other chemoconvulsant models and more similar to those that prevent tonic hind limb extension in the maximal electroshock seizure test. The test is useful to differentiate the mode of action of anticonvulsant drugs.

Modifications of the Method

Morales-Villagran et al. (1996) described protection against seizures induced by intracerebral or intra-cerebroventricular administration of 4-aminopyridine by NMDA receptor antagonists.

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3-Nitropropionic Acid-Induced Seizures in Mice

Purpose and Rationale

3-Nitropropionic acid is a naturally occurring toxin demonstrated to impair energy metabolism via irreversible inhibition of a mitochondrial complex II component, succinate dehydrogenase (Alston et al. 1977; Ludolph et al. 1991). 3-Nitropropionic acid evokes seizures in mice after i.p. injection of 100–200 mg/kg (Urbańska et al. 1998, 1999). Urbańska et al. (1998) and Zuchora et al. (2005) evaluated anticonvulsants for their protective effect against 3-nitropropionic acid-induced seizures.

Procedure

Male albino Swiss mice weighing 20–25 g were injected i.p. with 210 mg/kg 3-nitropropionic acid, which is equal to the ED_{97} dose (i.e., the dose required to evoke seizures in 97 % of the animals). Groups of eight mice received in addition various doses of the anticonvulsant drugs. Percentage of animals with seizures and latency until occurrence of seizures were determined. Mortality rate was determined 2 h after injection of 3-nitropropionic acid.

Evaluation

ED_{50} and LD_{50} values together with their confidence limits were estimated by computerized fitting of the data by linear regression analysis according to Litchfield and Wilcoxon. Statistical comparisons of latency data were performed by means of one-way analysis of variance (ANOVA) followed by adjustment of P value by the Bonferroni method.

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Epilepsy Induced by Focal Lesions

Purpose and Rationale

Intrahippocampal injections of noxious agents or certain cerebral lesions can induce seizures in animals. Cavalheiro et al. (1982) studied the long-term effects of intrahippocampal kainic acid injections in rats.

Procedure

Adult male Wistar rats are anesthetized with a chloral hydrate/Nembutal mixture and placed in a stereotactic apparatus. For injections, a 0.3 mm cannula is inserted through a burr hole in the calvarium. The coordinates for hippocampal injections are based on a stereotactic atlas, e.g., Albe-Fessard et al. (1971). Kainic acid is dissolved in artificial serum and infused in various doses (0.1–3.0 μg) in a volume of 0.2 μl over a period of 3 min. For recording, bipolar twisted electrodes (100 μm) are positioned stereotaxically and fixed on the skull with dental acrylic cement. Depth recording sites include the dorsal hippocampus and amygdala ipsilateral to the injected side. Surface electrodes are guided from jeweler's screws over the occipital cortex. An additional screw in the frontal sinus serves as indifferent electrode for grounding. Signals are recorded by an EEG polygraph.

Evaluation

EEG recordings and observations of convulsive seizures are performed during the acute phase and during the chronic phase (up to 2 months) with and without drug treatment.

Modifications of the Method

Several agents have been used as convulsants after topical administration, e.g., application of alumina cream (Kopeloff et al. 1942, 1955; Ward 1972; Feria-Velasco et al. 1980), implantation of cobalt powder (Dow et al. 1962; Fischer et al. 1967), injection of a colloidal gel of tungstic acid (Blum and Liban 1960; Black et al. 1967), topical application of penicillin (Matsumoto and Marsan 1964), subpial injection of saturated $FeCl_3$ solution (Reid et al. 1979; Lange et al. 1980), intracerebral injections of zinc sulfate (Pei et al. 1983), intracerebral injection of antibodies to brain gangliosides (Karpiak et al. 1976, 1981), microinjections of cholinergic agonists (Ferguson and Jasper 1971; Turski et al. 1983), topical application of atropine (Daniels and Spehlman 1973), injection of tetanus toxin into the hippocampus (Mellanby et al. 1984; Hawkins and Mellanby 1987), injection of strychnine in the visual or somatosensory cortex (Atsev and Yosiphov 1969), and electrophoretic application of bicuculline from a fluid-filled microelectrode (Campell and Holmes 1984).

Bernhard and Bohm (1955) and Bernhard et al. (1956) evaluated the anticonvulsive effect of local anesthetics in cats and monkeys. The head was fixed in light Nembutal anesthesia, the parietal areas exposed and covered with paraffin oil. Stimulating electrodes were placed at the surface of the parietal region. The cortex was stimulated with repetitive square wave shocks (duration 1–3 ms) with a frequency of 25 per s for 5 s. In order to avoid muscular movements, D-tubocurarine was given. Cortical after discharge was registered before and after injection of local anesthetics.

Cortical epileptic lesions were produced by local freezing (Stalmaster and Hanna 1972; Hanna and Stalmaster 1973; Loiseau et al. 1987).

Repetitive electrical stimulation of discrete regions of the central nervous system has been used as a convenient method for reproduction of the ictal phenomena of epilepsy (Marsan 1972; Racine 1972).

Remler and Marcussen (1986) and Remler et al. (1986) studied the pharmacological response of systemically derived focal epileptic lesions. A defined area of left hemisphere of rats was radiated by α-particles from a cyclotron destroying the blood–brain barrier. After a period of 150 days following irradiation, bicuculline was injected intraperitoneally resulting in focal lesions with EEG spikes and convulsions. Anticonvulsant drugs decreased these effects.

Walton and Treiman (1989) and Walton et al. (1994) described a model of cobalt-lesioned rats in which status epilepticus was induced by injection of homocysteine thiolactone.

Anderer et al. (1993) pointed out that restriction to a limited set of EEG-target variables may lead to misinterpretation of pharmaco-EEG results.

Krupp and Löscher (1998) developed a cortical ramp-stimulation model allowing repeated determinations of seizure threshold at short time intervals in individual rats without inducing postictal threshold increases.

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Kindled Rat Seizure Model

Purpose and Rationale

Kindling, first described by Goddard et al. (1969), results from repetitive subconvulsive electrical stimulation of certain areas of the brain. Initially, local afterdischarge is associated with mild behavioral signs; however, with continued stimulation, electrical activity presumably spreads, and generalized convulsions occur. Although the pathogenesis of kindled seizures is not fully understood, it serves as a useful tool for investigating the efficacy of experimental anticonvulsant agents.

Procedure

Adult female Sprague–Dawley rats (270–400 g) are used. The rats are implanted with an electrode in the right amygdala according to the coordinates of Pellegrino et al. (1979): frontal, 7.0; lateral, −4.7; and horizontal, 2.5. At least 1 week has to elapse before electrical stimulation of the brain is started. Afterdischarge threshold is determined for each rat. Duration and amplitude, behavioral seizure duration, and seizure stage are recorded with increased stimuli afterdischarges. Seizure severity is classified into five stages (Racine 1972). Rats are considered to be kindled on the first stimulation causing a stage 5 seizure which is followed by at least two consecutive stage 5 seizures.

The animals are tested on the day before and after treatment with the test compound (i.p. or orally). Amygdala stimulation is applied at various time intervals.

Evaluation

The occurrence and the degree of seizures are compared between control results and those after administration of the test compound.

Critical Assessment of the Method

The kindled seizure model offers an approach to study anticonvulsive drugs on the basis of a pathophysiological model. This method may give more relevant results than the simpler methods using maximal electroshock or chemically induced convulsions.

Modifications of the Method

Generalized convulsive seizures have been induced by daily amygdaloid stimulation in **baboons** (Wada and Osawa 1976) and in **rhesus monkeys** (Wada et al. 1978).

The kindling effect can be produced by intermittent administration of small doses of pentylenetetrazol (Mason and Cooper 1972).

Dürmüller et al. (1994) tested a competitive (NBQX) and a noncompetitive (GYKI 52446) AMPA antagonist and a competitive NMDA antagonist (D-CPPene) against the development of kindling and against fully kindled seizures in amygdala-kindled rats.

Croucher et al. (1996) described a chemical kindling procedure in rats by daily focal microinjection of NMDA into the right basolateral amygdala and the inhibition of seizures by an NMDA receptor antagonist.

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Löscher et al. (1993), Ebert et al. (1997), and Ebert and Löscher (1999) studied the effect of phenytoin on the spread of seizures in the amygdala kindling model in rats. Sprague–Dawley rats implanted with a stimulation and recording electrode in the basolateral amygdala showed an increase in current intensity necessary for eliciting afterdischarges of about 200 % after administration of phenytoin, while seizure severity at threshold was increased compared to controls. Phenytoin-resistant kindled rats are considered as a model of drug-resistant epilepsy.

Löscher (1998) discussed the pharmacology of glutamate receptor antagonists in the kindling model of epilepsy.

The kindling procedure can also be used to evaluate antidepressant drugs (Babington 1975).

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Posthypoxic Myoclonus in Rats

Purpose and Rationale

The syndrome of posthypoxic myoclonus in man was described by Lance and Adams (1963). Lance (1968), Fahn (1986), Truong et al. (1994), and Jaw et al. (1994, 1995, 1996) reported on a model in rats resembling this human disorder.

Procedure

Male Sprague–Dawley rats which fasted 12–24 h prior to surgery are anesthetized with 100 mg/kg ketamine i.p., supplemented by 0.4 mg/kg atropine. The animal is placed on a circulating water pad and kept at a constant body temperature by a heating lamp. The rat is intubated and ventilated with 30 % O_2 in N_2O. The femoral artery and vein are cannulated for monitoring blood pressure and delivery of drugs, respectively. Electrocardiogram and blood pressure are recorded with a polygraph. The rat is then paralyzed with 2 mg/kg succinlycholine i.v., and ventilator settings are

adjusted to a rate of 60 strokes/min and a volume of 7.5 ml/kg, which yields blood gases of >150 mmHg pO_2, 35–40 mmHg pCO_2, and a pH of 7.35–7.40. N_2O is replaced with N_2 and an equilibrium period of 5 min is allowed.

Cardiac arrest is accomplished with a transthoracic intracardiac injection of KCl and cessation of the respiration. Resuscitation is begun 10 min after the arrest by turning on the ventilator (100 % O_2), manual thoracic compressions, and i.v. injections of 20 µg /kg epinephrine hydrochloride and sodium bicarbonate (4 mEq/kg). The rat is then weaned from the ventilator over 2–4 h and extubated.

Auditory-induced myoclonus: Rats are presented with a series of 45 clicks from a metronome (1 Hz, 95 dB, 40 ms), and the response to each click is scored as follows: 0 = no response, 1 = ear twitch, 2 = ear and head jerk, 3 = ear, head, and shoulder jerk, 4 = whole body jerk, 6 = whole body jerk of such severity that it causes a jump. The total myoclonus score of each rat is determined by summing up the scores yielded over 45 clicks.

Since rats ranging from 3 to 14 days post cardiac arrest show similar susceptibility to audiogenic stimulation, animals within this period are used for pharmacological tests. Myoclonus scores are assessed 30 min before and 60 min after intraperitoneal drug application.

Evaluation

Changes in myoclonus scores are analyzed by paired two-tailed Student's *t*-test.

Critical Assessment of the Test

Some anticonvulsant drugs, such as clonazepam and valproic acid, were reported to be active in this test; however, phenytoin is not. Posthypoxic myoclonus may present a special pathological condition.

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Rat Kainate Model of Epilepsy

Purpose and Rationale

Temporal lobe epilepsy is characterized by complex partial seizures that involve and apparently originate in the mesial temporal structures of the limbic system. These complex partial seizures can evolve into secondarily generalized, tonic–clonic seizures. Patients become resistant to the treatment with the usual antiepileptic drugs. The kainate-treated rat is one of several models used to study temporal lobe epilepsy. Examination of the hippocampus and dentate gyrus from kainate-treated rats has revealed a similar pattern of neurodegeneration in the hippocampus and the presence of mossy fiber sprouting in the inner molecular level of the dentate gyrus. Several authors used this model to find drugs for treatment-resistant epilepsy (Bolanos et al. 1998; Hellier et al. 1998; Longo and Mello 1998; Maj et al. 1998; Bouilleret et al. 1999; Pitkänen et al. 1999; Cilio et al. 2001; Madsen et al. 2001; Ebert et al. 2002; Tamagami et al. 2004). Maj et al. (1998) tested the activity of several drugs

against kainate-induced status epilepticus and hippocampal lesions in the rat.

Procedure

Male Wistar rates weighing 225–250 g are anesthetized with sodium pentobarbital (50 mg/kg i.p.). They are implanted extradurally with electrodes over the frontal and parietal cortex and with a reference electrode on the cerebellum. Caution is taken not to break the inner table of the diploe. All the electrodes are connected to plugs and held to the skull with dental acrylic cement. At least 7 days after surgery, rats are treated with either saline or test drugs intraperitoneally. Then, 15 min later, the rats receive a single i.p. dose of kainic acid (10 mg/kg). EEG recordings and behavioral observations are performed up to 240 min after kainic acid administration. Status epilepticus is defined as a sustained ictal EEG pattern lasting 20 min or longer without any interruption longer than 1 min.

Seven days later, the rats are sacrificed, the brains removed and immersed for 48 h in 10 % formalin. Coronal sections (4 μm) are stained with hematoxylineosin. Hippocampal injury is assessed by counting the number of histologically normal CA4 pyramidal neurons.

Evaluation

The percentage of animals protected from status epilepticus is analyzed using Fisher's exact test. For calculation of the latency to status epilepticus (min) and duration of status epilepticus (min), all animals are included regardless of whether they showed status epilepticus or not. The data are evaluated by analysis of variance (ANOVA) followed by Dunnett's test. Neuronal counts are analyzed using the Mann–Whitney nonparametric test.

Modifications of the Method

Cilio et al. (2001) used immature rats to test the anticonvulsant action and long-term effects of gabapentin.

Hellier et al. (1998) used repeated low-dose systemic treatment in order to reduce the mortality associated with single injections with kainate.

Since intracerebroventricular administration of kainic acid decreases hippocampal neuronal number and increases dopamine receptor binding in the nucleus accumbens, kainic lesions have been discussed as an animal model of schizophrenia (Bardgett et al. 1995; Csernansky et al. 1998).

Humphrey et al. (2001) described methods for inducing neuronal loss in preweanling rats using an intracerebroventricular infusion of kainic acid.

Hu et al. (1998) investigated neuronal stress and injury in C57/BL **mice** after systemic kainic acid administration.

Bouilleret et al. (1999) tested recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult **mice**.

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Pilocarpine Model of Epilepsy

Purpose and Rationale

Several post-status models are described in which epilepsy develops after a chemically induced status epilepticus, such as the kainate, the pilocarpine, and the lithium–pilocarpine model (Löscher 2002). Several modifications of the pilocarpine and the lithium–pilocarpine model are reported in the literature (Cavalheiro et al. 1991; Leite and Cavalheiro 1995; André et al. 2001; Biagini et al. 2001; Klitgaard et al. 2002; Leite et al. 2002; Wallace et al. 2003; Arida et al. 2004; Leroy et al. 2004; Lyon et al. 2004; Rigoulot et al. 2004; Setkowicz et al. 2004). When rats are pretreated with lithium chloride, status epilepticus can be produced with a substantially lower dose of pilocarpine, and rats display the same clinical and EEG features of status epilepticus as with pilocarpine alone (Honchar et al. 1983). André et al. (2001) and Rigoulot et al. (2004) tested antiepileptic drugs in the lithium–pilocarpine model of epilepsy.

Procedure

Male Wistar rats weighing 225–250 g were anesthetized for electrode implantation by an i.p. injection of 2.5 mg/kg diazepam and 1 mg/kg ketamine hydrochloride. Two single-contact recording electrodes were placed on the skull, one on each side of the parietal cortex, and one bipolar deep-recording electrode was placed in the right hippocampus (Vergnes et al. 1982).

One week after surgery, rats received 3 mEq/kg lithium chloride i.p. On the following day, 1 mg/kg methylscopolamine bromide was administered s.c. to limit the peripheral effects of the convulsant. Status epilepticus was induced by injecting pilocarpine (25 mg/kg s.c.) 30 min after methylscopolamine. Various doses of test drug (i.p.) or 2.5 mg/kg diazepam (i.m.) were injected at 1 h after the onset of status epilepticus. The onset of status epilepticus corresponds to the moment at which rats experience successive seizures without recovery. Continuous spiking of the EEG occurs 30–60 min after pilocarpine

administration. The bilateral EEG cortical activity and the unilateral EEG hippocampal activity were recorded during the whole duration of status epilepticus, and concurrent behavioral changes were noted.

Quantification of neuronal damage was performed 14 days after status epilepticus. Brains of rats sacrificed in pentobarbital anesthesia were removed, and coronal sections containing the hippocampus from the anterior to the posterior level were prepared. Quantification of cell density was performed with a microscopic grid. The numbers of cells obtained in 12 counted fields were averaged.

Evaluation

Statistical analysis of neuronal damage and epilepsy between the different groups was performed by means of analysis of variance followed by a post hoc Dunnett's test for multiple comparisons.

Modifications of the Method

Hort et al. (1999) studied the relation between spontaneous recurrent seizures and the derangement of cognitive function in pilocarpine-induced status epilepticus,

Tang et al. (2004) recorded EEG in freely moving mice after pilocarpine-induced status epilepticus. A transmitter (TSE Systems, Bad Homburg, Germany) was fixed on the electrode socket by plug connection with wires attached to the skull by two screws 3 days before pilocarpine induction. The EEG signals were telemetrically received via an HF receiver which passed the signals to the computer.

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Self-Sustained Status Epilepticus

Purpose and Rationale

Status epilepticus causes neuronal damage that is associated with cognitive impairment. Self-sustained status epilepticus (SSSE) can be induced in rats by electrical stimulation of the perforant pathway (Halonen et al. 1996, 1999, 2001; Pitkänen et al. 1996; De Vasconcelos et al. 1999; Mazarati et al. 1999, 2004). This model is used to find antiepileptic drugs for patients with therapy-resistant epilepsy. Pitkänen et al. (1996), Halonen et al. (1996, 1999, 2001), and Mazarati et al. (2004) studied the effect of drugs on status epilepticus in rats.

Procedure

Under ketamine (60 mg/kg) and xylazine (15 mg/kg) anesthesia, male Wistar rats weighing 260–280 g were implanted with a bipolar stimulation electrode into the angular bundle of the perforant path (0.5 mm anterior and 4.5 mm left to lambda) and a bipolar recording electrode into the ipsilateral dentate gyrus (3 mm posterior and 2.5 mm left to bregma). The depth of the electrode was 3.5–4 mm from the brain surface and was optimized by finding the maximal population spike evoked from the dentate gyrus by stimuli applied to the perforant path.

For induction of self-sustained status epilepticus, perforant path stimulation was delivered using a Grass stimulator model 8800, for 30 min with the following parameters: 10-s, 20-Hz trains for 1 ms, 30-V pulses delivered every minute, together with continuous 2 Hz stimulation using the same parameters.

Test drugs were injected i.v. into the tail vein either 20 min before perforant path stimulation, or 10 or 40 min after the end of perforant path stimulation. Control animals were treated with saline.

Electrographic activity was acquired and analyzed off-line using Harmonie software (Stellate Systems, Montreal), configured for automatic detection and saving spikes and seizures. Analysis of EEG was performed by a “blinded” unbiased investigator. All seizure EEGs were reviewed manually.

Evaluation

The following indices were used to quantify seizure activity: duration of self-sustained status epilepticus (= time between the end of perforant path stimulation and the end of the last electrographic seizure), cumulative seizure time (the sum of the duration of all individual seizures), number of seizure episodes, average duration of individual seizures (cumulative seizure time

divided by number of seizures), and number of spikes per hour. Statistical analysis was carried out with one-way ANOVA followed by Newman–Keuls post hoc test, or, if the normality test failed, ANOVA on ranks followed by Mann–Whitney post hoc test.

Modifications of the Method

Brown et al. (1953) and Barton et al. (2001) characterized the 6 Hz psychomotor seizure model of partial epilepsy in rats.

Nissinen et al. (2000) described a model of chronic temporal lobe epilepsy induced by electrical stimulation of the lateral nucleus of the amygdala in rats.

Walton et al. (1996) induced status epilepticus in rats with actively epileptogenic cortical cobalt lesions by administration of homocysteine thiolactone.

Laurén et al. (2003) described selective changes in gamma-aminobutyric acid type A receptor subunits in the hippocampus in spontaneously seizing rats with chronic temporal lobe epilepsy.

Brandt et al. (2003) studied epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats.

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Rat Model of Cortical Dysplasia

Purpose and Rationale

Epilepsy becomes drug resistant in 20–30 % of patients. Cortical dysplasia is implicated as a major contributing factor of many types of epileptic disorders that are resistant to pharmacological intervention (Becker 1991; Aicardi 1994). Several animal models of cortical dysplasia with specific clinical pathologies have been described (Amano et al. 1996; Jacobs 1996; Jacobs et al. 1999; Lee et al. 1997; Hirotsune et al. 1998; Chevassus au Louis et al. 1999; Zhu and Roper 2000; Wenzel et al. 2001; Benardete and Kriegstein 2002; Morimoto et al. 2004; Jacobs and Prince 2005).

Baraban and Schwartzkroin (1995, 1996), Baraban et al. (2000), and Smyth et al. (2002) exposed rats in utero to methylazoxymethanol (MAM).

Procedure

Dysplastic and control rats were generated by injecting pregnant Sprague–Dawley rats on day 15 of gestation with 25 mg/kg i.p. MAM or vehicle (10 % DMSO in 0.3 ml 0.9 % saline).

For in vitro studies, recordings were performed using acute hippocampal slices from adult vehicle or MAM-treated rats. Hippocampi were not dissected out, and all slices included entorhinal cortex and other overlying cortical structures. After cutting, slices remained submerged in a holding chamber containing oxygenated recording medium (NaCF) consisting of (in mM): 124 NaCl, 3 KCl, 1.25NaH_2PO_4, 2$MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose. A slice was then transferred to a gas interface recording chamber and perfused with oxygenated NaCF at a flow rate of 2.5 ml/min at 33.5 °C. Borosilicate glass electrodes were pulled, filled with 2 M NaCl (2–8 MΩ) and placed in the CA1 region of stratum pyramidale and/or within neuronal heterotopias under visual microscopic control. A monopolar-stimulating electrode was placed in stratum radiatum. Voltage was recorded with a Neurodata IR-283 amplifier and monitored on a PC running pCLAMP software. Spontaneous field activity and evoked population spikes were stored on hard disk for later blinded analysis. Interictal epileptiform burst activity was initiated with perfusion of NaCF containing 4-aminopyridine (100 μM), a potassium channel blocker known to cause seizures in humans, and spontaneous epileptiform activity in hippocampal slice preparations. The 4-aminopyridine in vitro seizure model is based on blockade of A-type potassium channels leading to the appearance of giant excitatory postsynaptic potentials generated by the prolonged firing of pyramidal neurons in CA3 burst-generating regions of the hippocampus. Burst frequency was determined by counting the number of interictal epileptiform events per second during a 3-min epoch before and after 60 min of antiepileptic drug co-perfusion and was expressed as Hz. Burst amplitude (1.5–6 mV) was determined by measuring the average peak-to-peak interval for ten consecutive representative bursts during the same epoch. Evoked synaptic responses were analyzed by averaging the number of population spikes obtained on ten consecutive sweeps recorded after stratum radiatum stimulation (0.3–3-mA pulses 100 μs pulse width). A downward voltage deflection $\geq$0.5 mV superimposed on the population excitatory postsynaptic potential (EPSP) was defined as a "population spike"; the number of population spikes was compared for each slice during perfusion with normal ACSF (baseline), ACSF plus 4-aminopyridine, and ACSF plus 4-aminopyridine and antiepileptic drug. For each slice experiment, the population spike was monitored every 15 min.

For in vivo studies, control and MAM-exposed rats were administered with 15 mg/kg kainic acid, a concentration that reliably produces acute seizure activity. Behavioral activity was scored on a six-stage scale (Germano and Sperber 1997). Animals were treated with 400 mg/kg i.p. valproate 30 min before kainate injection. Latencies to the first sign of hyperexcitability and to the first tonic–clonic seizure were recorded.

Evaluation

Dates were plotted graphically as "survival" curves, and differences in mean latencies were ranked and analyzed using a nonparametric Kruskal–Wallis one-way ANOVA.

Critical Assessment of the Test

Since the MAM-exposed rats exhibit a dramatically reduced sensitivity to commonly prescribed antiepileptic drugs, this model is considered to be relevant for drug-resistant epilepsy.

Modifications of the Method

Leré et al. (2002) described a model of "epileptic tolerance" for investigating neuroprotection, epileptic susceptibility, and gene expression-related plastic changes. Expression of status epilepticus was triggered by infusion of the excitotoxic agent kainate in the right hippocampus of adult rats. An appropriate dose of kainate was used to cause brain damage to the homolateral, but not contralateral, hippocampus. At various times following the preconditioning insult, kainate was then readministered into the lateral ventricle, and neuroprotection was observed in the contralateral side between 1 and 15 days later.

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Genetic Animal Models of Epilepsy

Purpose and Rationale

Several animal species exhibit epilepsy with spontaneous recurrent seizures such as dogs, rats, and mice (Löscher 1984). Serikawa and Yamada (1986) described spontaneous epileptic rats which are double mutants and exhibit both tonic and absence-like seizures.

Procedure

Spontaneous epileptic rats are obtained by mating the tremor heterozygous rat (*tm*/+) with the zitter homozygous rat (*zi*/*zi*) found in a Sprague–Dawley colony. The behavior of the spontaneous epileptic rats is recorded weekly for 2 h on videotapes. The frequency of tonic convulsions and wild jumping occurring in the absence of external stimuli are recorded. Under anesthesia, silver ball-tipped and monopolar stainless steel electrodes are chronically implanted in the left frontal cortex and hippocampus. An indifferent electrode is placed on the frontal cranium. The frequency of absence-like seizures and tonic convulsions, as well as the duration of each seizure, are measured on the EEG. A mild tactile stimulus is given on the back of the animal every 2.5 min to induce consistent tonic convulsions. Compounds are given i.p. or orally.

Evaluation

The number of seizures and the duration of each seizure are obtained, and the total duration of the seizures (number × duration) is calculated every 5 min before and after injection of the drug. Percent changes between values before and after drug administration are calculated.

Critical Assessment of the Test

Studies in spontaneous epileptic rats and other genetic models are of value for an in-depth investigation of a potential antiepileptic drug.

Modifications of the Method

The **tremor rat (*tm*/*tm*)** was described as a model of petit mal epilepsy (Serikawa and Yamada 1986; Serikawa et al. 1987). Seki et al. (2002) attempted to determine whether gene transfer of aspartoacyclase inhibited absence-like seizures in tremor rats using recombinant adenovirus. Noda et al. (1998) and Iida et al. (1998) described the **NER rat strain** (Noda epileptic rat) as a genetic model in epilepsy research, which was developed by inbreeding rats with spontaneous tonic–clonic seizures in a stock of Crj:Wistar.

The **genetic epileptic WAG/Rij rat** has been recommended as a useful model for general absence epilepsy in humans (Van Luijtelaar and Coenen 1986; Coenen et al. 1992; Budziszewska et al. 1999; Van Luijtelaar et al. 2003; Sarkisova et al. 2003; Bouwman and van Rijn 2004). Danober et al. (1995, 1998), Deransart et al. (2000), Lakaye et al. (2002), and Nehling and Boehrer (2003) studied the **GAERS rat**, the genetic absence epilepsy rat from Strasbourg, which shows generalized nonconvulsive absence seizures characterized by the occurrence of synchronous and bilateral spike and wave discharges.

Amano et al. (1996) developed an **epileptic rat mutant with spontaneous limbic-like seizures** by successive mating and selection from an inherited cataract rat.

Racine et al. (1999) reported selective breeding of **kindling-prone** and kindling-resistant **rats**. The selection of these strains was based on their rates of amygdala kindling. From a parent population of Long–Evans hooded and Wistar rats, the males and females that showed the fastest and slowest amygdala kindling rates were selected and bred.

Sarkisian et al. (1999) described seizures in the **flathead (FH) rat** as a genetic model in early postnatal development.

Tsubota et al. (2003) identified the **Wakayama epileptic rat (WER)** in a colony of Wistar rats, a mutant exhibiting both tonic–clonic seizures and absence-like seizures

Several other genetic animal models have been described (Löscher and Frey 1984; Löscher and Meldrum 1984) showing epilepsy with spontaneous recurrent seizures, such as:

Dogs (Cunningham 1971; Edmonds et al. 1979)
Rats with petit mal epilepsy (Vergnes et al. 1982); rats with two mutations, zitter, and tremor (Serikawa and Yamada 1986; Xie et al. 1990); and rats with absence-like states and spontaneous tonic convulsions (Sasa et al. 1988)
Tottering mice (Green and Sidman 1962; Noebels 1979; Noebels and Sidman 1979; Fletcher et al. 1966; Tehrani et al. 1997)
Leaner mutant mice with severe ataxia and atrophic cerebellum (Herrup and Wilczynsnki 1982; Heckroth and Abbott 1994),
The quaking mouse (Sidman et al. 1966; Chermat et al. 1981) having deficiencies in myelinization in the nervous system (Hogan 1977; Li et al. 1993; Bartoszewicz et al. 1995) and alterations in the dopaminergic (Nikulina et al. 1995) and α_2-adrenergic (Mitrovic et al. 1992) brain system
The stargazer mutant mouse which shows generalized non-convulsive spike–wave seizures with behavioral arrest that resembles the clinical phenotype of general absence epilepsy (Noeberls et al. 1990; Di Pasquale et al. 1997) with a disrupted Cacng2 gene (Letts et al. 2005)
The lethargic (lh/lh) mouse as a model of absence seizures (Hosford et al. 1999)

There are models of epilepsy with reflex seizures, such as:

Baboons with photomyoclonic seizures (Killam et al. 1966, 1967; Stark et al. 1970; Naquet and Meldrum 1972; Killam and Killam 1984; Smith et al. 1991; Chapman et al. 1995)
Photosensitive fowls (Crawford 1969, 1970)
The Fayoumi strain of chickens (Fepi) (Batini et al. 2004)
Audiogenic seizure-susceptible mice (Collins 1972; Seyfried 1979; Chapman et al. 1984; Stenger et al. 1991)
Mechanically stimulated mice (Imaizumi et al. 1959; Oguro et al. 1991)
The EL mouse which is a strain highly susceptible to convulsive seizures after repeated sensory stimulation (Seyfried et al. 1986; King and LaMotte 1989; Green and Seyfried 1991; Wang et al. 1997; Suzuki 2004)
Audiogenic seizure-susceptible rats (Wistar audiogenic rats WAR) (Consroe et al. 1979; Reigel et al. 1986; Smith et al. 1991; Patel et al. 1990; Scarlatelli-Lima et al. 2003; Galvis-Alonzo et al. 2004; Magalhães et al. 2004)

The genetically epilepsy-prone rat GEPR responding to acoustic stimulation has been described by Ko et al. (1982), Dailey and Jobe (1985), Dailey et al. (1989), Faingold (1988), Faingold and Naritoku (1992), Faingold et al. (1994), Jobe et al. (1992, 1995), and Laird (1989). The inferior colliculus is strongly implicated as a critical initiation site within the neuronal network for audiogenic seizures. Two strains were characterized: **GEPR-3** exhibiting moderate or clonic convulsions and **GEPR-9** exhibiting more severe tonic extensor convulsions (Dailey et al. 1996; Kurtz et al. 2001; Moraes et al. 2005).

Gerbils with reflex seizures were described by Thiessen et al. (1968), Loskota et al. (1974), Majkowski and Kaplan (1983), Lee and Lomax (1984), Bartoszyk and Hamer (1987), and Lee et al. (1987).

Löscher et al. (1989) discussed **the sz mutant hamster** as a genetic model of epilepsy or of paroxysmal dystonia.

Quesney (1984) reported **generalized photosensitive epilepsy in cats** after long-term intramuscular administration of low-dose penicillin.

Famula et al. (1997) and Oberbauer et al. (2003) described the epidemiology of epilepsy in **tervurens (Belgian shepherd dogs)** and Srenk et al. (1994) in **golden retrievers**.

Seizure susceptibility was described in *Drosophila* (Kuebler and Tanouye 2000; Kuebler et al. 2001; Zhang et al. 2002).

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Transgenic Animals as Models of Epilepsy

Purpose and Rationale

The availability of transgenic animals has stimulated research on pathogenesis of epilepsy. Several surveys on this topic are available (Allen and Walsh 1999; Meldrum et al. 1999; Noebels 1999; Prasad et al. 1999; Toth and Tecott 1999; Schauwecker 2002; Weinshenker and Szot 2002; Upton and Stratton 2003; Giorgi et al. 2004; Yang and Frankel 2004). Several studies contribute to the understanding of pathology of epilepsy (Butler et al. 1995; Zeng et al. 1997; Campbell et al. 2000; Liang et al. 2000; Musumeci et al. 2000; Viswanath et al. 2000; Kearney et al. 2001; Knuesel et al. 2002; Potschka et al. 2002; Shimizu et al. 2002; Ludwig et al. 2003; Ferri et al. 2004; Diano et al. 2005; Peters et al. 2005).

Lüthi et al. (1997) found that mutant mice overexpressing protease nexin-1 (PN-1) in brain under the control of the Thy-1 promoter (Thy 1/PN-1) or lacking PN-1 (PN-1 −/−) develop epileptic activity in vivo. An endogenous serine protease inhibitor modulated epileptic activity and hippocampal long-term potentiation.

Kunieda et al. (2002) recommended mice with systemic overexpression of the alpha 1B-adrenergic receptor as an animal model of epilepsy.

Some of the studies gave hints for further development of antiepileptic drugs, such as the neuropeptide galanin (Kokaia et al. 2001; Mazarati et al. 2004) or the neuropeptide Y (Shannon and Yang 2004). Several studies were

devoted on the role of brain-derived neurotrophic factor (BDNF) (Lahteinen et al. 2002, 2003, 2004).

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Excitatory Amino Acid Transporters

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Electroshock in Mice

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Hypnotic Activity

Mary Jeanne Kallman

Contents

General Considerations

The term "hypnotic" has to be defined. In man, the purpose of taking hypnotics is to obtain a "normal" night's sleep from which the patient can be aroused without any subsequent hangover. In animal experiments, the term "hypnotic" has been applied to a much deeper stage of central depression of drug induced unconsciousness associated with loss of muscle tone and of righting reflexes. Therefore, most of the pharmacological models are questionable in regard to their predictivity to find an ideal hypnotic for human therapy. Many of the pharmacological tests are based on the potentiation of sleeping time induced by barbiturates or other sedative agents.

Since the biochemical events during sleep are rather unknown no in vitro method exists for testing compounds with potential hypnotic activity.

In Vivo Methods

Potentiation of Hexobarbital Sleeping Time

Purpose and Rationale

The test is used to elucidate CNS-active properties of drugs. Not only hypnotics, sedatives, and tranquilizers but also antidepressants at high doses are known to prolong hexobarbital induced sleep after a single dose of hexobarbital. The loss of righting reflex is measured as criterion for the duration of

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hexobarbital-induced sleeping time. Mice are used in this test, since metabolic elimination of hexobarbital is rapid in this species.

Procedure

Groups of 10 male NMRI-mice with an average weight of 18–22 g are used. They are dosed orally, i.p. or s.c. with the test compound or the reference standard (e.g., 3 mg/kg diazepam p.o.) or the vehicle. Thirty min after i.p. or s.c. injection or 60 min after oral dosing 60 mg/kg hexobarbital is injected intravenously. The animals are placed on their backs on a warmed (37 °C) pad and the duration of loss of the righting reflex (starting at the time of hexobarbital injection) is measured until they regain their righting reflexes. Injection of 60 mg/kg hexobarbital usually causes anesthesia for about 15 min. If there is any doubt as to the reappearance of the righting reflex, the subject is placed gently on its back again and, if it rights itself within 1 min, this time is considered as the endpoint.

Evaluation

Mean values of duration of anesthesia (min) are recorded in control and experimental groups. The percent change in duration of anesthesia is calculated in the experimental groups as compared to those of the controls. ED_{50} values can be calculated. ED_{50} is defined as the dose of drug leading to a 100 % prolongation in duration of anesthesia in 50 % of the animals.

Critical Assessment of the Method

The anxiolytic agents of the benzodiazepine type show an uniform pattern with oral ED_{50} values of less than 1 mg/kg. This is in agreement with the fact that barbiturates also show anxiolytic activity in anti-anxiety tests with animals as well as in patients. Neuroleptics, such as chlorpromazine and haloperidol, also prolong hexobarbital sleeping time in low doses. The test is considered to be unspecific since compounds which inhibit liver metabolism of hexobarbital also prolong time of anesthesia. Balazs and Grice (1963) discussed the relationship between liver necrosis, induced by CCl_4 or nitrosamines, and pentobarbital sleeping time in rats.

Other Uses of the Test

Hexobarbital sleeping time is not only prolonged by the simultaneous administration of many compounds but also shortened under special conditions. Several CNS-active compounds (analeptics and stimulants like amphetamine and related compounds and methylxanthines) reduce hexobarbital sleeping time. Standard compounds for this kind of procedure are pentylenetetrazol, methamphetamine and aminophylline.

After repeated administrations, induction of metabolic enzymes in the liver is caused by many compounds and leads to an increased destruction of hexobarbital. Due to the accelerated metabolism of hexobarbital, sleeping time is reduced (Remmer 1972).

Modifications of the Test

Instead of hexobarbital, another barbiturate, thiopental can be used which has been proven in clinical use to be a short acting anesthetic. Test compounds or the standard are given 60 min before i.v. injection of 25 mg/kg thiopental to mice with a weight between 18 and 22 g. The animals are placed on their backs and the reappearance of the righting reflex is observed. The ED_{50} which results in a 100 % prolongation in duration of anesthesia is between 2.5 and 4.0 mg/kg diazepam p.o.

Simon et al. (1982) tested the interaction of various psychotropic agents with sleep induced by barbital or pentobarbital in mice. Pentobarbital (50 mg/kg) or barbital (180 mg/kg) were injected i.p. and the latency and duration of sleep (loss of righting reflex) were recorded. The test compound was usually administered i.p. 30 min before the injection of the barbiturate. The test was recommended for detecting sedative or anti-sleep activity. Since pentobarbital is metabolized by the liver whereas barbital is not, a comparative study using the two compounds can be useful for determining whether an eventual potentiation or antagonism can be ascribed to enzymatic inhibition or induction.

Fujimori (1965) recommended the use of barbital-Na instead of hexobarbital for the sleeping time test since barbital is not biotransformed by the liver microsomal system.

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Experimental Insomnia in Rats

Purpose and Rationale

James and Piper (1978) described a method for evaluating potential hypnotic compounds in rats. Usually, the compounds are tested in normal animals where they do not significantly decrease wakefulness. Footshock induced "insomnia" in rats is proposed as suitable model for insomnia in patients.

Procedure

Male Wistar rats (200–275 g) are prepared for chronic electroencephalographic and electromyographic recordings. Four silver/silver chloride epidural electrodes and two disc nuchal electrodes are implanted. A minimum of 10 days is allowed for recovery from surgery. The animals are placed into soundattenuated recording chambers with grid floors. The frontal-occipital electroencephalogram and the electromyogram are recorded via nonrestraining recording leads on a polygraph and a tape recorder.

On the control day, the animals are dosed with the vehicle and a control nonstress recording is obtained for 8 h. On the next day, the animals are again injected with the vehicle and then exposed to electric footshocks for 8 h. The footshock is delivered through the grid floor of the recording chamber using the EMG leads as indifferent electrodes, in the form of a 0.5 mA pulse of 15 ms width for 30 s at 1 Hz. During the footshock the EEG and EMG recording circuits are automatically interrupted. The delivery of electric footshock is triggered automatically by two adjustable timers. In this way, each shock period of 30 s is followed by an interval of 30 min. On the next day the rats are dosed with the test compound or the standard and recordings are obtained during a shock session of 8 h.

Evaluation

The sleep-wake cycle is definitely altered by the stress procedure. The amounts of arousal and of slow wave sleep I are increased, whereas slow wave sleep II and paradoxical sleep are decreased. Phenobarbital and benzodiazepines antagonize these changes at least partially.

Critical Assessment of the Method

For screening procedures, the method is too expensive and time-consuming. However, the EEG-parameters in a situation of insomnia similar to men can indicate the usefulness of a new compound.

Modifications of the Method

Gardner and James (1987) described a modified shortened protocol in which a 2.5-h nonstressed control period is followed by drug or vehicle administration and a further 5.5-h recording of the electrocorticogram in the presence of intermittent footshock.

Laval et al. (1991) studied the effect of anxiolytic and hypnotic drugs on sleep circadian rhythms in the rat.

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EEG Registration in Conscious Cats

Purpose and Rationale

The effect of hypnotics on sleep pattern of EEG tracings can be studied in conscious, freely moving cats with chronically implanted electrodes (Heinemann et al. 1970, Heinemann and Stock 1973; Wallach et al. 1976; Hirotsu et al. 1988).

Procedure

Female cats weighing 2.5–3.5 kg are anesthetized and prepared with bipolar subcortical electrodes in the reticular formation (A3, L3, H −1), dorsal hippocampus (A5, L −5, H8), and either amygdala (A12, L −9, H −5), or caudate nucleus (A11, L9.5, H −2). Cortical screw electrodes are placed over the anterior suprasylvian, lateral, medial suprasylvian and ectosylvian gyri. Two Teflon coated steel wires are placed in the cervical neck muscles. All wires are connected to a subminiature socket and implanted in dental acrylic. Cats of this chronic colony are then intermittently utilized for drug experiments at interdrug intervals of at least 2 weeks.

On experimental days, the cats are taken into an experimental chamber 70 × 80 × 80 cm high. The box is lighted and ventilated with room air at 21 °C. The cat is immediately connected to a cable which exits through the top center of the cage into a mercury swivel. This prevents the cable from becoming twisted and restricting the cat's movement. Recordings of the cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are obtained. Continuous recordings for up to 96 h are amplified and stored in a recorder. The recordings of cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are analyzed for REM sleep, slow wave sleep, and wakefulness. Undefined periods which can not be identified either as slow wave sleep or as wakefulness are included in the awake total. Since a first night effect was observed (Wallach et al. 1976) drugs are given at the 3rd or 4th day.

Evaluation

The data are analyzed by analysis of variance with subjects, days, and drug as factors.

Modifications of the Method

Schallek and Kuehn (1965) measured the effects of benzodiazepines on spontaneous EEG and arousal responses in cats with implanted electrodes.

In addition to EEG and electromyogram, Holm et al. (1991) registered the electro-oculogram in conscious cats.

EEG studies in immobilized cats were performed by Ongini et al. (1982) for evaluation of a benzodiazepine hypnotic. Adult mongrel cats of both sexes were anesthetized with halothane. A tracheal cannula was inserted and artificial respiration was maintained throughout the experiment. The spinal cord was transected at C_2 level (Encephalè isolè preparation). The femoral vein was cannulated for i.v. injection of drugs. Cortical electrodes were inserted into the skull in the frontal, parietal and occipital areas. All incisions were infiltrated with mepivacaine 1 % to produce local anesthesia. The body temperature was maintained at 36.5–38.0 °C by an electrical heat pad. After recovery from surgery and anesthesia, a continuous EEG recording of 2 h was taken prior to drug administration. Test drugs were injected intravenously at various doses. Electrocortical activity was recorded using a

8-channel electroencephalograph. In addition, two electrodes were connected with an EEG-analyzer for the on-line evaluation of the EEG power spectrum. This was computed by the Fast Fourier Transform at a frequency range of 0–32 Hz. Power spectral plots averaging 30 s of electrocortical activity were derived during the experiment.

Shibata et al. (1994) administered various local anesthetics intravenously with constant rates of equipotent doses to cats with implanted electrodes until EEG seizures appeared. During slow rates of infusion, a tetraphasic sequence of changes was found.

Wetzel (1985) evaluated EEG recordings in freely moving **rats** by visual analysis for wakefulness, slow wave sleep or paradoxical sleep.

Krijzer et al. (1991) presented a subclassification of antidepressants based on the quantitative analysis of the electrocorticogram in the rat.

Sarkadi and Inczeffy (1996) described an integrated quantitative electroencephalographic system for pharmacological and toxicological research in the rat. Peak latencies and amplitudes of visual-evoked potentials, occurrence, duration, and linear excursions of photically evoked afterdischarges, activity, mobility, complexity according to Hjorth (1970), and absolute spectral powers of delta, theta, alpha, and beta frequency bands of background activity of visual cortex and frontal-visual leads were measured in freely moving rats.

Rinaldi-Carmona et al. (1929) performed temporal EEG analysis of the sleep-waking cycle in rats with implanted electrodes after administration of a 5-hydroxytryptamine$_2$ receptor antagonist.

Lozito et al. (1994) compared loss of righting with EEG changes in rats with implanted electrodes after single and multiple infusions of fentanyl analogues.

Jones and Greufe (1994) described a quantitative electroence phalographic method in **dogs**.

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Automated Rat Sleep Analysis System

Purpose and Rationale

Ruigt et al. (1989a, b, 1993) described an automated rat sleep classification system in rats which allows classification of psychotropic drugs such as potential antidepressants, antipsychotics and stimulants (Ruigt and van Proosdij 1990; de Boer and Ruigt 1995).

The system records and analyzes bioelectrical signals from several animals over extended periods of time. The analysis is based on three signals, the parietooccipital EEG, nuchal EMG and a movement indicator signal.

Procedure

Epidural screw electrodes are implanted over the parieto-occipital cortex of male rats weighing 250–300 g for the recording of EEG against a frontal electrode. Stainless-steel wire electrodes are inserted in the dorsal neck musculature for recording the electromyogram (EMG). After recovery from surgery animals are separately housed in a light- (12:12 h light–dark cycle) and temperature- (21 °C) controlled room. Twenty-nine hour EEG and EMG recordings are made in sound-attenuated Faraday cages from 32 rats simultaneously. Movements of the rats are detected as capacitative artefacts generated in an open-ended wire of the nonrestraining flat cable connecting the rats to a swivel commutator and to

amplification and A/D conversion units, which are hooked up through a data controller to a dedicated PDP-11/83 minicomputer system for online spectral EEG analysis and data compression.

Off-line sleep staging on a micro VAX is done per 2-s epoch based on 5 spectral EEG band values (1.0–3.0, 3.0–6.0, 6.0–9.0, 9.5–20.0, 20.0–45.0Hz), the integrated EMG level and the movement level. A first sleep stage assignment per epoch is done by application of a discriminant function to these epoch values. The discriminant function is derived from a discriminant analysis of visually classified representative recording segments from different sleep stages recorded during a separate calibration experiment for each rat. A moving average EEG smoothing procedure and a set of syntactic classification rules are then used to give a final sleep stage assignment to each specific EEG epoch.

Six sleep-wake stages are distinguished including 2 waking stages: (1) active waking characterized by movement, theta activity and high EMG, and (2) quiet waking without movement. Four sleep stages are discriminated: (3) quiet sleep, characterized by EEG spindles; (4) deep slow-wave sleep with prominent delta activity; (5) pre-REM sleep with spindles against a background of theta activity and low EMG, and (6) REM sleep with theta activity and low EMG.

Each experiment consists of 32 rats divided over maximally 4 groups, including various drug treatment groups (generally several doses of the same drug) and always one placebo group. Drug administration is done at the beginning of the light cycle of the rats. After each experiment 2–3 weeks are allowed for wash-out. Drug effects on sleep-waking behavior are assessed on several parameters extracted from the hypnogram, among which percentage time spent in each of the sleep stages per 30-min period and per rat. This gives for each compound a profile of changes over sleep stages and over time.

Evaluation

Sleep stage-dependent and sleep-independent parts of the EEG power spectrum are defined by a procedure originally developed by Fairchild et al. (1969, 1971, 1975). First, a normal canonical discriminant analysis is done on 4 EEG frequency bands (1–3, 3–6, 6–9, 9.5–20 Hz) from representative segments of only 3 visually classified sleep stages (quiet waking, deep sleep and REM sleep), the sleep stage being the dependent variable. This results in 2 sleep stage-dependent canonical variables covering 100 % of the variance in the data set and two residual canonical variables which are independent of sleep stage assignment. These 2 residual variables are subsequently used in a second canonical discriminant analysis in which the presence or absence of the drug is used as the dependent variable, resulting in a single canonical variable (the drug score) associated with the drug effect on the sleep stage-independent variance of the EEG spectral parameters.

Critical Assessment of the Method

According to the author's own judgment, antidepressants, antipsychotics and stimulants can be discriminated from each other and from placebo successfully from each other and from placebo by this method, whereas nootropics classified as placebo. Unfortunately, anxiolytics, hypnotics and anticonvulsants are classified poorly.

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Neuroleptic Activity

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Contents

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General Considerations

Neuroleptics have been defined as therapeutics effective against schizophrenia. One has to bear in mind that the effect of certain drugs has not been predicted by pharmacological tests but has been found in clinical trials by serendipity. The clinical discoveries were followed by pharmacological studies in many laboratories (Courvoisier 1956).

Various studies have demonstrated the blockade of postsynaptic catecholamine receptors, especially D_2-receptors, to be the main mode of action of most neuroleptics. Several in vitro methods measure the receptor blockade by neuroleptics.

Pharmacological models in the development of antipsychotic drugs were reviewed by Costall et al. (1991).

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In Vitro Methods

D_1 Receptor Assay: [^{3}H]-SCH 23390 Binding to Rat Striatal Homogenates

Purpose and Rationale

Dopamine receptors are the primary targets in the development of drugs for the treatment of schizophrenia, Parkinson's disease, and Huntington's chorea (Seeman and Van Tol 1994).

Reviews on dopamine receptors and their subtypes were given by Baldessarini and Tarazi (1996; Missale et al. 1998) and by the NC-IUPHAR subcommittee on dopamine receptors (Schwartz et al. 1998).

Multiple dopamine receptors are known. Two groups are most studied, designated as D_1 and D_2. In the group of D_1-like dopamine receptors, the subtypes D_{1A} and D_5/D_{1B} have been described. To D_2-like dopamine receptors belong the D_{2S}, the D_{2L}, the D_3, and the D_4 receptor (Sokoloff et al. 1990; Civelli et al. 1991; Grandy et al. 1991; Van Tol et al. 1991; Lévesque et al. 1992; Baldessarini et al. 1993; Ginrich and Caron 1993; Todd and O'Malley 1993; Waddington and Deveney 1996).

D_1 receptors are positively linked to adenylate cyclase, and the D_2 receptor has been shown to be negatively linked to adenylate cyclase. For typical neuroleptic agents, like butyrophenones, a good correlation was found between D_2 receptor binding and clinically effective doses. Atypical neuroleptics, like clozapine, were found to be potent inhibitors of D_1 and D_4 receptor binding, renewing interest in these receptor types. The compound SCH 23390 was found to be selective for the D_1 receptor.

Procedure

Reagents

[*N*-Methyl-^{3}H] Sch 23390 (Amersham Lab., specific activity 67–73 Ci/mmol). For IC_{50} determinations, ^{3}HSch 23390 is made up to a concentration of 10 nM and 50 µl is added to each tube. This yields a final concentration of 0.5 nM in the assay.

d-Butaclamol (Ayerst Laboratories). A 1 mM stock solution is made and diluted 1:20.

20 µl are added to three tubes for the determination of nonspecific binding.

For the test compounds α, 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assays ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, striata dissected, and weighed. The striata are homogenized in 100 volumes of 0.05 M Tris buffer, pH 7.7, using a Tekmar

homogenizer. The homogenate is centrifuged at 40,000 g for 20 min, and the final pellet is resuspended in the original volume of 0.05 Tris buffer, pH7.7, containing physiological ions (NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM, and $CaCl_2$ 2 mM).

Assay

50 µl	0.5 M Tris buffer, pH7.7, containing physiological ions
380 µl	H_2O
20 µl	Vehicle or butaclamol or appropriate concentration of test compound
50 µl	^{3}H-SCH 23390
500 µl	Tissue suspension

The tubes are incubated at 37 °C for 30 min. The assay is stopped by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. The filter strips are then washed three times with ice-cold 0.05 M Tris buffer, pH7.7, and counted in 10 ml Liquiscint scintillation cocktail.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 1 µM butaclamol. IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the average of duplicate determinations.

Modifications of the Method

Wamsley et al. (1992) recommended the radioactive form of a dopamine antagonist, [^{3}H] SCH39166, as ligand for obtaining selective labeling of D_1 receptors.

Sugamori et al. (1998) characterized the compound NNC 01–0012 as a selective and potent D_{1C} receptor antagonist.

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D_2 Receptor Assay: [^{3}H]-Spiroperidol Binding

Purpose and Rationale

The neuroleptic compound haloperidol has been used as binding ligand to study the activity of other neuroleptics. The use of haloperidol has been superseded by spiroperidol. Dopamine receptor binding assays employing dopaminergic antagonists in mammalian striatal tissue, a dopamine-enriched area of the brain, have been shown to be predictive of in vivo dopamine receptor antagonism and antipsychotic activity. Significant correlations exist between neuroleptic binding affinities and their molar potencies in antagonism of apomorphine- or amphetamine-induced stereotypy, apomorphine-induced emesis in dogs, and antipsychotic activity in man. Spiroperidol is considered to be an antagonist specific for D_2 receptors.

Procedure

Tissue Preparation

Male Wistar rats are decapitated, their corpora striata removed, weighed, and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH7.7. The homogenate is centrifuged at 40,000 g for 15 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 40,000 g. The final pellet is then resuspended in Tris buffer containing physiological salts (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) resulting in a concentration of 10 mg/ml.

Assay

The membrane preparations are incubated with ^{3}H-spiroperidol (0.25 nM) and various concentrations of test drug at 37 °C for 20 min. in a K/Na phosphate buffer (50 mM, pH7.2), followed by

cooling in an ice bath for 45 min. To determine nonspecific binding, samples containing 10 mM (+)-butaclamol are incubated under identical conditions without the test compound.

Bound ligand is separated by rapid filtration through Whatman GF/B glass fiber filters. The filters are washed three times with ice-cold buffer, dried, and shaken thoroughly with 3.5 ml scintillation fluid. Radioactivity is determined in a liquid scintillation counter. Specific binding is defined as the difference between total binding and the binding in the presence of 2.0 mM (+)-butaclamol.

Evaluation

The following parameters are determined:

- Total binding of ^{3}H-spiroperidol
- Nonspecific binding: binding of samples containing 2 mM butaclamol
- Specific binding: total binding nonspecific binding
- Percent inhibition: 100-specific binding as percentage of the control value

IC_{50} values are determined using at least 3–4 different concentrations of the test compound in triplicate. Results are presented as mean ± standard deviation.

Dissociation constants (K_d) are determined, using ^{3}H-spiroperidol concentrations ranging between 0.1 and 1.0 nM. *K*i values (inhibitory constants) are calculated using the following equation:

$$K_i = \frac{IC_{50}}{1 + c/K_d}$$

c = ^{3}H-spiroperidol concentrations used to determine IC_{50}.

Standard values: K_i of haloperidol = 6.0 ± 1.2 nM.

Modifications of the Method

Two isoforms of the D_2 receptor were found by alternative splicing: the long (D_{2L}) and the short (D_{2S}) isoform (Dal Toso et al. 1989; Giros et al. 1989; Monsma et al. 1989; Itokawa et al. 1996).

Niznik et al. (1985) recommended [^{3}H]-YM-09151–2, a benzamide neuroleptic, as selective ligand for dopamine D2 receptors.

Hall et al. (1985) used [^{3}H]-eticlopride, a substituted benzamide, selective for dopamine D_2 receptors, for in vitro binding studies.

Radioactive ligands for the D_2 and the D_3 receptor were described by Seeman and Schaus (1991), Chumpradit et al. (1994), Booze and Wallace (1995), Gackenheimer et al. (1995), Seeman and van Tol (1995), and Van Vliet et al. (1996).

Vessotskie et al. (1997) characterized binding of [^{125}I]S(−)5-OH-PIPAT to dopamine D_2-like receptors.

Neve et al. (1992) used a special apparatus, the "cytosensor microphysiometer," which measures the rate of proton excretion from cultured cells (McConnell et al. 1991, 1992; Owicki and Parce 1992). In C_6 glioma cells and L fibroblasts expressing recombinant dopamine D_2 receptors, the dopamine D_2 receptor agonist, quinpirole, accelerated the rate of acidification of the medium dose-dependent up to 100 nM quinpirole. The response was inhibited by the D_2 antagonist spiperone. The D_2 receptor-stimulated acidification was due to transport of protons by a Na^+/H^+ antiporter which was verified by the inhibition with amiloride or methylisobutyl amiloride.

The isolated rabbit ear artery was recommended as a useful model to characterize dopamine D_2 agonists and antagonists (Hieble et al. 1985).

Human Recombinant Dopamine D_{2A} and D_{2B} Receptors

Hayes et al. (1992) described functionally distinct human recombinant subtypes of the dopamine D_2 receptor, D_{2A} and D_{2B}.

D_{2A} Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2A} receptor is measured.

Twenty mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 2.0 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 mM haloperidol. Membranes

are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

D_{2B} Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2B} receptor is measured.

Fifteen mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 37 °C. Nonspecific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

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Dopamine D_2 Receptor Autoradiography (^{3}H-Spiperone Binding)

Purpose and Rationale

Autoradiography of ^{3}H-spiperone binding sites using selective labeling conditions permits the visualization of the anatomical locations of D_2-dopamine receptors (Palacios et al. 1981). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Altar et al. 1984; 1985). Using autoradiographic techniques, it has been demonstrated that striatal D2 receptors are present on intrinsic neurons (Trugman et al. 1986; Joyce and Marshall 1987) and that the distribution of D_2 receptors within the striatum is not homogeneous (Joyce et al. 1985). Anatomically discrete interactions of drugs with D_2 receptors can be examined in vitro with inhibition experiments and ex vivo following acute or chronic drug treatment of the whole animal.

Since ^{3}H-spiperone labels serotonin-2 (5-HT_2) sites in many brain regions, a masking concentration of a 5-HT_2 receptor blocker, e.g., ketanserin, is included to selectively define binding to D_2 receptors. This is necessary if the test compound

inhibits 5-HT_2 binding or if the brain region of interest has a low D_2 receptor density.

The assay is used to determine potential antipsychotic activity of compounds via direct interaction with the D_2 dopamine recognition site in discrete regions of the rat brain.

Procedure

Reagents

1a. 0.5 M Tris + 1.54 M NaCl, pH7.4.

1b. 0.05 M Tris + 0.154 M NaCl, pH7.4.

2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham (TRK.818).

   For IC_{50} determinations, ^{3}H-spiperone is prepared at a concentration of 8 nM, and 0.55 ml is added to each slide mailer (yields a final concentration of 0.4 nM in the 11.0 ml assay volume).

   For saturation experiments, ^{3}H-spiperone is prepared at a concentration of 20 nM. The final concentrations should range from 0.2 to 1.0 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).

3. Sulpiride is obtained from sigma. A stock solution of 5×10^{-4} M is made by dissolving the sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 10 μM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).

4. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin in 0.5 ml 1 N acetic acid and bringing the final volume to 10 ml with distilled water. The tartrate salt is water-soluble. This is further diluted to 5×10^{-6} M (50 μl q.s. to 10 ml). 0.22 ml is added to all mailers.

5. Test compounds (for IC_{50} determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Rat brain sections are collected from plates 9 (rostral nucleus accumbens) through plate 17 (caudal striatum) of *The Rat Brain Atlas in Stereotaxic Coordinates* by Paxinos and Watson.

1. For in vitro inhibition experiments, 3–5 sets of 10 slides are collected with 3–4 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 3–4 sections per slide.
3. For ex vivo inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer).

   Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover two sections. A proportional adjustment of the volumes to be pipetted is made.

   (a) In vitro inhibition experiments

   Separate mailers are prepared for total binding, nonspecific binding, and 7–8 concentrations of test compound. Ketanserin is included in all mailers to mask binding of [^{3}H]-spiperone to 5-HT_2 sites so that inhibition of binding is D_2-selective.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 0.4 nM final concentration
3.96 ml	Distilled water
0.22 ml	Ketanserin, 5×10^{-6} M, final concentration 100 nM or vehicle
0.22 ml	Test compound, final concentration 10^{-8} to 10^{-5} M or sulpiride 5×10^{-4}, final conc. 10 μM or vehicle

(b) Ex vivo inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including ketanserin to mask 5-HT_2 receptor binding.

(c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Ketanserin is not included in the mailers, in saturation experiments, since specific binding is defined as sulpiride-displaceable.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[3H]-spiperone, final concentration 0.2–1.0 nM
4.18 ml	Distilled water
0.22 ml	5 × 10–4 M sulpiride, final concentration 10 μM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH7.4 for 5 min, and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, rinsed in buffer 1b for 2 × 5 min, and dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter, and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually over night). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrofilm for 14–17 days.

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Binding to the D_3 Receptor

Purpose and Rationale

Sokoloff et al. (1990) reported molecular cloning and characterization of a dopamine receptor (D_3) as a potential target for neuroleptics. The D_3 receptor is localized in limbic areas of the brain which are associated with cognitive, emotional, and endocrine functions. Together with the D_{2S}, the D_{2L}, and the D_4 receptor, the D_3 receptor belongs to the group of D_2-like dopamine

receptors (Ginrich and Caron 1993). 7-[^{3}H] hydroxy-*N*,*N*-di-n-propyi-2-aminotetralin (Lévesque et al. 1992), R(+)-7-OH-DPAT (Baldessarini et al. 1993), and [^{125}I]trans-7-OHPIPAT-A (Kung et al. 1993) have been recommended as ligands for receptor binding studies.

Chio et al. (1993) compared the heterologously expressed D_3 dopamine receptors with D_2 receptors in Chinese hamster ovary cells.

Damsma et al. (1993) described R-(+)-7-OH-DPAT (R-(+)-7-hydroxy-2-(*N*,*N*-di-n-propylamino)tetralin) as a putative dopamine D_3 receptor ligand.

Functional correlates of dopamine D_3 receptor activation in the rat in vivo and their modulation by the selective agonist, (+)-S 14297, have been described by Millan et al. (1995).

Isoforms of the D_3 receptor have been described (Pagliusi et al. 1993).

Akunne et al. (1995) described binding of the selective dopamine D_3 receptor agonist ligand [^{3}H]PD 128907 = 4aR,10bR-(+)-trans-3,4,4a,10b-tetrahydro-4-n-propyl-2H,5H-[1] benzopyrano[4,3-b]1,4-oxazin-9-ol.

Procedure

Human dopamine D_3 receptor is expressed in Chinese hamster ovary cells. Cells are grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum. Cells are harvested by trypsin treatment (0.25 %) for 4–5 min and centrifugation at 2000 *g* for 5 min. They are homogenized with a Polytron in 10 mM Tris–HCl (pH7.5) containing 1 mM EDTA and are centrifuged at 35,000 *g* for 15 min. The pellet is then resuspended by sonication in a buffer containing 50 mM NaHepes, 1 mM EDTA, 50 µM 8-hydroxyquinoline, 0.005 % ascorbic acid, and 0.1 % bovine serum albumin (pH7.5) (incubation buffer). Membrane suspensions (15–25 µg protein) are added to polypropylene test tubes containing [^{3}H]7-OH-DPAT (7-[^{3}H] hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin) for the D_3 receptor assay. Competing drugs are dissolved in incubation buffer, the final volume being 1 ml. Tubes are incubated in triplicate for 1 h at room temperature. The incubations are stopped by rapid filtration under reduced pressure through Whatman GF/C glass filters coated with 0.1 % bovine serum albumin, followed by three rinses with 3–4 ml ice-cold buffer. Nonspecific binding is measured in the presence of 1 µM dopamine.

Evaluation

Saturation curves are analyzed by computer nonlinear regression using a one-site cooperative model to obtain equilibrium dissociation constants (K_D) and maximal density of receptors (B_{max}). Inhibition constants (K_i) are estimated according to the equation

$$K_i = IC_{50}/1 + L/K_D$$

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Binding to D_4 Receptors

Purpose and Rationale

Van Tol et al. (1991) reported cloning of the gene of a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. Together with the D_{2S}, the D_{2L}, and the D_3 receptor, the D_4 receptor belongs to the group of D_2-like dopamine receptors (Ginrich and Caron 1993). Recognition and characterization of this dopamine binding site may be useful in the design of new types of antipsychotic drugs.

Dopamine D_4 receptors have been localized in GABAergic neurons of the primate brain (Mrzljak et al. 1996).

Procedure

A plasmid construct of a 3.9-kb gene-cDNA hybrid subcloned into the expression vector pCD-PS is introduced into COS-7 cells by calcium phosphate-mediated transfection. Cells are cultivated and homogenized (Teflon pestle) in 50 mM Tris–HCl (pH7.4 at 4 °C) buffer containing 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM KCl, and 120 mM NaCl. Homogenates are centrifuged for 15 min at 39,000 g, and the resulting pellets resuspended in buffer at a concentration of 150–250 μg/ml. For saturation experiments, 0.25 ml of tissue homogenate are incubated in duplicate with increasing concentrations of [^{3}H]-spiperone (70.3 Cl mmol^{-1}; 10–3000 pM final concentration) for 120 min at 22 °C in a total volume of 1 ml. For competition binding experiments, assays are initiated by the addition of 0.25 ml membrane and incubated in duplicate with various concentrations of competing ligands (10^{-14}–10^{-3} M) and [^{3}H]spiperone (150–300 μM) either in the absence or the presence of 200 μM Gpp(NH)p for 120 min at 22 °C. Assays are terminated by rapid filtration through a Titertek cell harvester and filters then monitored for tritium. For all experiments, specific binding is defined as that inhibited by 10 μM (−)sulpiride.

Evaluation

Both saturation and competition binding data are analyzed by the nonlinear least-square curve-fitting program ligand run on a suitable PC.

Modifications of the Method

Human Recombinant Dopamine $D_{4,2}$, $D_{4,4}$, $D_{4,7}$, and D_5 Receptors

Van Tol et al. (1992) described multiple dopamine D_4 receptor variants in the human population.

Sunahara et al. (1991) reported the cloning of the gene for a human D_5 receptor.

Human Recombinant Dopamine $D_{4,2}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,2}$ receptor is measured.

Fifteen µg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine $D_{4,4}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,4}$ receptor is measured.

Twenty-five µg of membrane are incubated with [^{3}H]-spiperone at a concentration of 1.0 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine $D_{4,7}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine $D_{4,7}$ receptor is measured.

Fifteen µg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine D_5 Receptor

In a radioligand binding assay, the binding of [^{3}H]SCH 23390 to membranes prepared from COS cells expressing a recombinant human dopamine D_5 receptor is measured.

First, 40 µg of membrane is incubated with [^{3}H]SCH 23390 at a concentration of 2 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 µM *cis*-flupentixol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]SCH 23390 bound.

Several selective dopamine D_4 antagonists were described: Hidaka et al. (1996), Merchant et al. (1996), Rowley et al. (1996), and Birstow et al. (1997).

Some radioligands were proposed as being selective for dopamine D_4 receptors: [^{3}H]clozapine (Ricci et al. 1997a, b), [^{3}H]NGD 94–1 (Thurkauf 1997; Primus et al. 1997), and RBI-257 (Kula et al. 1997).

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Determination of Dopamine Autoreceptor Activity

Purpose and Rationale

The method describes the procedure to determine if a compound possesses autoreceptor blocking activity without the interference from postsynaptic effects. Striatal DOPA (3,4-dihydroxyphenylalanine), DOPAC (3,4-dihydroxyphenylacetic acid), and DA (dopamine) are quantitated following in vivo treatment with drug, apomorphine, gamma butyrolactone, and NSD-1015. Antipsychotic compounds that block striatal dopaminergic presynaptic autoreceptors are believed to possess a greater liability for producing EPS.

Procedure

Reagents

1. 0.1 M HCl
2. 1 N NaOH
3. 0.1 M perchloric acid (PCA) containing 4.3 mM EDTA
4. 2 mM solutions of DOPAC, DA, and DOPA in 0.1 M HCl, with 0.5 ml aliquots stored at −60 °C until use
5. Preparation of 2° standard mixture

   10 μM solution of DOPAC, DA, and DOPA diluted from reagent 4 with 0.1 M PCA/EDTA

   The 2° standard solution is used for the preparation of standard curves.

6. Mobile phase/MeOH-buffer (4: 96, v/v) buffer: 0.012 mM sodium acetate, 0.036 M citric acid, and 152 µM sodium octane sulfonate (mobile phase); methanol/buffer (80 ml + 1920 ml) filtered through a 0.2 pm nylon 66 filter
7. Preparation of dosing solutions
   (a) Apomorphine (2 mg/kg) is prepared in saline containing 1 % Tween 80 + 0.1 % ascorbic acid to prevent oxidation.
   (b) GBL (750 mg/kg) is prepared as a solution in saline containing 1 % Tween 80.
   (c) NSD-1015 (100 mg/kg) is prepared as a solution in saline containing 1 % Tween 80.

HPLC-Instrumentation

Consists of the following:

- Pump, model SP8810 (Spectra Physics)
- Injector, WISP 710B (Waters Associates)
- Detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA)
- Integrator, D-2000 (Hitachi), used as a backup for the data collection/integrator, CS 9000 (IBM) system
- Analytical column: C18-ODS Hypersil, 3 pm, 100 × 4.6 mm (Shandon)

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation at the predetermined time. The brain is rapidly removed; the striatum is dissected on ice and frozen on dry ice. The tissue is analyzed by HPLC the same day.

Tissue is homogenized in 500 µl 0.1 M PCA/EDTA. The homogenate is centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatant is transferred to 0.2 pm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

Five µl of the striatum homogenate is injected into the HPLC column.

HPLC flow rate is 1.5 ml/min; run time is 20 min. Helium flow is constant in mobile phase.

For protein analysis, 1.0 ml 1 N NaOH is added to the tissue pellet. The next day, the protein analysis is performed as described by Bradford (1976) using the BioRad Assay Kit.

Evaluation

Peak area is used for quantitation. The mg of protein and pmoles of DOPAC, DA, and DOPA are calculated from linear regression analyses using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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Dopamine-Sensitive Adenylate Cyclase in Rat Striatum

Purpose and Rationale

Agonist stimulation of dopamine D_1 receptors leads to increased cAMP formation mediated by a guanine nucleotide-binding regulatory protein. This effect is blocked by selective antagonists like SCH 23390.

Agonist stimulation of the dopamine D_2 receptor leads to a decreased cAMP formation mediated by a guanine nucleotide-binding protein. Apomorphine is a potent agonist with full intrinsic activity at D_2 receptors. Phenothiazines block both D_1 and D_2 receptors, whereas butyrophenones and related drugs are very potent antagonists at D_2 receptors.

Studies on cAMP formation may be useful for differentiation of antipsychotic drugs.

Procedure

Tissue Preparation

Male Wistar rats are sacrificed by decapitation, the brains removed, and the striata dissected out, and weighed. Striatal tissue from two rats is homogenized in 25 volumes of ice-cold 0.08 M Tris-maleate buffer, pH7.4, containing 2 mM EGTA. Protein content of an aliquot is determined. A 50 µl aliquot is used in the cyclase enzyme assay.

Enzyme Assay

The following volumes are placed in conical centrifuge tubes kept in an ice-water bath:

200 µl	Incubation buffer (equal amounts of 0.8 mM Tris-maleate, pH 7.4; 60 mM $MgSO_4$; 100 mM theophylline and 4 mM EGTA)
50 µl	1 mM dopamine HCl or water
25 µl	Test drug or water
125 µl	Distilled water
50 µl	Tissue homogenate

After incubation for 20 min at 0 °C, the enzyme reaction is started by addition of 50 µl of 15 mM ATP solution. The tube rack is placed in a shaking water bath preset at 30 °C for 2.5 min. The reaction is terminated by placing the tube rack in a boiling water bath for 4 min. Then, the tubes are centrifuged at 1000 g for 10 min.

A 25 µl aliquot of the supernatant in each tube is removed and the cAMP determined using a commercial RIA kit (Amersham).

Evaluation

Results are expressed as pmoles cAMP/mg protein of dopamine-stimulated versus nondopamine-stimulated level. Percentage inhibition of this dopamine-stimulated level by test drugs is calculated and IC_{50} values determined by log-probit analysis.

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α_1-Adrenergic Receptor Binding in Brain

Purpose and Rationale

The use of neuroleptic and antidepressant drugs is sometimes limited by their side effects, such as orthostatic hypotension and sedation. These side effects are attributed to blockade of central and peripheral adrenergic α-receptors. For neuroleptics the ratio between their dopamine antagonistic and their receptor antagonistic potencies should be taken into account rather than their absolute α-blocking effect. WB-4101 is a specific and potent antagonist of the α_1-adrenoreceptor, characterized in vitro in rat brain, heart, vascular smooth muscle, and gastrointestinal smooth muscle.

The in vitro [^{3}H]-WB 4101 receptor binding assay quantitates the α-adrenergic blocking properties of psychoactive agents and is used to assess a compound's potential to cause orthostatic hypotension and sedation as well as primary blood pressure lowering effects through α_1-receptor blockade.

Procedure

Reagents

[Phenoxy-3-^{3}H(N)]-WB 4101 = (2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane, New England Nuclear (specific activity 20–35 Ci/mmol).

For IC_{50} determinations, [^{3}H]-WB 4101 is made up to a concentration of 2 nM in Tris buffer and 500 µl is added to each tube (yields a final concentration of 0.5 nM in the 2 ml assay).

L-norepinephrine bitartrate (Sigma Chemical Company). A 800 µM solution is prepared in Tris buffer and 250 µl is added to each of three tubes to determine nonspecific binding. This yields a final concentration of 100 µM in the 2 ml assay.

Test compounds: A 80 µM stock solution is made up in a suitable solvent and serially diluted with Tris buffer, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Usually, seven concentrations are studied for each assay.

Tissue Preparation

Male Wistar rats (100–150 g) are sacrificed by decapitation. The whole brain minus cerebellum is homogenized in 75 volumes of ice-cold 0.05 M Tris buffer, pH7.7. The homogenate is centrifuged at 40,000 g at 4 °C for 15 min. The supernatant is discarded and the pellet is rehomogenized in fresh Tris buffer and recentrifuged at 40,000 g at 4 °C for 15 min. The final pellet is resuspended in the original volume of ice-cold 0.05 M Tris buffer. The final tissue concentration in the assay is 10 mg/ml. Specific binding is approximately 80 % of total bound ligand.

Assay

1200 µl	Tissue suspension
500 µl	3H-WB 4101
250 µl	Vehicle (for total binding) or
800 µM	L-norepinephrine bitartrate (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept in ice for additions, then vortexed, and incubated for 15 min at 25 °C. The binding is terminated by rapid vacuum filtration through Whatman GF/B filters, followed by three 5 ml washes with ice-cold 0.05 M Tris buffer. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation

Specific WB 4101 binding is defined as the difference between the total binding and that bound in the presence of 100 µM norepinephrine. IC_{50} calculations are performed using computer-derived log-probit analysis.

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[^{3}H]Spiroperidol Binding to 5-HT$_2$ Receptors in Rat Cerebral Cortex

Purpose and Rationale

The purpose of this assay is to determine the antiserotonin activity of neuroleptics, antidepressants, and antihypertensive compounds, by measuring the displacement of [^{3}H]spiroperidol from serotonergic antagonist binding sites in cerebral cortical membranes. The regulation of 5-HT2 receptor density by chronic antidepressant treatment is discussed in a separate protocol (see chapter "► Antidepressant Activity").

The receptor binding of serotonergic sites in the CNS has been investigated using [^{3}H]serotonin (5-HT) (Bennett and Snyder 1976), [^{3}H]LSD (Peroutka and Snyder 1979), and [^{3}H] spiroperidol (Peroutka and Snyder 1979; List and Seeman 1981; Leysen et al. 1978) as the radioligand. Receptor sites have been defined kinetically and classified as 5-HT$_1$ sites (labeled by [^{3}H]5-HT and displaced by agonists) and 5-HT$_2$ sites (labeled by [^{3}H]-spiroperidol and displaced by antagonists). [^{3}H]LSD labels both 5-HT1 and 5-HT2 binding sites (Peroutka and Snyder 1979). Of the brain regions tested, the frontal cerebral cortex contained the greatest density of 5-HT2 binding sites. Lesioning studies indicate that 5-HT$_2$ binding sites are postsynaptic and not linked to adenyiate cyciase (Peroutka et al. 1979).

The inhibition of 5-HT$_2$ binding correlates with the inhibition of quipazine-induced head twitch, which may reflect decreased behavioral excitation. The physiological and pharmacological role of these receptors is not clear. Although numerous neuroleptics and antidepressants of varying chemical structures are potent inhibitors of 5-HT$_2$ binding, there is no clear-cut relationship to the efficacy of these drugs. Methysergide and cyproheptadine are both potent inhibitors of 5-HT$_2$ binding without having neuroleptic or antidepressant effects. However, potent interaction with 5-HT$_2$ receptors may indicate a reduced potential for catalepsy, since methysergide blocks catalepsy induced by haloperidol (Rastogi et al. 1981). The interaction of serotonergic neurons with cholinergic neurons in the striatum (Samanin et al. 1978) may also be decreased by potent 5-HT2 antagonists. In addition, the ratio of activity at D2 and 5-HT2 receptors may be useful in the screening of atypical antipsychotic agents (Meltzer et al. 1989). Furthermore, it has been shown that ketanserin, a selective 5-HT$_2$ antagonist, is an effective hypotensive agent which blocks peripheral vascular 5-HT receptors.

5-HT$_2$ receptors have been subdivided into 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. The new 5-HT receptor classification has been

published by the VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin) (Hoyer et al. 1994). Further comments were given by Humphrey et al. (1993), Martin and Humphrey (1994), Saxena (1994), and Tricklebank (1996).

Several compounds with HT_{2A} antagonistic activity are described, such as trazodone (Clements-Jewery et al. 1980; Hingtgen et al. 1984; Stryjer et al. 2003), MDL 100,907 (Kehne et al. 1996; Moser et al. 1996), and sarpogrelate (Hayashi et al. 2003).

McCullough et al. (2006) described the 5-HT_{2B} antagonist and 5-HT_4 agonist activities of tegaserod in the anesthetized rat.

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.7
   (a) 57.2 g Tris–HCl.
   16.2 g Tris base
   q.s. to 1 l (0.5 M Tris buffer, pH7.7)
   (b) Make a 1:10 dilution in distilled H_2O (0.05 M Tris buffer, pH7.7).
2. Tris buffer containing physiological ions
   (a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g

   q.s. to 100 ml in 0.5 M Tris buffer.
   (b) Dilute 1:10 in distilled H_2O.
   (c) This yields 0.05 M Tris–HCl, pH7.7; containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (1 mM).
3. [Benzene-^{3}H] spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations, ^{3}H-spiroperidol is made up to a concentration of 30 nM in 0.01 N HCl and 50 µl added to each tube (yields a final concentration of 1.5 nM in the 1 ml assay).
4. Methysergide maleate is obtained from Sandoz. Methysergide maleate stock solution is made up to 0.25 mM for determination of nonspecific binding. The final concentration in the assay is 5 µM, when 20 µl of the stock solution is added to the reaction tube.
5. Test compounds. For most assays, α 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay, and higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated, and the cerebral cortical tissue is dissected, weighed, and homogenized in 50 volumes of 0.05 M Tris buffer, pH 7.7 (buffer 1b) with the Brinkman Polytron and then centrifuged at 40,000 *g* for 15 min. The supernatant is discarded and the pellet resuspended and recentrifuged as described above. This pellet is resuspended in 50 volumes of buffer 2b and stored in an ice bath. The final tissue concentration is 10 mg/ml. Specific binding is 7 % of the total added ligand and 50 % of total bound ligand.

Assay

50 µl	0.5 M Tris-physiological salts (buffer 2a)
380 µl	H_2O
20 µl	Vehicle (for total binding) or 0.25 mM methysergide (for nonspecific binding) or appropriate drug concentration
50 µl	[^{3}H] spiroperidol
500 µl	Tissue suspension

The samples are incubated for 10 min at 37 °C and then immediately filtered under reduced pressure using Whatman GF/B filters. The filters are washed with three 5 ml volumes of ice-cold 0.05 M Tris buffer, pH 7.7 mM methysergide.

Evaluation

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

Modification of the Method

The receptor binding properties of the 5-HT_2 antagonist ritanserin were reported by Leysen et al. (1985).

Preclinical characterization of a putative antipsychotic as a potent 5-HT_{2A} antagonist was reported by Kehne et al. (1996).

Using [^{125}I]LSD and [^{3}H]5-HT binding assays, Siegel et al. (1996) characterized a structural class of 5-HT_2 receptor ligands.

[^{3}H]Ketanserin has been described as a selective ^{3}H-ligand for 5-HT_2 receptor binding sites (Leysen et al. 1981).

[^{3}H]RP 62203, a potent and selective 5-HT_2 antagonist, was recommended for in vivo labeling of 5-HT_2 receptors (Fajolles et al. 1992).

Other selective 5-HT_2 receptor radioligands were recommended:

[^{125}I]-EIL (radioiodinated D-(+)-N1-ethyl-2-iodolysergic acid diethylamide) (Lever et al. 1991); [^{3}H]MDL100,907 (Lopez-Gimenez et al. 1998).

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Serotonin 5-HT_2 Receptor Autoradiography (^{3}H-Spiperone Binding)

Purpose and Rationale

Autoradiography of ^{3}H-spiperone binding sites with selective labeling conditions permits the visualization of the anatomical locations of 5-HT_2 receptors (Palacios et al. 1981; Pazos et al. 1985; Altar et al. 1985). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Pazos et al. 1985; Altar et al. 1984). Using autoradiographic techniques, it has been demonstrated that there is a heterogeneous distribution of 5-HT_2 receptors, with much higher levels in telencephalic areas such as the neocortex and the claustrum than in meso- or metencephalic areas. Within the cortex, 5-HT_2 receptors are abundant in layers IV and V (Pazos et al. 1985). The high concentration of 5-HT_2 receptors in the frontoparietal motor area and the claustrum which connects to the motor cortex and other motor areas suggests a physiological role for 5-HT_2 receptors in some motor syndromes (Cadet et al. 1987; Costall et al. 1975; Kostowski et al. 1972). The high affinity of the atypical antipsychotic clozapine for 5-HT_2 receptors (Fink et al. 1984; Altar et al. 1986) and the downregulation of 5-HT_2 receptors following chronic administration of clozapine (Reynolds et al. 1983; Lee and Tang 1984; Wilmot and Szczepanik 1989) suggest that 5-HT_2 receptor interaction may be a significant factor in the lack of extrapyramidal side effects and tardive dyskinesias with its clinical use.

Since^3 H-spiperone labels a_1-noradrenergic sites in the cerebral cortex, a masking concentration of the a_1-blocker prazosin is included to selectively define binding to 5-HT_2 receptors (Morgan et al. 1984). This is necessary if the test compound also inhibits *a*1receptors which may be present in the brain region of interest.

The assay is used to determine the direct interaction of potential antipsychotic compounds with the serotonin-5-HT_2 recognition site in discrete regions of the rat brain either in vitro or after ex vivo treatment of the whole animal.

Procedure

Reagents

1a. 0.5 M Tris + 1.54 M NaCl, pH7.4.

1b. 0.05 M Tris + 0.154 M NaCl, pH7.4.

2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham.
   - For IC_{50} determinations, ^{3}H-spiperone is made up to a concentration of 20 nM, and 0.55 ml is added to each slide mailer (yields a final concentration of 1.0 nM in the 11.0 ml assay volume).
   - For saturation experiments, ^{3}H-spiperone is made up to a concentration of 20 nM. The final concentration should range from 0.5 to 2.5 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Methysergide is used to determine nonspecific binding in brain sections of the frontal cortex. Methysergide maleate is obtained from Sandoz. A stock solution of 2.5×10^{-4} M is

made by dissolving in distilled water. A volume of 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 5 μM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).

4. Ketanserin is used to determine nonspecific binding in those slide mailers containing sections with the nucleus accumbens and striatum. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin (free base) in 0.05 N acetic acid or the tartrate salt in distilled water. This is further diluted to 5×10^{-6} M (50 μM q.s 10 ml with distilled water). A volume of 0.22 ml is added to the slide mailers to give a final concentration of 100 nM.
5. Prazosin is used to mask α_1-receptors in cortical brain section.

   Prazosin HCl is obtained from Pfizer. A stock solution of 10^{-4} M is made by dissolving prazosin in 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. This is further diluted to 5×10^{-6} M (100 μM q.s 10 ml). A volume of 0.22 ml is added to those slide mailers to be used for cortical brain sections to give a final concentration of 100 nM.
6. Sulpiride is used to mask D_2 receptor binding in brain sections from the nucleus accumbens and striatum.

   Sulpiride is obtained from sigma. A stock solution of 10^{-4} M is made by dissolving sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. A volume of 0.22 ml is added to the appropriate slide mailers to give a final concentration of 10 μM.
7. Test compounds (for in vitro IC_{50} determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Frontal cortical brain sections are collected from plates 5 through 8, and nucleus accumbens/striatal sections are collected from plates 9 (rostral n. accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

1. For in vitro inhibition experiments, 3–5 sets of 10 slides are collected with 4–5 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 4–5 six sections per slide.
3. For ex vivo inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer).

   Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover two sections. A proportional adjustment of the volumes to be pipetted is made.

   (a) In vitro inhibition experiments

   Separate mailers are prepared for total binding, nonspecific binding, and 7–8 concentrations of test compound.

   1. For frontal cortical brain sections, prazosin is included in all mailers to mask the binding of [^{3}H]-spiperone to α_1-receptors, and nonspecific binding is defined with 5 μM methysergide.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 1.0 nM final concentration
3.96 ml	Distilled water
0.22 ml	Prazosin 5×10^{-6} M, final concentration 100 nM or vehicle
0.22 ml	Test compound, final concentration 10^{-8}–10^{-5} M or methysergide 2.5×10^{-4} M, final concentration 5 μM or vehicle

2. For brain sections with the nucleus accumbens and striatum in which there is negligible binding of [^{3}H]-spiperone to α_1-receptors, prazosin is not included. Since levels of 5-HT$_2$ receptors in these brain areas are low, 10 μM sulpiride is included in all mailers to mask the binding of [^{3}H]-spiperone to D$_2$ receptors.

   Ketanserin, final concentration of 100 nM, is used to determine nonspecific binding since methysergide has a weak affinity for D$_2$ receptors (IC_{50} approximately 1–5 μM).

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 1.0 nM final concentration
3.96 ml	Distilled water
0.22 ml	Sulpiride 5×10^{-4} M, final concentration 10 μM or vehicle
0.22 ml	Test compound, final concentration 10^{-8} to 10^{-5} M or ketanserin 5×10^{-5} M, final concentration 100 nM or vehicle

(b) Ex vivo inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including sulpiride to mask D$_2$ receptor binding with brain sections through the nucleus accumbens and striatum and prazosin to mask α_1-receptors in cortical brain sections.

(c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Prazosin is not included in the mailers in saturation experiments, since specific binding is defined by methysergide which has negligible affinity for α_1-receptors.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, final concentrations 0.5–2.5 nM
4.18 ml	Distilled water
0.22 ml	2.5×10^{-4} M methysergide, final concentration 5 μM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH7.4 for 5 min, and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, 2 × 5 min rinsed in buffer 1b, dipped in distilled water.

   Slides used for wipes: both sections are wiped with one Whatman GF/B filter, and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually overnight). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

   In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrofilm for 14–17 days.

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Binding to the Sigma Receptor

Purpose and Rationale

Sigma receptors, as a class of binding sites in the brain, were originally described as a subtype of the opiate receptors. Efforts to develop less addicting opiate analgesics led to the study of several benzomorphan derivatives which produce analgesia without causing the classical morphine-induced euphoria. Unfortunately, these compounds, like *N*-allylnormetazocine (SKF 10,047), produced a variety of psychotic symptoms. This psychotomimetic effect is thought to be mediated by sigma receptors. This binding site is sensitive to many neuroleptics, most notably the typical antipsychotic haloperidol, leading to the hypothesis that drug interactions with the sigma site may be a new approach for the discovery of novel antipsychotics which are not dopamine receptor antagonists. D_2 receptor antagonism is thought to be linked with the occurrence of extrapyramidal symptoms in the form of hyperkinesia and Parkinson symptoms or tardive dyskinesia, limiting the therapeutic use of traditional antipsychotic medication. It is hoped that ligands to the sigma receptor do not produce these adverse reactions. The sigma site is believed to be distinct from the binding site for the psychotomimetic drug phencyclidine.

Procedure

Reagents

(+)-SKF 10,047 is prepared as a stock solution of 5×10^{-3} M with distilled water. 130 µl added to the 6.5 ml assay yields a final concentration of 10^{-4} M.

^{3}H-(+)-SKF 10,047 (specific activity 40 Ci/mmol) is obtained from New England Nuclear. A 200 nM stock solution is made up with distilled water for IC_{50} determinations. 325 µl added to each tube yields a final concentration of 10 nM in the 6.5 ml assay.

Test Compounds

A 5 mM stock solution is prepared in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

The assay utilizes slide-mounted cross sections of brain tissue from male Hartley guinea pigs. Whole brain sections of 10 pm thickness are obtained from the hippocampus, thaw-mounted onto gel-chrome alum subbed slides, freeze-dried, and stored at −70 °C until use. On the day of the assay,

the sections are thawed briefly at room temperature until the slides are dry and then used in the assay at a final volume of 6.5 ml.

Assay

Incubation solutions are prepared in plastic slide mailer containers as follows:

3.250 ml	0.05 M Tris buffer, pH 7.7
2.470 ml	Distilled water
0.325 ml	0.5 M Tris buffer, pH 7.7
0.130 ml	(+)-SKF 10,047 or vehicle
0.325 ml	[^{3}H](+)-SKF 10,047

Dried slides with tissue sections are added to the slide mailers and incubated at room temperature for 90 min. Non-bound radioligand is removed by rinsing the slides sequentially in two 5-min rinses in ice-cold 0.05 M Tris buffer and a dip in ice-cold distilled water. The sections are either swabbed with Whatman GF/B filters for scintillation counting of tissue-bound radioligand or exposed to tritium-sensitive film for autoradiography of the binding sites.

Evaluation

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M (+)SKF 10,047 and is typically 60–70 % of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Modifications of the Method

[^{3}H]-(+)-pentazocine has been recommended as a highly potent and selective radioligand for μ receptors (de Costa et al. 1989; DeHaven-Hudkins et al. 1992).

Classification of sigma binding sites into α_1 and α_2 receptors has been proposed (Walker et al. 1990; Quirion et al. 1992; Abou-Gharbia et al. 1993).

Hashimoto and London (1993) characterized [^{3}H]ifenprodil binding to σ_2 receptors in rat brain.

Ganapathy et al. (1999) provided evidence for the expression of the type 1 sigma receptor in the Jurkat human T lymphocyte cell line.

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Simultaneous Determination of Norepinephrine, Dopamine, DOPAC, HVA, HIAA, and 5-HT from Rat Brain Areas

Purpose and Rationale

To measure the effects of potential antipsychotic drugs on catecholamines and indols, a quantitative method for the determination of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindolacetic acid (5HIAA), and 5-hydroxytryptamine (5-HT) from rat brain regions is used. These catecholamines and indols are measured in rat brain prefrontal cortex, nucleus accumbens, and striatum.

Procedure

Reagents

1. 0.1 M HCl.
2. 1 N NaOH.
3. 2 mM solutions of DOPAC, DA, and DOPA in 0.1 M HCl;
   0.5 ml aliquots are stored at −60 °C until use.
4. Preparation of 2° standard mixture
   – 10 μM solution of NE, DOPAC, DA, HVA, 5HIAA, and 5-HT (diluted from reagent 3) in mobile phase (reagent 5).

- The 2° standard solution is used for the preparation of standard curves.

5. Mobile phase/MeOH: buffer (7.5:92.5, v/v).
   - Buffer: 0.07 M sodium acetate, 0.04 M citric acid, 130 µM EDTA, and 230 µM sodium octane sulfonate
   - Mobile phase: methanol/buffer (150 ml + 1850 ml) is filtered through a 0.2 pm nylon 66 filter.

HMLC-Instrumentation

- Pump, model SP8810 (Spectra Physics).
- Injector, WISP 710B (Waters Associates).
- Detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA).
- Integrator, D-2000 (Hitachi), used as a backup for the data collection/integrator, CS 9000 (IBM) system.
- Analytical column: C18-ODS Hypersil, 3 pm, 100 × 4.6 mm (Shandon).

Animal Treatment

Six rats per group (150–250 g) are dosed with 4–5 different concentrations of the putative antipsychotic drug; usual concentrations range from 0.03 to 30 mg/kg. At a predetermined time, usually 60 min, the rats are sacrificed.

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation. The brain is rapidly removed and placed on ice. The striatum, nucleus accumbens, and prefrontal cortex are dissected and placed in 1.5 ml microcentrifuge tubes. The tubes are capped and immediately placed in dry ice. The frozen brain sections are stored at −60 °C until HPLC analysis.

Tissue is homogenized in mobile phase (striatum, in 600 µl, nucleus accumbens and prefrontal cortex, in 300 µl). The homogenates are centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatants are transferred to 0.2 pm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

The following volumes are injected to the HPLC column:

- Striatum, 5 µl; nucleus accumbens, 20 µl; prefrontal cortex, 50 µl.
- HPLC flow rate is 1.0 mi/min; run time is 25 min.

Helium flow is constant in mobile phase.

For protein analysis, 1 N NaOH is added to the tissue pellets as follows:

- Striatum: 1.0 ml
- Nucleus accumbens and prefrontal cortex: 0.5 ml

The next day, the protein analysis is run in duplicate with 5 µl of striatum and 20 µl of nucleus accumbens and prefrontal cortex as described by Bradford (1976) using the BioRad Assay Kit.

Evaluation

Peak area is used for quantitation. The mg of protein and pmoles of NE, DOPAC, DA, HVA, 5HIAA, and 5-HT are calculated from linear regression analysis using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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Measurement of Neurotransmitters by Intracranial Microdialysis

Purpose and Rationale

Methods to measure neurotransmitters and their metabolites in specific areas of the brain by microdialysis were introduced by Ungerstedt and his group (Ungerstedt et al. 1982; Zetterström et al. 1982, 1983; Zetterström and Ungerstedt 1983; Ungerstedt 1984; Stähle et al. 1991; Lindefors et al. 1989; Amberg and Lindefors (1989) and by Imperato and di Chiara 1984, 1985). In brain dialysis, a fine capillary fiber is implanted in a selected brain area. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into a physiological salt solution that flows through the capillary fiber at a constant rate. The fluid is collected and analyzed.

Procedure

Several designs of dialysis probes have been used (Santiago and Westerink 1990; Kendrick 1991):

1. *Horizontal Probe*

   A straight tube (Vita Fiber, 3 × 50 Amicon) with an outer diameter of 0.34 mm and a molecular weight cutoff of 50,000 is used. The outer surface of the tube is porous and can easily be sealed by epoxy which is applied by passing the tube through a droplet of epoxy and then through a narrow hole corresponding to the outer diameter of the tube. The wall of the tube is sealed in this way except for the area where the dialysis is intended to take place. The length of this region can be varied from 2 to 8 mm depending upon which structure of the brain will be perfused. During the coating and all other handling of the tube, it is supported by a thin tungsten or steel wire inserted into its lumen. One end of the tube is glued into a steel cannula (6 mm long, outer diameter 0.64 mm).

   Male Sprague Dawley rats weighing 250–300 g are anesthetized with halothane and held in a stereotactic instrument. The animals are maintained under halothane anesthesia during the entire experiment.

   Holes are drilled bilaterally (5.7 mm below and 1.5 mm in front of bregma) in the temporal bones after the temporal muscles have been retracted from the bones and folded away.

   During the implantation, the cannula is held by the micromanipulator of the stereotactic instrument, and the dialysis tube is passed horizontally through the brain through the holes drilled on both sides of the skull. A polyethylene tubing carrying the perfusion fluid is connected to the steel cannula. The perfusate is collected at the other end.
2. *Loop Probe*

   The probe is made of a flexible cellulosic tubing (Dow 50, outer diameter 0.25 mm).

Both ends of the tube are inserted into 0.64 mm diameter steel tubes, one of which is bent in an angle. A very thin microsuture (0.1 mm in diameter) is inserted into the tube and positioned half between the steel tubes. Before implantation, the tube is moistened and bent in such a way that the two steel tubes are held closely together in the micromanipulator of the stereotactic instrument. A tungsten wire is inserted into the straight steel tube and passed down the lumen of the dialysis tube in order to stretch it and make it rigid enough to be implanted into the brain. The tube is implanted vertically, and the steel cannulae are attached to the skull by dental cement. The tungsten wire is removed before starting the experiment. The cellulosic tube is flexible enough to withstand the bending at the lower end. The microsuture keeps the bend open.

Loop-shaped or U-shaped microdialysis probes have been used by several authors, e. g., Ichikawa and Meltzer (1990), Jordan et al. (1994), Westerink and Tuinte (1985), and Auerbach et al. (1994).

3. *Vertical Probe*

The probe is sealed at one end by epoxy. The other end is glued into a 0.64-mm-diameter steel tube. A thin inner cannula made of a steel tube or a glass capillary carries the fluid to the bottom of the dialysis tube where it leaves the inner capillary and flows upwards and leaves the probe by a lateral tube. This vertical probe can also be coated with epoxy. It is especially suited for reaching ventral parts of the brain and performing dialysis in small nuclei of the brain.

A similar device has been described for continuous plasma sampling in freely moving rats by Chen and Steger (1993).

Most of the commercially available microdialysis probes are based on this principle.

4. *Commercially Available Microdialysis Probes*

The microdialysis probes CMA/10, manufactured by Carnegie Medicine, Stockholm, Sweden, consist of a tubular membrane (polycarbonate; length: 3 mm; outside diameter: 0.50 mm; and inside diameter: 0.44 mm) glued to a cannula (outside diameter, 0.60 mm) and sealed with a glue at the tip (Stähle et al. 1991). The perfusion medium is carried to the dialyzing part of the probe by a thin cannula inside the probe. The medium leaves the inner cannula through two holes, flows back between the membrane and the inner cannula, and is collected at the outlet of the probe. The perfusion medium is delivered by means of a high precision microsyringe pump.

This probe was used by several authors, e. g., Wood et al. (1988), Benveniste et al. (1989), Rollema et al. (1989), Scheller and Kolb (1991), Wang et al. (1993), Kreiss and Lucki (1995), and Fink-Jensen et al. (1996).

CMA/11 probes were used by Boschi et al. (1995), Romero et al. (1996), and Gobert et al. (1997).

Dialysis fibers with a semipermeable membrane AN 69-HF, Hospal-Dasco, Bologna, Italy, were used by de Boer et al. (1994), Rayevsky et al. (1995), Arborelius et al. (1996), Gainetdinov et al. (1996), and Tanda et al. (1996).

Evaluation

Samples of the dialyzate are collected for different time intervals and analyzed for neurotransmitters. For the **evaluation of neuroleptics**, most authors measured dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC), and homovanillinic acid (HVA) by HPLC using appropriate detectors. See and Lynch (1996) analyzed dialysis samples for glutamate and GABA concentrations.

For the **evaluation of antidepressants**, the concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxy indole acetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), or noradrenaline (NA) were measured in the effluent by HPLC. Wood et al. (1988) and Egan et al. (1996) used 3-methoxytyramine accumulation as an index of dopamine release.

Critical Assessment of the Method

The results obtained from brain dialysis depend on at least three variables: type of probe, post-implantation interval, and whether

anesthetized or freely moving animals are used (Di Chiara 1990).

Several authors analyzed the diffusion processes underlying the microdialysis technique and described the limitations of the experiments (Jacobson et al. 1985; Amberg and Lindefors 1989; Benveniste et al. 1989; Scheller and Kolb 1991; Le Quellec et al. 1995).

As a matter of fact, brain microdialysis has been used for the evaluation of many drugs in various indications, such as:

- For neuroleptics by Ichikawa and Meltzer (1990), Meil and See (1994), Hernandez and Hoebei (1994), See et al. (1995), Schmidt and Fadayel (1995), Semba et al. (1995), Rayevsky et al. (1995), Fink-Jensen et al. (1996), See and Lynch (1996), Gainetdinov et al. (1996), Egan et al. (1996), and Klitenick et al. (1996)
- For **antidepressants** by de Boer et al. (1994), Jordan et al. (1994), Arborelius et al. (1996), Ascher et al. (1995), Auerbach et al. (1994), de Boer (1995, 1996), Casanovas and Artigas (1996), Gobert et al. (1997), Ichikawa and Meltzer (1995), Kreiss and Lucki (1995), Petty et al. (1996), Potter (1996), Romero et al. (1996), Sharp et al. (1996), and Tanda et al. (1996a, b)
- For studies in **Parkinson** models by Rollema et al. (1989) and Parsons et al. (1991)

Modifications of the Method

Ferraro et al. (1990) continuously monitored ethanol levels in the brain by microdialysis.

Hernandez and Hoebei (1994) performed simultaneous cortical, accumbens, and striatal microdialysis in freely moving rats.

Hegarty and Vogel (1995) assayed dopamine, DOPAC, and HVA in the brain of rats after acute and chronic diazepam treatment and immobilization stress.

Casanovas and Artigas (1996) implanted microdialysis probes simultaneously in six different brain areas of rats (frontal cortex, dorsal striatum, ventral hippocampus, dorsal hippocampus, dorsal raphe nucleus, median raphe nucleus).

Beneviste et al. (1984) determined extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis.

Boschi et al. (1995) showed that microdialysis of small brain areas in mice is feasible using the smallest commercially available probes.

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Use of Push–Pull Cannulae to Determine the Release of Endogenous Neurotransmitters

Purpose and Rationale

Originally reported by Gaddum (1961), the push–pull cannula has become recognized and utilized as a powerful tool in conjunction with sufficiently sensitive assays to measure low levels of neuroregulator release in distinct brain areas in vivo (Philippu 1984).

This method has been used for various purposes, e.g.:

- To perfuse the ventricles of the brain with drugs or to determine the release of labeled or endogenous compounds in the CSF (Bhattacharya and Feldberg 1958; Korf et al. 1976)
- To perfuse distinct brain areas with drugs and to study their effects on functions of the central nervous system (Myers et al. 1976; Bhargava et al. 1978; Ruwe and Myers 1978)
- To inject labeled monoamines or amino acids and to investigate the resting or induced release of radioactive compounds and their metabolites (Sulser et al. 1969; Strada and Sulser 1971; Kondo and Iwatsubo 1978)

- To perfuse distinct brain areas with labeled transmitter precursors and to determine the patterns of release of the newly synthesized transmitters (Philippu et al. 1974; Chéramy et al. 1977; Nieoullon et al. 1977; Gauchy et al. 1980)
- To perfuse distinct brain areas of anesthetized and conscious animals and to determine the release of endogenous neurotransmitters in the perfusate (Dluzen and Ramirez 1991)

Procedure

The superfusion of the hypothalamus of the conscious, freely moving rabbit has been described by Philippu et al. (1981) and Philippu (1984). Rabbits of both sexes are anesthetized with 40 mg/kg sodium pentobarbital i.p. Guide cannulae are mounted on a metal plate which is fixed on the skull with screws and dental cement. Some days after the operation, the guide cannulae are replaced with push–pull cannulae which are 4 mm longer than the guide cannulae, thus reaching the areas which are intended for superfusion. The push–pull cannulae are connected by tubing to two peristaltic pumps: one to push and another one to pull the fluid. The second pump is essential, because the superfusate is not directly collected from the side branch of the push–pull cannula but from tubing which is connected to the side branch. The superfusate is automatically collected every 10 s in fraction collectors.

Evaluation

The concentrations of neurotransmitters, e.g., epinephrine, norepinephrine, or dopamine, are determined with appropriate analytical methods (Wolfensberger 1984) before and after stimulation.

Modifications of the Method

Experiments in cats were described by Dietl et al. (1981) and in rats by Tuomisto et al. (1983).

The cortical cup technique for collection of neurotransmitters has been described by Moroni and Pepeu (1984).

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Fos Protein Expression in Brain

Purpose and Rationale

The proto-oncogene c-fos encodes a 55,000 mol wt, 380 amino acid phosphoprotein (FOS), which after translation in the cytoplasm reenters the nucleus and binds to DNA (Morgan and Curran 1989). C-fos induction can occur as a consequence of synaptic activation. An increase in fos immunoreactivity is associated with an increased metabolic demand on a neuron, i.e., a marker for neurons that is metabolically activated. Intermediate early genes such as c-fos have been tentatively classified or linked to third messengers, whose function is to produce a long-term effect on the recipient neuron.

Acute administration of antipsychotics induces c-fos expression in several areas of the rat forebrain as was shown with immunocytochemical methods (Dragunow et al. 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994). Fos protein is believed to act as an initiator of long-term cellular changes (neural plasticity) in response to a variety of extracellular stimuli, including drugs (Graybiel et al. 1990; Rogue and Vicendon 1992). Typical (e.g., haloperidol) and atypical (e.g., clozapine) neuroleptic drugs have different antipsychotic effects and side effects. A differential FOS-protein induction in rat forebrain regions after haloperidol and clozapine treatment was found (Deutch et al. 1992; Fibiger 1994; Fink-Jensen and Kristensen 1994; Merchant et al. 1994; Sebens et al. 1995). The induction pattern of Fos-like immunoreactivity in the forebrain could serve as predictor of atypical antipsychotic drug activity (Robertson et al. 1994).

Procedure

Groups of 4–6 male Wistar rats weighing 350–450 g are injected subcutaneously with saline

(control) or with various doses of the standard drugs or compounds with putative antipsychotic activity. After 2 h, the animals are deeply anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital and perfused with 200 ml saline followed by 200 ml of 4 % paraformaldehyde in phosphate buffer solution (PBS). Each brain is removed immediately after perfusion and placed in fresh fixative for at least 12 h.

After the post-fixative period, 30-pm sections are cut from each brain using a vibratome. Several antisera to detect Fos can be used, such as a sheep polyclonal antibody directed against residues 2–16 of the N-terminal region of the Fos molecule or a polyclonal antiserum raised in rabbits against Fos peptide (4–17 amino acids of human Fos).

Sections are washed three times with 0.02 mM PBS and then incubated in PBS containing 0.3 % hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections are then washed three times in PBS and incubated in PBS containing 0.3 % Triton X-100, 0.02 % azide, and Fos primary antisera (diluted 1:200) for 48 h. The sections are then washed three times with PBS and incubated with a biotinylated rabbit antisheep secondary antibody (diluted 1:200) for 1 h. The sections are washed three times with PBS and incubated for 1 h with PBS containing 0.3 % Triton X-100 and 0.5 % avidin-biotinylated horseradish peroxidase complex. After three washes in PBS, the sections are rinsed in 0.1 M acetate buffer, pH6.0. Fos immunoreactivity is revealed by placing the sections in a solution containing 0.05 % 3,3′-diaminobenzidine, 0.2 % ammonium nickel sulfate, and 0.01 % H_2O_2. The reaction is terminated with a washing in acetate buffer. The sections are mounted on chrome-alum-coated slides, dehydrated, and prepared for microscopic observation.

Drug-induced changes in Fos-like immunoreactivity are quantified by counting the number of immunoreactive nuclei in the medial prefrontal cortex, nucleus accumbens, medial and dorsolateral striatum, and the lateral septal nucleus. The number of Fos-positive nuclei is counted with a 550 × 550 pm grid placed over each of these regions with a 100 × magnification.

Typical and atypical antipsychotics can be classified on the basis of difference between Fos-like immunoreactivity in the nucleus accumbens and lateral striatum. For this purpose, the data are corrected for the effects which are produced by the injection procedure itself. The injection-corrected value for the dorsolateral striatum is subtracted from the corresponding accumbal value for each drug dose.

This manipulation yields a value termed the atypical index, i.e., number of Fos-positive neurons in the nucleus accumbens minus the number in the lateral striatum = atypical index. A negative index indicates the probability of side effects, like extrapyramidal syndrome, exerted by the typical neuroleptics, a positive value to be devoid of it.

Evaluation

A one-way analysis of variance is performed on the cell count data for each dose and the corresponding vehicle control. If the analysis of variance is significant, multiple comparisons are performed by using the Newman–Keuls test.

Modifications of the Method

Graybiel et al. (1990) reported a drug-specific activation of c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum by amphetamine and cocaine.

Deutch et al. (1991) found that stress selectively increases Fos protein in dopamine neurons innervating the prefrontal cortex.

Gogusev et al. (1993) described modulation of c-fos and other proto-oncogene expression by phorbol diester in a human histiocytosis DEL cell.

Deutch et al. (1995) studied the induction of Fos protein in the thalamic paraventricular nucleus as locus of antipsychotic drug action.

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Neurotensin

General Considerations on Neurotensin and Neurotensin Receptors

Neurotensin is a 13-amino acid peptide originally isolated from calf hypothalamus (Carraway and Leeman 1973). It is secreted by peripheral and neuronal tissues and produces numerous

pharmacological effects in animals, suggesting **analgesic** (Coguerel et al. 1988; Clineschmidt and McGuffin 1977; Smith et al. 1997), **wound healing** (Brun et al. 2005), **cardiovascular** (Carraway and Leeman 1973; Schaeffer et al. 1998; Seagard et al. 2000), **endocrine** (Rostene and Alexander 1997), **hypothermic** (Bissette et al. 1976; Benmoussa et al. 1996; Tyler-McMahon et al. 2000), and **antipsychotic** (Nemeroff 1986; Sarhan et al. 1997; Feifel et al. 1999; Kinkead et al. 1999; Cusack et al. 2000) actions. Neurotensin is even considered to be an endogenous neuroleptic (Ervin and Nemeroff 1988; Gully et al. 1995). Radke et al. (1998) studied synthesis and efflux of neurotensin in different brain areas after acute and chronic administration of typical and atypical antipsychotic drugs.

Neurotensin affects **gastrointestinal functions**, such as stimulating the growth of various gastrointestinal tissues (Feurle et al. 1987), modulating pre- and postprandial intestinal motility (Pellissier et al. 1996), inhibiting gastric acid secretion (Zhang et al. 1989a), stimulating responses in rat stomach strips (Quirion et al. 1980), inducing contractile responses in intestinal smooth muscle (Unno et al. 1999), and maintaining gastric mucosal blood flow during cold water restraint (Zhang et al. 1989b; Xing et al. 1998).

Neurotensin acts as a **growth factor** on a variety of normal and cancer cells (Wang et al. 2000).

Like other neuropeptides, neurotensin is synthesized as part of a larger precursor which also contains neuromedin N, a six amino acid neurotensin-like peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.8).

Several peptidic and non-peptidic neurotensin agonists and antagonists have been synthesized and analyzed in pharmacological tests as potential drugs mainly in psychopharmacology (Gully et al. 1995, 1996, 1997; Azzi et al. 1996; Castagliuolo et al. 1996; Chapman and See 1996; Mule et al. 1996; Hong et al. 1997; Johnson et al. 1997; Sarhan et al. 1997; Betancur et al. 1998; Gudasheva et al. 1998; Schaeffer et al 1998; Kitabgi 2002). Furthermore, inhibitors of neurotensin-degrading enzymes were described (Bourdel et al. 1996). Binder et al. (2001) reviewed neurotensin and dopamine interactions.

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Neurotensin Receptor Binding

Purpose and Rationale

Neurotensin interacts with two cloned receptors that were originally differentiated on the basis of their affinity to the antihistaminic drug levocabastine (Schotte et al. 1986). The high sensitive, levocabastine-insensitive rat neurotensin receptor (*NTR1*) was cloned first (Tanaka et al. 1990) and shown to mediate a number of peripheral and central neurotensin responses, including the neuroleptic-like effects of the peptide (Labbé-Jullié et al. 1994). The human NTR1 has been cloned from the colonic adenocarcinoma cell line HT29 (Vita et al. 1993) and shown to consist of a 416 amino acid protein that shares 84 % homology with rat NTR1. A second human NTR1 receptor differing only in one amino acid has been cloned from substantia nigra by Watson et al. (1993).

The lower-affinity, levocabastine-sensitive neurotensin receptor (*NTR2*) was cloned by Chalon et al. (1996) and Mazella et al. (1996) and characterized by Yamada et al. (1998). Studies by Dubuc et al. (1999) indicate that NTR2 mediates neurotensin-induced analgesia.

A third neurotensin receptor (*NTR3*) was cloned from a human brain cDNA library (Mazella et al. 1998; Vincent et al. 1999; Mazella 2001; Mazella and Vincent 2006). It is identical with sortilin, a receptor-like protein, cloned from human brain (Petersen et al. 1997, 1999). The NT3/gp95/sortilin protein is a transmembrane

neuropeptide receptor which does not belong to the superfamily of G-protein-coupled receptors.

Gully et al. (1997) described a binding assay for the neurotensin1 receptor.

Procedure

Cell Culture

CHO cells transfected with cDNA of the human neurotensin receptor cloned from HT 29 cells (h-NTR1-CHO cells) are cultured at 37 °C in modified Eagle's medium without nucleosides, containing 10 % fetal calf serum, 4 mM glutamine, and 300 μg /ml geneticin (G418), in a humidified incubator under 5 % CO_2 in O_2. The colonic adenocarcinoma HT 29 cell line (American Type Culture Collection, Rockville, MD) is cultured under similar conditions in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10 % fetal calf serum, 4 mM glutamine, 200 IU/ml penicillin, and 200 mg/ml streptomycin. One week after seeding, confluent monolayer cultures are washed three times with 3 ml PBS and harvested by enzymatic dissociation with trypsin. After dilution with PBS, cells are resuspended in the same culture medium at a density of 5×10^4 cells/ml and are plated into 35-mm diameter, fibronectin-coated Petri culture dishes.

Membrane Homogenate Preparation and Binding Assay

Whole brains of male Sprague Dawley rats albino guinea pigs or cell pellets are homogenized in 10 volumes of ice-cold 50 mM Tris–HCl buffer (pH7.4) for 30 s, using a polytron homogenizer (setting 5). After 20 min. centrifugation at 30,000 g, the pellet is washed; centrifuged again under the same conditions; resuspended in a storage buffer containing 50 mM Tris–HCl (pH7.4), 1 mM EDTA, 0.1 % BSA, 40 mg/l bacitracin, 1 mM 1,10-orthophenanthroline, and 5 mM dithiothreitol; and stored as aliquots in liquid nitrogen until used.

Aliquots of membranes (10, 50, 300, and 500 μg of protein for h-NTR1-CHO cells, HT 29 cells, rat brain, and guinea pig brain, respectively) are incubated for 20 min at 20 °C in the incubation buffer (0.5 ml final volume) containing appropriate concentrations of [^{125}I-Tyr^3] neurotensin (25–100 pM) and unlabeled drugs. After incubation, the assay medium is diluted with 4 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) supplemented with 0.1 % BSA and 1 mM EDTA, and the mixture is rapidly filtered under reduced vacuum through Whatman GF/B glass fiber filters that have been pretreated with 0.1 % polyethyleneimine. The filters are washed under the same conditions three times and radioactivity is measured. Nonspecific binding is determined in the presence of 1 μM unlabeled neurotensin. All experiments are performed in triplicate, and data are expressed as the mean ± SEM of at least three separate determinations.

Evaluation

The IC_{50} is the value of ligand that inhibits 50 % of the specific binding and is determined using an iterative nonlinear regression program (Munson and Rodbard 1980).

Modifications of the Method

Cusack et al. (1995) studied species selectivity of neurotensin analogs at the rat and two human NTR1 receptors.

Lugrin et al. (1991) produced a series of pseudopeptide analogs of neurotensin by systematically replacing peptide bonds in neurotensin with CH_2NH bonds. The compounds were screened in vitro for agonist or antagonist activity and for metabolic stability.

Le et al. (1997) cloned the human neurotensin receptor gene and determined the structure.

Labbé-Jullié et al. (1998) attempted to identify residues in the rat NTR1 that are involved in binding of a nonpeptide neurotensin antagonist.

Souazé et al. (1997) and Najimi et al. (1998) studied the effects of a neurotensin agonist and showed in human colonic adenocarcinoma HT 29 cells after short incubation an increase, after prolonged exposure a decrease of mRNA levels, and in the human neuroblastoma cell line CHP 212 a high-affinity neurotensin receptor gene activation.

Ovigne et al. (1998) described a monoclonal antibody specific for the human NTR1.

Nouel et al. (1999) found that both NT2 and NT3 neurotensin receptor subtypes were expressed by cortical glial cells in culture.

Cusack et al. (2000) developed a neurotensin analog, NT34, which can distinguish between rat and human neurotensin receptors and exhibits more than a 100-fold difference in binding affinities.

Neuromedin N, a peptide belonging to the gastrin-releasing peptide/bombesin family (see chapter "▶ Pharmacological Effects on Gastric Function"), shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

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Genetically Altered Monoamine Transporters

Monoamine transporters, such as the dopamine transporter, 5-hydroxytryptamine transporter, and noradrenaline transporter, in the plasma membrane provide effective control over the intensity of monoamine-mediated signaling by recapturing neurotransmitters released by presynaptic neurons (Gainetdinov et al. 2002). These transporters act also as molecular gateways for neurotoxins (Uhl and Kiayama 1993; Miller et al. 1999; Vincent et al. 1999).

Takahashi et al. (1997) found that heterozygote animals of VMAT2 knockout mice display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity.

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Dopamine Transporter Knockout Mice

Many drugs exert their psychotropic action via dopamine transporters (Amara and Kuhar 1993; Giros and Caron 1993).

Dopamine transporter knockout mice, which are generated by disruption of the gene encoding the dopamine transporter by homologous recombination (Giros et al. 1996; Sora et al. 1998), have a distinct biochemical and behavioral phenotype. At the neurochemical level, the homoeostasis of dopamine-containing neurons is altered markedly, including disrupted clearance of dopamine, an elevated extracellular concentration of dopamine, and dramatically decreased intraneuronal storage of dopamine (Jones et al. 1998; Gainetdinov et al. 1998; Benoit-Marand et al. 2000).

In response to the elevated dopamine-mediated tone, both presynaptic and postsynaptic dopamine receptors are downregulated (Giros et al. 1996), but although autoreceptor functions are lost (Jones et al. 1999), some postsynaptic responses appear to be enhanced (Gainetdinov et al. 1999a; Fauchey et al. 2000).

Dopamine transporter knockout mice are hyperactive (Gainetdinov et al. 1999b; Spielewoy et al. 2000) and have a much reduced body size (Bossé et al. 1997). These animals have cognitive deficits (Gainetdinov et al. 1999a, b), disrupted sensorimotor gating (Ralph et al. 2001), and sleep dysregulation (Wisor et al. 2001). Dopamine transporter knockout mice appear to provide a model of some aspects of manic behavior (Ralph-Williams et al. 2003).

Abnormalities in skeletal structure (Bliziotes et al. 2000) and altered regulation of gastrointestinal tract motility (Walker et al. 2000) are also observed.

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Serotonin Transporter Knockout Mice

The serotonin transporter has a key role in regulating the intensity of 5-HT-mediated transmission and is the primary target for several antidepressants and psychostimulants (Amara and Kuhar 1993; Bengel et al. 1996).

Disruption of 5-HT uptake in serotonin transporter knockout mice increases the extracellular concentration of 5-HT sixfold and reduces intracellular concentration by 60 % – 80 % (Fabre et al. 2000).

Holmes et al. (2003) found that mice lacking the serotonin transporter exhibit 5-HT_{1A} receptor-mediated abnormalities in tests for anxiety-like behavior.

Lira et al. (2003) reported altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin-transporter-deficient mice.

Marked desensitization of both presynaptic and postsynaptic 5-HT_{1A} receptors is observed in electrophysiological studies (Gobbi et al. 2001).

There is a significant decrease in 5-HT_{1A} receptor binding sites, mRNA, and protein in some, but not all, 5-HT-containing brain areas. Altered hypothermic and neuroendocrine responses to 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) are also reported (Li et al. 1999).

Thermal hyperalgesia in mice after chronic constrictive sciatic nerve injury was absent in serotonin transporter-deficient mice (Vogel et al. 2003).

Decreases in 5-HT_{1A} and 5-HT_{1B} receptor coupling are observed, accompanied by disruption of the neurochemical responses to the 5-HT1A receptor agonist ipsapirone and the 5-HT_{1A}/5-HT_{1D} receptor agonist GR127935 (Fabre et al. 2000).

The hyperlocomotor effect of MDMA, but not that of high doses of d-amphetamine, is disrupted in serotonin receptor knockout mice (Bengel et al. 1996).

In double knockout mice that lack the dopamine transporter and have no or one copy of the gene that encodes the serotonin transporter, no place preference for cocaine was observed (Sora et al. 2001).

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Noradrenaline Transporter Knockout Mice

The noradrenaline transporter has a role similar to that of the dopamine transporter and the serotonin transporter with respect to noradrenaline-mediated transmission (Blakely et al. 1994).

Noradrenaline-transporter knockout mice have been generated using homologous recombination (Xu et al. 2000).

Wang et al. (1999) reviewed genetic approaches to studying norepinephrine function using knockout of the mouse norepinephrine transporter gene.

The prolonged synaptic lifetime of noradrenaline in noradrenaline transporter knockout mice results in elevation of the extracellular concentration of noradrenaline and depletion of the intraneuronal stores. In addition, in noradrenaline transporter knockout mice, the α_1-adrenoceptor decreased in the hippocampus (Xu et al. 2000), although α_{2A}-adrenoceptor density did not change in the spinal cord (Bohn et al. 2000).

Noradrenaline-transporter knockout mice have a lower body weight and reduced locomotor responses to novelty. In the tail-suspension test used for screening antidepressant drugs, noradrenaline transporter knockout mice behaved like antidepressant-treated, wild-type animals, and no additional effects of the antidepressants desipramine, paroxetine, and bupropion were observed in mutant mice in this test (Xu et al. 2000).

In the tail-flick assay, morphine induced greater analgesia in noradrenaline transporter knockout mice compared with wild-type mice (Bohn et al. 2000).

In synaptosomes from the frontal cortex of noradrenaline transporter knockout mice, cocaine and nisoxetine had no inhibitory effect on the uptake of dopamine, whereas in the nucleus accumbens, the effectiveness of cocaine was somewhat reduced. Uptake of dopamine in brain

regions that have low levels of dopamine transporter may depend primarily on the noradrenaline transporter (Morón et al. 2002).

Locomotor responses to cocaine and amphetamine are elevated in noradrenaline knockout mice, and chronic administration of cocaine did not induce further sensitization. The enhanced responses to psychostimulants in noradrenaline transporter knockout mice correlate with the suppression of presynaptic dopamine function and supersensitivity to postsynaptic D2 and D3 receptors (Xu et al. 2000).

Haller et al. (2002) studied behavioral responses to social stress in noradrenaline transporter knockout mice.

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In Vivo Tests

Golden Hamster Test

Purpose and Rationale

"Innate behavior" of many species including man has been described by Lorenz (1943, 1966). The "golden hamster test" (Ther et al. 1959) uses the innate behavior of this species (*Mesocricetus auratus*) for differentiation between neuroleptic and sedative – hypnotic activity. The aggressive behavior of male golden hamsters is suppressed by neuroleptics in doses which do not impair motor function.

Procedure

Ten to 20 male golden hamsters with an average weight of 60 g are crowded together in Makrolon$^{(R)}$ cages for at least 2 weeks. During this time, the animals develop a characteristic fighting behavior. For the test, single animals are placed into glass jars of 2 l. In this situation, the hamsters assume a squatting and resting position during the day. If the animals are touched with a stick or a forceps, they wake up from their daytime sleep and arouse immediately from the resting position. If one tries to hold the hamster with a blunted forceps, a characteristic behavior is elicited: The hamster throws himself onto his back, tries to bite and to push the forceps away with his legs, and utters angry shrieks. Touching the animals is repeated up to six times followed by punching with the forceps. Only animals responding to the stimulus with all three defense reactions (turning, vocalizing, and biting) are included into the test.

The test compounds are applied either subcutaneously, intraperitoneally, or orally. Six animals are used for each dose.

Evaluation

The stimuli are applied every 20 min for 3 h. The number of stimuli until response is recorded. Furthermore, the suppression of the defense reactions (turning, biting, and vocalizing) is evaluated. An animal is regarded to be completely "tamed" if all defense reactions are suppressed even after

punching with the forceps at least once during the test period.

After each stimulation, the "tamed" animal is placed on an inclined board with 20° inclination. Normal hamsters and hamsters tamed by neuroleptics are able to support themselves or to climb on the board. Impaired motor function causes sliding down. This experiment is repeated three times after each testing of the defense reactions. An animal's coordination is considered to be disturbed if it falls three times during two tests of the experiments.

For each dose, the number of tamed hamsters and the number of animals with impaired motor function are recorded. Using different doses, ED_{50} values can be calculated for the taming effect and for impairment of motor function.

The ED_{50} values of taming were 1.5 mg/kg for chlorpromazine s.c. and 0.2 mg/kg for reserpine s.c. Much higher doses (ten times of chlorpromazine and five times of reserpine) did not elicit motor disturbances. On the contrary, while ED_{50} values of 10 mg/kg phenobarbital s.c. and 180 mg/kg meprobamate p.o. for the taming effect were found, these doses already caused severe motor disturbances. The taming dose of diazepam was 10 mg/kg p.o. which already showed some muscle-relaxing activity. The term "neuroleptic width" indicates the ratio between the ED_{50} for taming and the ED_{50} for motor disturbances. Only for neuroleptic drugs are ratios found between 1:5 and 1:30.

Critical Assessment of the Method

The method has the advantage that neuroleptics can easily be differentiated from sedative and hypnotic drugs. Anxiolytics with pronounced muscle-relaxing activity also show no significant differences between taming and impaired motor function. Moreover, the method has the advantage that no training of the animals and no expensive apparatus are needed.

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Influence on Behavior of the Cotton Rat

Purpose and Rationale

The "cotton-rat test" is another attempt to use the innate behavior as described for several animal species by Lorenz (1943, 1966) for the differentiation of psychotropic drugs (Vogel and Ther 1960). The cotton rat (*Sigmodon hispidus*) is a very shy animal which conceals himself at any time. This innate flight reflex is suppressed by centrally active drugs. Simultaneous evaluation of motor function allows the differentiation between neuroleptic and sedative drugs.

Procedure

Cotton rats are bred in cages equipped with a clay cylinder of 20 cm length and 10 cm diameter. This cylinder is used by the animals for hiding, sleeping, and breeding. Moreover, the animals which bite easily can be transported from one cage to another just by closing the cylinder on both ends. For the test, young animals with a body weight of 40 g are used. Young animals are as shy as the old ones but less vicious. Nevertheless, leather gloves have to be used for handling of cotton rats. Normal cages (25 × 30 × 20 cm) with a wire lid are used. A tunnel of sheet metal (half of a cylinder) 20 cm long and 7 cm high is placed into the cage. The cotton rats hide immediately in this tunnel. If the tunnel is lifted and placed on another site of the cage, the cotton rats immediately hide again.

Three rats are placed in one cage and tested for their behavior. Selective shaving of the fur enables the observer to recognize each animal. If the rats behave as described, they are then treated with the test compound subcutaneously or orally. At least six animals divided in two cages are used for each dose of test compound or standard. Fifteen min after application of the drug, the test period of three h is started. The tunnel is lifted and placed to another site. If the animals do not show the immediate flight reflex, an airstream of short duration is blown through the wire lid. If the animal still does not respond with the flight reflex, it is considered to be positively influenced. Afterwards, the animal is placed on an inclined board with 35° of inclination and tested for disturbance of motor coordination. A normal animal is able to climb upwards. If coordination is disturbed, the rat slides down.

Evaluation

The test procedure is repeated every 15 min over a period of 3 h. The animals which show at least one suppression of the flight reflex during the test period are counted as well as those who slide down on the inclined board. Using different doses, ED_{50} values are calculated for both parameters. The ratio between these two ED_{50} values is regarded as "neuroleptic width" which is 1:20 for chlorpromazine and 1:30 for reserpine, whereas ratios of 1:2 for phenobarbital and 1:1.5 for meprobamate indicate the absence of neuroleptic activity.

Critical Assessment of the Method

The method allows the differentiation of drugs with neuroleptic activity against other centrally active drugs. No training of the animals and no expensive equipment are necessary.

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Artificial Hibernation in Rats

Purpose and Rationale

Giaja (1938, 1940, 1953, 1954) studied the effects of reduced oxygen tension and cold environment on rats. The animals were placed in hermetically closed glass vessels which were submerged in ice water. Due to the respiratory activity, the oxygen tension diminishes and the carbon dioxide content increases. Under the influence of cooling and of hypoxic hypercapnia, the rectal temperature falls to 15 °C, and the animal is completely anesthetized and immobilized. The rat can survive in this poikilothermic state for more than 20 h. Complete recovery occurs after warming up. This kind of artificial hibernation was augmented by chlorpromazine (Courvoisier et al. 1953; Giaja and Markovic-Giaja 1954). Vogel (1959) and Ther et al. (1959, 1963) used these observations for evaluation of neuroleptics and opioid analgesics.

Procedure

Male Wistar rats weighing 100–150 g are deprived of food with free access to tap water overnight. The test compounds are injected subcutaneously 15 min prior to the start of the experiment. First, the rats are placed in ice-cold water to which surfactant is added in order to remove the air from the fur for 2 min. Then, the animals are placed into hermetically closed glass vessels of 750 ml volume which are placed into a refrigerator at 2 °C temperature. During the following hour, the vessels are opened every 10 min for exactly 10 s, allowing some exchange of air and reducing the carbon dioxide accumulation. At each time, animals are removed from the glass vessel and observed for signs of artificial hibernation which are not shown by control animals under these conditions. Treated animals, lying on the side, are placed on the back and further examined. An animal is considered positive, when it remains on the back, even if the extremities are stretched out. In this state, cardiac and respiration frequency are reduced, and the rectal temperature has fallen to 12–15 °C. The rigor of the musculature allows only slow movements of the extremities. The animals recover completely within a few

hours if they are brought to their home cages at room temperature. Artificial hibernation is induced dose-dependent by neuroleptics of the phenothiazine type and by some opioid analgesics like meperidine and methadone. In contrast, morphine shows only slight activity.

Evaluation

Various doses are applied to groups of ten animals. Percentage of positive animals is calculated for each group, and ED_{50} values with confidence limits are estimated according to Litchfield and Wilcoxon.

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Catalepsy in Rodents

Purpose and Rationale

Catalepsy in rats is defined as a failure to correct an externally imposed, unusual posture over a prolonged period of time. Neuroleptics which have an inhibitory action on the nigrostriatal dopamine system induce catalepsy (Costall and Naylor 1974; Chermat and Simon 1975; Sandberg et al. 1986), while neuroleptics with little or no nigrostriatal blockade produce relatively little or no cataleptic behavior (Honma and Fukushima 1976). Furthermore, cataleptic symptoms in rodents have been compared to the Parkinson-like extrapyramidal side effects seen clinically with administration of antipsychotic drugs (Duvoisin 1976).

Procedure

Groups of six male Sprague Dawley or Wistar rats with a body weight between 120 and 250 g are used. They are dosed intraperitoneally with the test drug or the standard. Then, they are placed individually into translucent plastic boxes with a wooden dowel mounted horizontally 10 cm from the floor and 4 cm from one end of the box. The floor of the box is covered with approximately 2 cm of bedding material. White noise is presented during the test. The animals are allowed to adapt to the box for 2 min. Then, each animal is grasped gently around the shoulders and under the forepaws and placed carefully on the dowel. The amount of time spent with at least one forepaw on the bar is determined. When the animal removes its paws, the time is recorded, and the rat is repositioned on the bar. Three trials are conducted for each animal at 30, 60, 120, and 360 min.

Evaluation

An animal is considered to be cataleptic if it remains on the bar for 60 s. Percentage of cataleptic animals is calculated. For dose–response curves, the test is repeated with various doses and more animals. ED_{50} values can be calculated. A dose of 1 mg/kg i.p. of haloperidol was found to be effective.

Critical Assessment of the Method

The phenomenon of catalepsy can be used for measuring the efficacy and the potential side effects of neuroleptics.

Modifications of the Method

Catalepsy induced by neuroleptic drugs can also be measured by the **PAW test**, which measures increase in forelimb and hindlimb retraction time in rats (Ellenbroek et al. 1987, 2001; Ellenbroek and Cools 1988, 2000; Prinssen et al. 1994, 1995).

The test is performed 30 min after intraperitoneal injection of test drug. Male Wistar rats weighing 220–300 g are placed on a Perspex platform (30 × 30 cm with a height of 20 cm) containing two holes for the forelimbs (40 mm) and two for the hindlimbs (50 mm), and a slit for the tail. The distance between the right and left forelimb holes is 15 mm, and the distance between forelimb and hindlimb holes is 55 mm. The rat is held behind the forelimbs, and the hindlimbs are gently placed in the holes. The forelimb retraction time and the hindlimb retraction time are defined as the time the animal needs to withdraw one forelimb and one hindlimb, respectively. The average forelimb retraction time and hindlimb retraction time (the mean of three measurements) is calculated for each rat.

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Pole Climb Avoidance in Rats

Purpose and Rationale

The pole-climb avoidance paradigm is an avoidance escape procedure used to separate neuroleptics from sedatives and anxiolytics. Whereas sedative compounds suppress both avoidance and escape responding at approximately the same doses, neuroleptic drugs reduce avoidance responding at lower doses than those affecting escape responding (Cook and Catania 1964).

Procedure

Male rats of the Long-Evans strain with a starting body weight of 250 g are used. The training and testing of the rats is conducted in a 25 × 25 × 40 cm chamber that is enclosed in a dimly lit, sound-attenuating box. Scrambled shock is delivered to the grid floor of the chamber. A 2.8-kHz speaker and a 28-V light are situated on top of the chamber. A smooth stainless-steel pole, 2.5 cm in diameter, is suspended by a counterbalance weight through a hole in the upper center of the chamber. A microswitch is activated when the pole is pulled down 3 mm by a weight greater than 200 g. A response is recorded when a rat jumps on the pole and activates the microswitch. The rat cannot hold the pole down while standing on the grid floor because of the counterbalance tension and cannot remain on the pole any length of time because of its smooth surface. The activation of the light and the speaker together is used as the conditioning stimulus. The conditioning stimulus is presented alone for 4 s and then is coincident with the unconditioned stimulus, a scrambled shock delivered to the grid floor, for 26 s. The shock current is maintained at 1.5 mA. A pole climb response during the conditioned stimulus period terminates the conditioned stimulus and the subsequent conditioned and unconditioned stimuli. This is considered an avoidance response. A response during the time when both the conditioned and unconditioned stimuli are present terminates both stimuli and is considered an escape response. Test sessions consist of 25 trials or 60 min, whichever comes first. There is a minimum intertrial interval of 90 s. Any time remaining in the 30 s allotted to make the pole climb is added to the 90 s intertrial interval. Responses during this time have no scheduled consequences; however, rats having greater than ten intertrial interval responses should not be used in the experiment. Before testing experimental compounds, rats are required to make at least 80 % avoidance responses without any escape failures.

Evaluation

Data are expressed in terms of the number of avoidance and escape failures relative to the respective vehicle control data. ED_{50} values can be calculated using different doses.

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Footshock-Induced Aggression

Purpose and Rationale

The test as described by Tedeschi et al. (1959) using mice which fight after footshock-induced stimulation is useful to detect neuroleptics but also shows positive effects with anxiolytics and other centrally effective drugs. The method has been used by several authors to test drugs with neuroleptic activity. The test is described in chapter "Tests for Anxiolytic Activity".

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Brain Self-Stimulation

Purpose and Rationale

In several species, electrical stimulation of selected brain loci produces effects which are positively reinforcing and pleasurable (Olds and Milner 1954; Olds 1961, 1972). Most of the data available have been obtained from experiments using rats with electrodes chronically implanted in the median forebrain bundle at the level of hypothalamus. Minute electrical pulses sustain a variety of operant behaviors such as lever pressing. Neuroleptics have been shown to be potent blockers of self-stimulation (Broekkamp and Van Rossum 1975; Koob et al. 1978; Gallistel and Freyd 1987). Conversely, compounds that facilitate catecholaminergic transmission such as d-amphetamine and methylphenidate will increase responding for such stimulation.

Procedure

Male Wistar rats (350–400 g) are anesthetized with 50 mg/kg pentobarbital i.p. and their heads placed on a level plane in a Kopf stereotactic instrument. A midline incision is made in the scalp and the skin held out of the way by muscle retractors. A small hole is drilled in the scull with a dental burr at the point indicated by the stereotactic instrument for the structure it is desired to stimulate. Using bregma as a reference point, the electrode (Plastic Products MS303/1) is aimed at the medium forebrain bundle according to the atlas of Paxinos and Watson (1986), using the coordinates of AP $= -0.8$ mm, Lat $= +2.8$ mm, and DV $= -7.2$ mm below dura. The assembly is then permanently affixed to the scull using stainless-steel screws and bone cement.

After a minimum of 10 days for recovery, the animals are trained to bar press for electrical stimulation on a continuous reinforcement schedule in a standard operant box outfitted with a single lever. The reward stimulus is a train of biphasic square-wave pulses generated by a Haer stimulator (Pulsar 4i). The parameters are set at a pulse duration of 0.5 ms with 2.5 ms between each pulse pair. The train of pulses may vary between 16 and 30/s, and the intensity of the pulses that are delivered range from 0.1 to 0.5 mA using the lowest setting that will sustain maximal responding. After consistent baseline responding is obtained for five consecutive 30-min session, the animals are ready for testing with standard agents. Compounds are administered 60 min. prior to testing. All data are collected on both cumulative recorders and counters.

Evaluation

The number of drug responses is compared to the number of responses made during each animal's 30-min control session on the preceding day, which is considered to be equal to 100 %. Testing various doses, ED_{50} values with 95 % confidence limits can be calculated.

Critical Assessment of the Method

Since there is sufficient evidence that self-stimulation behavior is maintained by catecholamines, the method gives indirectly insight into the catecholaminergic facilitating or blocking properties of a compound. Active neuroleptic drugs inhibit the self-stimulation behavior in very small doses. The relative potency observed in this test of clinically efficacious drugs parallels their potency in the treatment of schizophrenia.

Modifications of the Method

Reinforcing brain stimulation by electrodes placed in the medial forebrain bundle of rats is decreased after lesion of the internal capsule in the region of the diencephalic–telencephalic border. This decrement in rewarding processing can be reversed by antidepressant drugs (Cornfeldt et al. 1982).

Depoortere et al. (1996) used electrical self-stimulation of the ventral tegmental area to study the behavioral effects of a putative dopamine D_3 agonist in the rat.

Anderson et al. (1995) examined the interaction of aversive and rewarding stimuli in self-stimulating rats in terms of duration and direction. The rats were implanted with two moveable electrodes, one in a region supporting self-stimulation (the ventral tegmental area) and another in a region supporting escape (the nucleus reticularis gigantocellularis).

Kokkinidis et al. (1986) used amphetamine withdrawal for a behavioral evaluation. Mice implanted with stimulating electrodes in the lateral hypothalamus demonstrated stable and reliable rates of self-stimulation responding. After exposure to a chronic schedule of amphetamine treatment, response rates were severely depressed.

Post-amphetamine depression of self-stimulation from the substantia nigra can be reversed by cyclic antidepressants (Kokkinidis et al. 1980).

Moreau et al. (1992) reported that antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation in rats.

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Prepulse Inhibition of Startle Response

Purpose and Rationale

Prepulse inhibition is a model of sensorimotor gating which can be assessed in both animals and humans using the startle reflex response. When a fixed startle-eliciting stimulus (i.e., the pulse) is preceded by 30–500 ms by a weak, non-startle-eliciting stimulus (i.e., the prepulse), the magnitude of the startle response is significantly reduced to the pulse alone. Schizophrenic patients have decreased prepulse inhibition relative to normal control subjects, and this is thought to reflect an impairment in their ability to filter irrelevant sensory stimuli (Braff and Geyer 1990; Geyer 1998). Similar reductions in prepulse inhibition are produced in rats by administration of psychotomimetic drugs such as the dopamine agonists amphetamine and apomorphine or the noncompetitive NMDA antagonists phencyclidine and dizocilpine (MK801) (Mansbach and Geyer 1989; Swerdlow et al. 1998; Geyer et al. 2001; Rowley et al. 2001; Weiss and Feldon 2001; Pouzet et al. 2002). Most antipsychotics tested are able to antagonize prepulse inhibition disruption produced by dopamine antagonists, whereas prepulse inhibition disruption by NMDA antagonists may be selectively sensitive to antipsychotics with atypical features (Bakshi and Geyer 1995; Bubenikova et al. 2005; Fox et al. 2005). Haloperidol failed to block the effects

of phencyclidine and dizocilpine prepulse inhibition of startle (Keith et al. 1991).

Feifel et al. (Feifel and Reza 1999; Feifel et al. 1999a, b) tested the effects of a neurotensin agonist on prepulse inhibition of startle in rats.

Procedure

Male Sprague Dawley rats were treated with various doses of test compound or saline s.c. Immediately afterwards, rats receive a second s.c. injection consisting of 2 mg/kg d-amphetamine, or 0.5 mg/kg apomorphine, or 0.1 mg/kg dizocilpine or saline. Then, 10 min later, animals were placed in special startle chambers (SR-LAB, San Diego Instruments, San Diego, Calif., USA). Startle chambers consist of a Plexiglas cylinder 8.2 cm in diameter, resting on a 12.5 × 25.5 cm Plexiglas frame within a ventilated enclosure housed in a sound-attenuated room exposed to 70-dB background noise. After a 5-min acclimation period, acoustic stimuli were presented via a speaker mounted 24 cm above the animal. Acoustic stimuli consisted of a 120-dB pulse by itself (pulse alone) or a 120-dB pulse preceded by 100 ms by prepulses 3, 5, and 10 dB above background noise. There was an average of 15 s between stimuli. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced the motion within the cylinder. Startle amplitude was defined as the degree of motion detected by this accelerometer. Each rat was tested on four separate occasions separated by 7 non-test days. On each test day, the dose of test compound was kept constant, but the specific psychotomimetic agent was alternated across test days in a counterbalanced fashion.

Evaluation

Prepulse inhibition was calculated as the percentage of the pulse-alone startle amplitude using the following formula: [1 (startle amplitude after prepulse–pulse pair/startle amplitude after pulse only)] × 100. Analysis of data was then carried out using a three-factor repeated-measures analysis of variance (ANOVA). Significant factor results from the ANOVA were followed up with separate one-way ANOVAs for each psychotomimetic agent and then, when indicated, with individual group mean comparisons using post hoc *t*-tests for multiple comparisons using the Bonferroni method.

Modifications of the Method

Sipes and Geyer (1995) studied the disruption of prepulse inhibition of the startle response in the rat by DOI [(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride], which is mediated by 5-HT_{2A} receptors. The authors suggested that studies of the serotonergic substrates of prepulse inhibition may provide a model of the possible serotonergic role in the sensorimotor gating abnormalities in patients with schizophrenia and with obsessive-compulsive disorder.

Ellenbroeck et al. (1998) described the effects of an early stressful life event on sensorimotor gating in adult rats.

Andersen and Pouzet (2001) compared the effects of acute versus chronic treatment with typical or atypical antipsychotics on d-amphetamine-induced sensorimotor gating deficits in rats.

Heidbreder et al. (2000) used the prepulse inhibition of acoustic startle for behavioral, neurochemical, and endocrinological characterization of the early social isolation syndrome.

Krebs-Thomson et al. (2001) reported that postweanling handling attenuates isolation-rearing disruption of prepulse inhibition in rats.

Weiss et al. (2001) studied the dissociation between the effects of preweaning and/or postweaning social isolation on prepulse inhibition and latent inhibition in adult Sprague Dawley rats.

Dirks et al. (2003) reported reversal of startle gating deficits in transgenic **mice** overexpressing corticotropin-releasing factor by antipsychotic drugs.

Andreasen et al. (2006) studied the effect of nicotinic agents on prepulse inhibition (PPI) in mice using a startle response/PPI system from TSE Systems, Bad Homburg, Germany.

Lind et al. (2004) described prepulse inhibition of the acoustic startle reflex in pigs and its disruption by d-amphetamine.

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N40 Sensory Gating

Purpose and Rationale

The N40 auditory-evoked potential has been used to develop an animal model for the study of sensory gating mechanisms (Boutros et al. 1997a; Boutros and Kwan 1998). The method has been applied to evaluation of psychotropic compounds (Adler et al. 1986; Boutros et al. 1994, 1997b). Bickford-Wimer et al. (1990) localized one possible source of the N40 waveform to the CA3 region of the hippocampus.

Fox et al. (2005) used the N40 sensory gating model in mice for evaluation of a potential antipsychotic drug.

Procedure

Male DBA/2 mice were stereotaxically implanted with tripolar stainless-steel wire head stages for EEG recordings in the CA3 region of the hippocampus. The mice were first anesthetized with a solution of 2.8 % ketamine, 0.28 % xylazine, and 0.05 % acepromazine. Three access holes for the electrodes were made at AP −1.8 mm from the bregma, and in a plane perpendicular to the suture, ML 0.6 (cortical electrode), 1.6 (reference electrode), and 2.6 mm electrode directed at the hippocampus). The depth of the hippocampal electrode tip was DV 1.65–1.70 mm below the surface of the cortex. The depths of the cortical and reference electrodes were DV 0.5 mm from the surface of the skull, resulting in contact, but not penetration, of the cortical tissue. The tripolar electrode was lowered into position with a stereotaxic electrode holder and affixed using cyanoacrylic gel and dental acrylic and two anchor screws. Mice were allowed to recover for 3 days before commencement of the experiments. Awake mice were recorded in acoustically isolated chambers. Flexible tethers and electrical swivels were used to convey EEG signals to differential AC EEG amplifiers and allowed the mice free movement within the chambers. The EEG was amplified 1000 × with a 50- to 60-Hz notch filter engaged, and high- and low-pass filters were set at 1 and 100 Hz, respectively. Hippocampal auditory-evoked potentials were generated by presentation of 60 sets of 3 kHz-paired tone bursts from a speaker within the recording chamber at a distance of 15–20 cm to the mouse. The first tone of the pair is referred to as the conditioning stimulus, and the second is referred to as the test stimulus. The duration of both the condition and test stimuli was 5 ms, with 0.5 s between the stimuli and 20 s between pairs. Data acquisition software recorded EEG signals 100 ms before and for 899 ms after the initial conditioning stimulus. The software averaged the 60-paired responses into one composite-evoked response. Various

doses of test drug were administered i.p. 20–30 min before mice were placed into the recording chambers and initiation of auditory-evoked potential recording. Recording of paired auditory potentials continued for two 20-min sessions, each comprised of 60 paired stimuli. Each mouse was administered every treatment dose and a control vehicle treatment in a balanced order on separate days with at least 48 h between treatments. This within-subject design allowed each mouse to serve as its own control. The hippocampal response to auditory stimuli was identified as the highest positive peak deflection in the ongoing EEG at a latency of 10–20 ms after the stimulus (P20), followed by the lowest negative peak deflection in the ongoing EEG at 20–45 ms after the stimulus (N40). The difference in amplitude between P20 and N40 was defined as the N40 amplitude in microvolts.

Evaluation

N40 amplitudes were determined for both the averaged conditioning and test-evoked potentials, and a ratio was derived between the two responses by dividing the test amplitude by the conditioning amplitude (T/C ratio).

Modifications of the Method

Flack et al. (1996) studied sensory gating in a computer model of the CA3 neural network of the hippocampus.

Stevens et al. (1998) investigated changes in auditory information processing after kainic acid lesions in adult rats used as a model of schizophrenia.

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Latent Inhibition

Purpose and Rationale

Latent inhibition has been recommended as an animal model of schizophrenia (Feldon and Weiner 1992; Swerdlow et al. 1996; Vaitl and

Lipp 1997; Moser et al. 2000; Bender et al. 2001). Latent inhibition refers to the retarded acquisition of a conditioned response that occurs if the subject being tested is first preexposed to the to-be-conditioned stimulus without the paired unconditioned stimulus. Because the "irrelevance" of the to-be-conditioned stimulus is established during non-contingent preexposure, the slowed acquisition of the conditioned stimulus-unconditioned stimulus association is thought to reflect the process of overcoming this learned irrelevance. Latent inhibition has been reported to be diminished in acutely hospitalized schizophrenia patients. Several authors used the latent inhibition model in rats to test psychotropic compounds (Solomon et al. 1981; Feldon and Weiner 1991; Moran and Moser 1992; De la Casa et al. 1993; Lacroix et al. 2000; Alves et al. 2002). Trimble et al. (2002) tested the effects of selective D1 antagonists on latent inhibition in the rat.

Procedure

Animals

Male Sprague Dawley rats weighing 300–400 g were housed two to a cage under a 12-h reversed cycle lighting with food and water ad libitum. All experimental manipulations were carried out in the dark phase of the dark/light cycle.

Apparatus

Modified metal Skinner boxes (24.5 × 24.5 × 21 cm measured from a raised grid floor) were located in darkened, sound-insulated, ventilated outer boxes. A removable water bottle was located on one side of each Skinner box through a hole of 1.0 cm diameter, positioned 2 cm above the grid floor. When water was not required, the water bottle was removed. Licks at the spout of each water bottle were recorded using a lickometer (model 453, Campden Instruments, London, UK). The preexposed stimulus was a flashing light (10 s duration with three light flashes per second) situated in the middle of the roof of each Skinner box. The grid floor consisted of steel bars (0.5 cm in diameter) spaced 1 cm apart. Shock generators with scramblers were calibrated to produce 0.5-mA shocks via the grid floor.

Procedure

Rats were randomly assigned to experimental groups and were allocated to a particular Skinner box. They had experience of only that box for the duration of the experiment. After adaptation to the housing conditions for 1 week, rats were placed immediately on a 23-h water deprivation schedule that continued until the end of the experiments. Food remained freely available.

Baseline Days (Days 15–19)

After 7 days on the water deprivation period, 5 days of pretraining commenced. Each rat was placed in a Skinner box for 15 min. The water bottle was present and each rat could drink freely. After the baseline session was over, each rat was returned to its home cage and allowed access to water for 45 min.

Preexposure (Day 20)

With the water bottle removed, each rat was placed in a Skinner box. Rats received ten stimulus (flashing house-light) presentations of 10 s duration (three light flashes per second) with a fixed stimulus interval of 50 s. Afterwards the rats were returned to their home cages and allowed access to water for 1 h.

Conditioning (Day 21)

With the water bottle removed, each rat was placed in a Skinner box. Then, 5 min later, each rat received the first of two light footshock pairings. House-light parameters were identical to those of the preexposure period. The house-light was immediately followed by the footshock (0.5 mA, 1 s). The second light-shock pairing was given 5 min later. After the conditioning period had terminated, animals were returned to their home cages and allowed access to water for 1 h.

Re-baseline Day (Day 22)

With the water bottle present, each rat was placed in a Skinner box and allowed to drink as in the baseline sessions.

Test Day (Day 23)

With the water bottle present, each rat was placed in a Skinner box and allowed to drink. When each rat

completed 75 licks, the flashing house-light was presented and continued until 5 min had elapsed from stimulus onset. Time bins of 30-s duration commenced from the time of stimulus presentation, and the number of licks made by each rat within every time bin was recorded. This measure allowed the pattern of drinking over the course of stimulus presentation to be shown. The amount of suppression of licking for each rat was assessed using a suppression ratio calculated from the time (in seconds) to complete licks 51–75 (pre-stimulus) divided by the time (in seconds) to complete licks 51–100 (pre-stimulus + stimulus on). A suppression ratio of 0.01 indicates total suppression of licking (no latent inhibition), while a ratio of 0.5 indicates no change in licking rate from the pre-stimulus period to the stimulus-on period (latent inhibition).

DRUG Treatment

Test drugs or vehicle was administered by subcutaneous injection in various doses 30 min prior to preexposure and conditioning.

Evaluation

Times to complete licks and the suppression ratios were analyzed independently using a 2×6 ANOVA with main factors of preexposure and drugs.

Modifications of the Method

Lehmann et al. (1998) studied the long-term effects of repeated maternal separation on three different latent inhibition paradigms.

Pouzet et al. (2004) reported that latent inhibition is spared by NMDA-induced ventral hippocampal lesions, but is attenuated following local activation of the ventral hippocampus by intracerebral NMDA infusion.

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Tests Based on the Mechanism of Action

Amphetamine Group Toxicity

Purpose and Rationale

It is well known that aggregation of mice in small cages greatly enhances the toxicity of amphetamine. The death rate can be reduced by pretreatment with neuroleptics. This phenomenon is generally accepted as an indicator of neuroleptic activity. The increased toxicity results from increased behavioral activation due to aggregation inducing an increase of circulating catecholamines. The mechanism can be understood by the fact that amphetamine is an indirectly acting sympathomimetic amine that exerts its effects primarily by releasing norepinephrine from storage sites in the sympathetic nerves. After administration of high doses of amphetamine, mice exhibit an elevated motor activity which is highly increased by aggregation. This increased behavioral activation is followed by death within 24 h in 80–100 % of control animals. Neuroleptics reduce this death rate. In contrast, non-neuroleptic sympatholytics and psychosedative agents like the barbiturates do not produce a dose-related protection. Moreover, anxiolytic agents like benzodiazepines are also found to be ineffective in the prevention of amphetamine group toxicity.

Procedure

Ten male mice of the NMRI-strain are used for each group. They are dosed with the test compound or the standard either orally or intraperitoneally and all placed in glass jars of 18 cm diameter. Untreated animals serve as controls. The test has to be performed at room temperature of 24 °C. Thirty min after i.p. or 1 h after oral administration, the mice receive 20 mg/kg d-amphetamine subcutaneously. The mortality is assessed 1, 4, and 24 h after dosing.

Evaluation

The mortality of amphetamine-only treated animals is at least 80 %. If less than 80 % die due to low ambient temperature, the test has to be repeated. The estimation of ED_{50} values for protection and their confidence limits are calculated by probit analysis of the data using the number of dosed versus the number of surviving animals. Doses of 10 mg/kg chlorpromazine p.o. and 1 mg/kg haloperidol have been found to be effective.

Critical Assessment of the Method

The amphetamine group toxicity test has been used by many investigators and has been found to be a reliable method for detecting neuroleptic activity.

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Inhibition of Amphetamine Stereotypy in Rats

Purpose and Rationale

Amphetamine is an indirect acting sympathomimetic agent which releases catecholamines from

its neuronal storage pools. In rats the drug induces a characteristic stereotypic behavior. This behavior can be prevented by neuroleptic agents.

Procedure

Groups of six Wistar rats with a body weight between 120 and 200 g are used. They are injected simultaneously with d-amphetamine (10 mg/kg s.c.) and the test compound intraperitoneally and then placed individually in stainless-steel cages (40 × 20 × 18 cm). The control groups receive d-amphetamine and vehicle. Stereotypic behavior is characterized by continuous sniffing, licking or chewing and compulsive gnawing. The animals are observed 60 min after drug administration. An animal is considered to be protected, if the stereotypic behavior is reduced or abolished.

Evaluation

The percent effectiveness of a drug is determined by the number of animals protected in each group. A dose–response is obtained by using ten animals per group at various doses. ED_{50} values can be calculated. The standard neuroleptic drugs have the following ED_{50} values: chlorpromazine 1.75 mg/kg i.p. and haloperidol 0.2 mg/kg i.p.

Critical Assessment of the Method

Inhibition of amphetamine-induced stereotypies in rats can be regarded as a simple method to detect neuroleptic activity. However, this may reflect the effects in the corpus striatum which are thought to be responsible for the Parkinsonism-like side effects of neuroleptics.

Modifications of the Method

Ljungberg and Ungerstedt (1985) described a rapid and simple behavioral screening method for simultaneous assessment of limbic and striatal blocking effects of neuroleptic drugs. A low dose of 2 mg/kg d-amphetamine i.p. induces both increased locomotion, thought to reflect an increased dopamine transmission in the nucleus accumbens, and weak stereotypies, thought to reflect an increased dopamine transmission in the neostriatum. The behavior is measured in a combined open-field apparatus with holes on the bottom to measure nose-pocking and registration of time spent in the corners. Neuroleptics with less propensity to induce unwanted extrapyramidal side effects can be differentiated from classical drugs with more extrapyramidal adverse reactions.

Segal and Kuczenski (1997) described an escalating dose "binge" model of amphetamine psychosis. Rats were exposed to escalating doses of amphetamine (1.0–8.0 mg/kg) before multiple daily injections of relatively high doses of the drug (8 mg/kg every 2 h × 4 injections).

Atkins et al. (2001) described stereotypic behaviors in mice selectively bred for high and low methamphetamine-induced stereotypic chewing.

Machiyama (1992) recommended chronic methylamphetamine intoxication in **Japanese monkeys** (*Macaca fuscata*) as a model of schizophrenia in animals.

Ellenbroek (1991) described the ethological analysis of **Java monkeys** (*Macaca fascicularis*) in a social setting as an animal model for schizophrenia.

Sams-Dodd and Newman (1997) described the effects of the administration regime on the psychotomimetic properties of d-amphetamine in the Squirrel monkey (*Saimiri sciureus*).

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Inhibition of Apomorphine Climbing in Mice

Purpose and Rationale

Administration of apomorphine to mice results in a peculiar climbing behavior characterized initially by rearing and then full-climbing activity, predominantly mediated by the mesolimbic dopamine system (Costall et al. 1978). The ability of a drug to antagonize apomorphine-induced climbing behavior in the mouse has been correlated with neuroleptic potential (Protais et al. 1976; Costall et al. 1978).

Procedure

Groups of ten male mice (20–22 g) are treated i.p. or orally with the test substance or the vehicle and placed individually in wire-mesh stick cages. Thirty min afterwards, they are injected s.c. with 3 mg/kg apomorphine. Ten, 20, and 30 min after apomorphine administration, they are observed for climbing behavior and scored as follows:

0 = four paws on the floor
1 = four feet holding the vertical bars
2 = four feet holding the bars

Evaluation

The average values of the drug-treated animals are compared with those of the controls, and the decrease is expressed as percent. The ED_{50}-values and confidence limits are calculated by probit analysis. Three dose levels are used for each compound and the standard with a minimum of ten animals per dose level.

Critical Assessment of the Test

Similar to the enhancement of compulsive gnawing of mice after apomorphine by antidepressant drugs, the suppression of climbing behavior of mice after apomorphine can be used for testing neuroleptic drugs. The test has been modified by various authors.

In contrast to other strains of mice, apomorphine climbing is not induced in DBA2 mice unless subchronic manipulations of brain dopamine transmission are performed (Duterte-Boucher and Costentin 1989).

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Inhibition of Apomorphine Stereotypy in Rats

Purpose and Rationale

Apomorphine induces a stereotyped behavior in rats, characterized by licking, sniffing, and gnawing in a repetitive, compulsive manner, which is an indication of striatal dopaminergic stimulation (Anden et al. 1967; Ernst 1967; Costall and Naylor 1973). Compounds which prevent apomorphine-induced stereotypy antagonize dopamine receptors in the nigrostriatal system (Ljungberg and Ungerstedt 1978; Tarsy and Baldessarini 1974). Furthermore, antagonism of this behavior is predictive of propensity for the development of extrapyramidal side effects and tardive dyskinesias (Klawans and Rubovits 1972; Tarsy and Baldessarini 1974; Christensen et al. 1976; Clow et al. 1980).

Procedure

For screening, groups of six male Wistar rats with a body weight between 120 and 200 g are used. The test drug or the standard is administered i.p. 60 min. prior to apomorphine dosage. Apomorphine HCl is injected s.c. at a dose of 1.5 mg/kg. The animals are placed in individual plastic cages. A 10 s observation period is used to measure the presence of stereotypic activity such as sniffing, licking, and chewing 10 min after apomorphine administration. An animal is considered protected if this behavior is reduced or abolished.

Evaluation

The percent effectiveness of a drug is determined by the number of animals protected in each group. With a group size of ten animals, dose–response curves are obtained and ED_{50} values calculated. ED_{50} values were found to be 0.2 mg/kg s.c. for haloperidol and 5.0 mg/kg for chlorpromazine, whereas clozapine was ineffective even at high doses.

Modifications of the Methods

Puech et al. (1978) studied the effects of several neuroleptic drugs on hyperactivity induced by a low dose of apomorphine in mice.

Apomorphine induces stereotypic behavior in a variety of species including pigeons. The symptoms in pigeons are manifested as pecking against the wall of the cage or on the floor. Akbas et al. (1984) described a method registering the pecking after apomorphine by a microphone, amplification through a pulse preamplifier, and registration with a polygraph. The effect of apomorphine was dose-dependent decreased by yohimbine and neuroleptics.

Stereotyped behavior in guinea pigs induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

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Yawning/Penile Erection Syndrome in Rats

Purpose and Rationale

Yawning is a phylogenetically old, stereotyped event that occurs alone or associated with stretching and/or penile erection in humans and in animals from reptiles to birds and mammals under different conditions (Argiolas and Melis 1998). The yawning–penile erection syndrome can be induced in rats by apomorphine and other dopamine autoreceptor stimulants (Ståhle and Ungerstedt 1983; Gower et al. 1984) and can be antagonized by haloperidol and other dopamine antagonists. Antagonism against this syndrome

can be regarded as indication of antipsychotic activity (Furukawa 1996).

Besides the **dopaminergic** system in this behavior (Mogilnicka and Klimek 1977; Baraldi et al. 1979; Benassi-Benelli et al. 1979; Nickolson and Berendsen 1980; Gower et al. 1984, 1986; Dourish et al. 1985; Doherty and Wisler 1994; Kurashima et al. 1995; Bristow et al. 1996; Fujikawa et al. 1996a; Asencio et al. 1999) also the **serotonergic** (Baraldi et al. 1977; Berendsen and Broekkamp 1987; Berendsen et al. 1990; Protais et al. 1995; Millan et al. 1997), the **cholinergic** (Yamada and Furukawa 1980; Fujikawa et al. 1996b), the **GABAergic** (Zarrindast et al. 1995), the **NO system** (Melis et al. 1995, 1996, 1997a, b), and **steroid** as well as **peptide hormones** (Bertolini and Baraldi 1975; Bertolini et al. 1978; Holmgren et al. 1980; Berendsen and Nickolson 1981; Berendsen and Gower 1986; Gully et al. 1995) are involved (Argiolas and Melis 1998).

Procedure

Naive male Wistar rats, weighing 220–280 g, are housed under controlled 12 h light–dark cycle with free access to standard food pellets and tap water. Rats are pretreated with subcutaneous injection of the antagonist 30 min prior to injections of the agonist, such as apomorphine (0.02 to 0.25 mg/kg s.c.) or physostigmine (0.02 to 0.3 mg/kg s.c. or i.p.). After administration of the agonist, rats are placed in individual transparent Perspex cages. A mirror is placed behind the row of observation cages to facilitate observation of the animals for penile erections and yawns. Yawning is a fixed innate motor pattern characterized by a slow, wide opening of the mouth. A penile erection is considered to occur when the following behaviors are present: repeated pelvic thrusts immediately followed by an upright position and an emerging, engorged penis which the rats proceed to lick while eating the ejaculate. The number of penile erections and yawns is counted for 30 min following the last injection.

Evaluation

The results are expressed as the mean number of yawns and of penile erections per group ± SEM. The statistical significance is determined by comparing the results of each group with the results of the relevant control group using a non-parametric rank sum test.

Critical Assessment of the Method

Ferrari et al. (1993) published some evidence that yawning and penile erection in rats underlie different neurochemical mechanisms. Nevertheless, the procedure can be regarded as a useful behavioral tool to study putative antipsychotic activity of new compounds.

Modifications of the Method

Two sublines of Sprague Dawley rats were bred for high- and low-yawning frequency in males (Eguibar and Moyaho 1997).

Apomorphine produced more yawning in Sprague Dawley rats than in F344 rats (Tang and Himes 1995).

Sato-Suzuki et al. (1998) evoked yawning by electrical or chemical stimulation in the paraventricular nucleus of anesthetized rats.

The yawning–penile erection syndrome in rats can be elicited by injections of 50 ng NMDA or AMPA (Melis et al. 1994, 1997b) into the paraventricular nucleus of the hypothalamus or intracerebroventricular injection of 50 ng oxytocin (Melis et al. 1997a) or ACTH (Genedani et al. 1994; Poggioli et al. 1998) or α-MSH (Vergoni et al. 1998).

Champion et al. (1997) and Bivalacqua et al. (1998) studied the effect of intracavernosal injections of adrenomedullin and other peptide hormones on penile erections in **cats**.

Dopaminergic influences on male sexual behavior of **rhesus monkeys** were studied by Pomerantz (1990, 1992).

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Inhibition of Mouse Jumping

Purpose and Rationale

Lal et al. (1975) described a jumping response in mice after administration of L-dopa in amphetamine pretreated animals where the number of jumps can be objectively counted. The mouse jumping is due to dopaminergic overstimulation similar to that seen in rats when stereotypy is induced by higher doses of amphetamine. The phenomenon can be blocked by neuroleptics.

Procedure

Male CD-1 mice weighing 22–25 g are injected with 4 mg/kg d-amphetamine sulfate, followed 15 min later by an i.p. injection of 400 mg/kg L-dopa. The mice spontaneously begin to jump at a high rate. A median of 175 jumps can be observed in these mice during 60 min. Since mice do not show any jumping after saline administration, the responses after drug administration are specific and can be measured automatically through a pressure-sensitive switch closure or properly positioned photoelectric beam disruptions. Test compounds are administered 60 min prior to L-dopa injection.

Evaluation

Jumps of mice treated with test drugs or standard are counted and expressed as percentage of jumps in amphetamine-/L-dopa-treated animals. Using various doses, ED_{50} values with 95 % confidence limits are calculated.

Critical Assessment of the Method

The method has been found to be sensitive and rather specific for neuroleptic drugs.

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Antagonism Against MK-801-Induced Behavior

Purpose and Rationale

Dizocilpine (MK-801), a noncompetitive NMDA antagonist, induces a characteristic behavior in rat and mice, which is regarded as a model of psychosis (Andiné et al. 1999). In mice MK-801 induces a characteristic stereotypy marked by locomotion and falling behavior through both dopamine-dependent and dopamine-independent mechanisms (Carlson and Carlson 1989; Verma and Kulkarni 1992). Antipsychotic agents dose-dependently antagonize this MK-801-induced behavior.

Procedure

Male CD-1 mice (20–30 g) are individually placed in activity boxes lined with wire-mesh flooring and allowed to acclimate for 60 min. The animals are then dosed with compounds 30 min prior to subcutaneous administration of MK-801 at 0.2 mg/kg. The mice are observed for locomotion and the presence of falling behavior 15 min following MK-801 administration.

Evaluation

ED_{50} values and 95 % confidence limits are calculated by the Litchfield and Wilcoxon method.

Modifications of the Method

Deutsch and Hitri (1993), Rosse et al. (1995), Deutsch et al. (2002, 2003), and Mastropaolo et al. (2004) described methods to measure the MK-801-induced explosive behavior in mice, called “popping.”

Farber et al. (1996) showed that neuroleptic drugs can prevent neuronal vacuolization and necrosis induced by MK-801 (Fix et al. 1993).

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Phencyclidine Model of Psychosis

Purpose and Rationale

Phencyclidine (PCP)-induced symptoms in rats are considered as a model of psychosis (Ogawa et al. 1994; Halberstadt 1995; Steinpreis 1996; Abi-Saab et al. 1998; Sams-Dodd 1998a; Jentsch and Roth 1999; Phillips et al. 2000; Farber 2003; Morris et al. 2005).

PCP-induced symptoms can be antagonized by neuroleptic drugs (Witkin et al. 1997; Sams-Dodd 1998b; Javitt et al. 2004).

Cartmell et al. (1999) found that metabotropic glutamate receptor agonists selectively attenuate phencyclidine versus d-amphetamine motor behaviors in rats.

Procedure

Behavior of male Sprague Dawley rats weighing 250–300 g was monitored while in transparent, plastic shoebox cages of the dimensions 45 × 25 × 20 cm, with 1 cm depth of wood clips as bedding, and a metal grill on the top of the cage. Motor monitors consisted of a rectangular rack of 12 photobeams arranged in an 8 × 4 formation. Shoe boxes were placed inside these racks, enabling the activity of the rat to be monitored in a home-cage environment. The lower rack was positioned at a height of 5 cm, which allowed the detection of PCP-induced head movements in addition to movements of the body of the rat. Rearing activity was detected by a second rack placed 10 cm above the first. Rats were placed in the cage for an acclimation period of 30 min, and then removed, administered the test compounds s.c. or sterile water, and then returned to the same cages. After 30 min, the rats were given an s.c. injection of PCP or amphetamine

or sterile water and once again returned to the cages. Motor activity was monitored for the following 60 min resulting in the measurement of three different parameters: ambulations (pattern of beam breaks indicating that the animal had relocated its entire body), fine movements (nonambulatory beam breaks), and time at rest. An indication of rearing activity was detected in the upper rack of photobeams.

Evaluation

Data were analyzed by a one-way ANOVA, and then post hoc comparisons for each dose group versus control or PCP alone or PCP and test compound were made using Newman–Keuls multiple comparison test.

Modifications of the Method

Furuya et al. (1998) investigated the effects of a strychnine-insensitive glycine site antagonist on the hyperactivity and the disruption of prepulse inhibition induced by phencyclidine (PCP) in rats.

Redmond et al. (1999) tested the effects of acute and chronic antidepressant administration on PCP-induced locomotor hyperactivity.

Boulay et al. (2004) tested a putative atypical antipsychotic for improvement of social interaction deficits induced by PCP in rats.

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Inhibition of Apomorphine-Induced Emesis in the Dog

Purpose and Rationale

The blockade of centrally acting dopaminergic mechanisms is considered to play a major role in suppression of psychotic reactions in schizophrenia. Apomorphine, regarded as a direct dopaminergic agonist, produces a pronounced emetic effect in dogs, and the blockade of apomorphine emesis is used as an indication of dopaminergic blockade. However, although both antiemetic activity and antipsychotic activity are thought to be due to dopaminergic blockade, the sites of action are in different brain areas, and there is a lack of complete correlation of these activities.

Procedure

Adult beagle dogs of either sex are used in treatment groups of three to nine dogs/dose. The dogs are given the test compounds in a gelatin capsule; they are then dosed with 0.15 mg/kg apomorphine s.c. at various intervals after administration of the test compound. The dogs are first observed for overt behavioral effects, e.g., pupillary response to light, changes in salivation, sedation, tremors, etc.; then, after the administration of apomorphine, the dogs are observed for stereotypic sniffing, gnawing, and the emetic response. Emesis is defined as retching movements followed by an opening of the mouth and either attempted or successful ejection of stomach content.

Evaluation

If the experimental compound is antiemetic in the primary screen, the dose is progressively lowered to obtain a minimal effective dose or an ED_{50} value. The ED_{50} values for haloperidol and chlorpromazine were found to be 0.06 mg/kg p.o. and 2.0 mg/kg p.o., respectively. Clozapine was not effective at doses between 2 and 10 mg/kg. p.o.

Critical Assessment of the Method

The method has been extensively used by several laboratories. However, since nonclassical neuroleptics like clozapine did not show pronounced activity, the test has been abandoned. Moreover, tests in higher animals like dogs are limited due to regional regulations.

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Purposeless Chewing in Rats

Purpose and Rationale

Purposeless chewing can be induced in rats by directly acting cholinergic drugs or cholinesterase inhibitors (Rupniak et al. 1983), which can be blocked by antimuscarinic agents. The chewing behavior has been proposed to be mediated through central M_2 receptors rather than via

central M_1 sites (Stewart et al. 1989). Chewing can also be induced by chronic administration of neuroleptics in rats (Clow et al. 1979; Iversen et al. 1980). Purposeless chewing is mediated by dopaminergic and nicotinic mechanisms.

Procedure

Male albino rats are housed 10 per cage at room temperature and kept on a 12 h light–dark cycle. For the experiments, rats are placed individually in a large glass cylinder (height 30 cm, diameter 20 cm) at 21 ± 1°C and allowed to habituate for 15 min before injection of drugs. The antagonists, e.g., sulpiride or mecamylamine as standards, are given at different doses 30 min before treatment either with 0.01 mg/kg nicotine or 1 mg/kg pilocarpine i.p. Number of chewings are counted by direct observation immediately after drug administration. The results are presented as number of chews in a 30-min period.

Evaluation

Analysis of variance (ANOVA), followed by Newman–Keuls tests, are used to evaluate the significance of the results obtained. $P < 0.05$ is considered as significant.

Modifications of the Method

Tremulous jaw movements induced by tacrine (Cousins et al. 1999) can be antagonized by antipsychotic drugs (Betz et al. 2005; Ishiwarii et al. 2005).

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Models of Tardive Dyskinesia

Purpose and Rationale

Tardive dyskinesia is a severe side effect of traditional neuroleptics affecting a considerable number of patients probably based on a genetic disposition, being characterized by involuntary movements of the oral region. Various authors used rats as animal model for tardive dyskinesia,

either after treatment with reserpine (Waddington 1990; Neisewander et al. 1994; Bergamo et al. 1997; Queiroz and Frussa-Filho 1999; Andreassen and Jorgensen 2000; Casey 2000; Van Kampen and Stoessl 2000; Calvente et al. 2002; Abílio et al. 2003; Peixoto et al. 2003) or haloperidol (Takeuchi et al. 1998; Harvey and Nel 2003; Naidu et al. 2003; Burger et al. 2005). Several authors compared the effects of different neuroleptics (See and Ellison 1990; Tamminga et al. 1994) or studied potential antagonistic effects (Takeuchi et al. 1998; Queiroz and Frussa-Filho 1999; Abílio et al. 2003; Naidu et al. 2003; Peixoto et al. 2003).

Burger et al. (2005) found that ebselen attenuates haloperidol-induced orofacial dyskinesia and oxidative stress in rat brain.

Procedure

Male Wistar rats weighing 270–320 g were injected s.c. once a week with 12 mg/kg haloperidol decanoate for 4 weeks. Another group was pretreated with 30 mg/kg ebselen and received in addition to haloperidol every other day an i.p. injection of 30 mg/kg ebselen.

The animals were observed for the quantification of orofacial dyskinesia just before haloperidol administration and on the 7th, 14th, 21st, and 28th day after the first administration of haloperidol.

Rats were placed individually in cages (20 × 20 × 19 cm) containing mirrors under the floor and behind the back wall of the cage to allow behavioral quantification when the animal was faced away from the observer. To quantify the occurrence of oral dyskinesia, the incidence of tongue protrusions, vacuous chewing movements frequency, and the duration of facial twitching were recorded for 15 min. Observers were blind to drug treatment.

Evaluation

Data were analyzed by a three-way ANOVA, followed, when appropriate, by univariate analysis and Duncan's post hoc test.

Modifications of the Method

Several authors used monkeys (*Cebus apella* or *Macaca speciosa*) to evaluate the effect of neuroleptics to induce tardive dyskinesia-like symptoms (Gunne and Barany 1979; Domino 1985; Werge et al. 2003).

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Single-Unit Recording of A9 and A10 Midbrain Dopaminergic Neurons

Purpose and Rationale

Interactions with central nervous system dopamine pathways are crucial for the expression of antipsychotic effects seen with clinically effective neuroleptics. These interactions also have a role in the expression of several of the neurological side effects seen with these agents. Extracellular single-unit recording techniques of rat A9 (substantia nigra) and A10 (ventral tegmental area) dopamine neurons show that after acute treatment with neuroleptics, the number of spontaneously firing cells is increased in both areas. After repeated treatment (21 days), a decrease was found with all neuroleptics in the A10 neurons, whereas in the A9 cell, only compounds with clinically evident extrapyramidal side effects induced a decrease. Clozapine which is believed not to produce extrapyramidal side effects resulted in the depolarization inactivation of A10 neurons but not A9 cells. The method provides a prediction of a compound's antipsychotic potential as well as potential neurological side effects (Chiodo and Bunney 1983).

Procedure

Male Wistar rats weighing 280–360 g are anesthetized with chloral hydrate intraperitoneally. The animal is mounted in a stereotaxic apparatus (Kopf, model 900). The cranium is exposed, cleaned of connective tissue, and dried. The skull overlying both the substantia nigra (A9: anterior (A) 3000–3400 μm, lateral (L) 1800–2400 μm from lambda) and the ventral tegmental area (A10: A 3000–3400 μm, L 400–1000 μm from lambda) (Paxinos and Watson 1986) is removed. Using the dura as point of reference, a micropipette driven by a hydraulic microdrive is lowered through the opening of the skull at vertical 6000–8500 μm. Spontaneously firing dopamine neurons within both the substantia nigra and the ventral tegmental area are counted by lowering the electrode into twelve separate tracks (each track separated from the other by 200 μm) in each region. The sequence of these tracks is kept constant, forming a block of

tissue which can be reproducibly located from animal to animal.

Extracellular neuronal signals are sampled using a single barrel micropipette approximately one μm at its tip and filled with 2 M NaCl saturated with 1 % pontamine sky blue dye (in vitro impedance between 5 and 10 MΩ). Electrical potentials are passed through a high-impedance preamplifier, and the signal is sent to a window discriminator which converts potentials above background noise levels to discrete pulses of fixed amplitude and duration. Only cell whose electrophysiological characteristics match those previously established for midbrain dopamine neurons are counted. In an anesthetized rat, a neuron is considered to be dopaminergic if it displays a triphasic positive–negative–positive spike profile of 0.4 to 1.5 mV amplitude and 2.5 ms duration, firing in an irregular pattern of 3 to 9 Hz with occasional bursts characterized by progressively decreasing spike amplitude and increasing spike duration.

At the end of each experiment, the location of the last recorded track tip is marked by passing 25 microampere cathodal current through the recording micropipette barrel for 15 min in order to deposit a spot of dye. The rat is sacrificed, and the brain is then removed, dissected, and frozen on a bed of dry ice. Frozen serial sections (20 μm in width) are cut, mounted and stained with cresyl violet, and examined using a light microscope.

Animals pretreated with vehicle prior to neuronal sampling serve as controls. For animals that are used in an acute single-unit dopamine neuron sampling assay, test compounds are administered intraperitoneally 1 h prior to the beginning of dopamine neuron sampling. For animals used in a chronic single-unit dopamine sampling assay, the compounds are administered once a day for 21 days, and dopamine neuron sampling is started 2 h after the last dose on the 21st day.

Evaluation

Drug treatment groups are compared to vehicle groups with a one-way ANOVA with a post hoc Newman–Keuls analysis for significance.

Modifications of the Method

Nyback et al. (1975) tested the influence of tricyclic antidepressants on the spontaneous activity of norepinephrine-containing cells of the locus coeruleus in anesthetized rats.

Scuvée-Moreau and Dreese (1979) studied the effect of various antidepressant drugs on the firing rate of locus coeruleus and dorsal raphe neurons of the anesthetized rat with extracellular microelectrodes.

Using the method of single-unit recording of spontaneous firing of locus coeruleus neurons in rats, Cedarbaum and Aghajanian (1977) studied the inhibition by microiontophoretic application of catecholaminergic agonists.

Marwaha and Aghajanian (1982) examined in single-unit studies the actions of adrenoceptor antagonists at *alpha-1* adrenoceptors of the dorsal raphe nucleus and the dorsal lateral geniculate nucleus and *alpha-2* adrenoceptors of the nucleus locus coeruleus.

Mooney et al. (1990) studied the organization and actions of the noradrenergic input to the superior colliculus of the hamster using microiontophoretic techniques together with extracellular single-unit recording.

Bernardini et al. (1991) studied in vitro with brain slices of mice the amphetamine-induced and spontaneous release of dopamine from A9 and A10 cell dendrites.

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In Vivo Voltammetry

Purpose and Rationale

Various groups (Lane et al. 1979, 1987, 1988; Blaha and Lane 1983, 1984, 1987; Crespi et al. 1984; Marsden et al. 1984; Maidment and Marsden 1987a, b; Armstrong-James and Millar 1979, 1984; Kawagoe et al. 1993) described in vivo voltammetry as an electrochemical technique that uses carbon fiber microelectrodes stereotactically implanted in brain areas to monitor monoamine metabolism and release. De Simoni et al. (1990) reported on a miniaturized optoelectronic system for telemetry of in vivo voltammetric signals in freely moving animals.

Procedure

Carbon fiber working electrodes are made from pyrolytic carbon fibers supported in a pulled glass capillary (Armstrong-James and Millar 1979; Sharp et al. 1984) and electrically pretreated for simultaneous recording of ascorbic acid DOPAC and 5-HIAA (Crespi et al. 1984).

Male Sprague Dawley rats weighing 270–340 g are anesthetized with a 2–3 % halothane O_2/NO_2 mixture (1:1) and held in a stereotactic frame. Reference and auxiliary electrodes

are positioned on the surface of the dura through 1 mm holes drilled in the cranium and held in place with dental cement. Holes, approx. 2 mm in diameter, are drilled in the cranium above the left or right nucleus accumbens and contralateral anterior striatum, and the underlying dura is broken with a hypodermic needle. A working electrode is lowered in one of the above regions and cemented in place. A second electrode is then implanted in the remaining structure. The coordinates, measured from the bregma, are as follows: nucleus accumbens–rostrocaudal +3.4 mm, mediolateral ± 1.4 mm, dorsoventral −7 mm, striatum–rostrocaudal +2.8 mm, mediolateral ± 2.6 mm, and dorsoventral −5.5 mm.

Drugs are injected subcutaneously. Voltammograms are recorded using a Princeton Applied Research 174A polarographic analyzer alternatively from each region every 5 min and after a 1 h stabilization period.

Evaluation

Voltammetric data are expressed as percentage changes from preinjection control values using the mean of the last six peak heights before administration of drug as the 100 % value. However, statistical analysis of the data is carried out on the absolute peak heights using a paired Student's *t*-test to compare six preinjection control peak heights with those after administration of drug at selected time points.

Modifications of the Method

Swiergiel et al. (1997) constructed voltammetric probes from stainless steel and fused silica tubing sheathing carbon fibers and compared them with commercially available glass-sealed IVEV-5 electrodes. This type of electrodes can be easily manufactured and does not require any special equipment.

Parada et al. (1994, 1995) described a triple-channel swivel suitable for intracranial fluid delivery and microdialysis experiments which can be equipped with three electrical channels for in vivo voltammetry and measurement of intracranial temperature with a thermocouple.

Frazer and Daws (1998) used electrodes coated with a perfluorinated ion exchange resin (Nafion) to assess serotonin transporter function in vivo by **chronoamperometry** whereby voltage is applied to the electrode in a pulsed manner and the current obtained measured as a function of time.

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Genetic Models of Psychosis

The Heterozygous Reeler Mouse

Purpose and Rationale

Reelin is an extracellular matrix protein secreted by GABAergic interneurons that, acting through pyramidal neuron integrin receptors, provides a signal for dendritic spine plasticity. The gene responsible for a mouse mutant strain is called reeler (D'Arcangelo and Curran 1998; Lombroso and Goldowitz 1998; Fatemi 2001; Pappas et al. 2003). Heterozygous reeler mice that exhibit a 50 % downregulation of reelin expression replicate the dendritic spine and GABAergig defects described in human schizophrenia (Larson et al. 2003). The heterozygote reeler mouse was recommended as a model for the development of a new generation of antipsychotics (Tueting et al. 1999; Rowley et al. 2001; Costa et al. 2002). This view has been challenged by Podhorna and Didriksen (2004).

Tomasiewicz et al. (1993) and Wood et al. (1998) proposed **NCAM-180 knockout mice** with a deletion of the neural cell adhesion molecule variant (NCAM-180) displaying increased lateral ventricle size and a reduced prepulse inhibition of startle response as model for schizophrenia.

Dirks et al. (2003) reported reversal of startle gating deficits in **transgenic mice overexpressing corticotropin-releasing factor** by antipsychotic drugs.

Van den Buuse (2003) showed deficient prepulse inhibition of acoustic startle in **Hooded-Wistar** rats compared with Sprague Dawley rats, suggesting that the Hooded-Wister line could be a useful genetic animal model to study the interaction of glutaminergic and dopaminergic mechanisms in anxiety and schizophrenia.

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The Hooded-Wistar Rat

Purpose and Rationale

Van den Buuse (2003), Lodge et al. (2003), and Martin et al. (2004) suggested that the Hooded-Wistar line (fawn-hooded rats) could be a useful genetic animal model to study the interaction of glutamatergic and dopaminergic mechanisms in anxiety and schizophrenia.

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Binding to D_4 Receptors

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General Considerations on Neurotensin and Neurotensin Receptors

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Models of Tardive Dyskinesia

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Antidepressant Activity

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Contents

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General Considerations

The first antidepressant drugs- were detected by serendipity in clinical trials. Iproniazid was developed for the treatment of tuberculosis. The observation of mood-elevating effects was followed by the detection of the inhibition of the enzyme monoamine oxidase. During clinical investigation of phenothiazine analogues as neuroleptics, imipramine was found to be relatively ineffective in agitated psychotic patients but showed remarkable benefit in depressed patients. Later on, inhibition of uptake of biogenic amines was found to be the main mechanism of action resulting in downregulation of β-receptors (Vetulani et al. 1976). Influence on α_2-adrenoreceptors (Johnson et al. 1980) was discussed as well. Several lines of preclinical and clinical evidence indicate that an enhancement of 5-HTmediated neurotransmission might underlie the therapeutic effect of most antidepressant treatments (Blier and de Montigny 1994).

Animal models of depression have been reviewed by Porsolt et al. (1991), Panksepp et al. (1991), Willner and Muscat (1991), and Cryan et al. (2002).

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In Vitro Methods

Inhibition of [^{3}H]-Norepinephrine Uptake in Rat Brain Synaptosomes

Purpose and Rationale

As shown by Hertting and Axelrod (1961) the neuronal reuptake mechanism for norepinephrine is the most important physiological process for removing and inactivating norepinephrine in the synaptic cleft. This uptake is inhibited by cocaine, certain phenylethylamines, and antidepressants. This mechanism is considered as one of the most important modes of action of antidepressants leading to receptor downregulation. In the brain, the hypothalamus shows the highest level and greatest uptake of noradrenaline. Therefore, this region is used for testing potential antidepressant drugs.

Procedure

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. The hypothalamic region is prepared, weighed, and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1000 g at 0–4 °C for 10 min. The supernatant is decanted and used for the uptake experiments.

Assay

Two hundred microliter of tissue suspension are incubated with 800 µl 62.5 nM ^{3}H-norepinephrine in Krebs-Henseleit bicarbonate buffer and 20 µl of the appropriate drug concentration (or the vehicle) at 37 °C under a 95 % O_2/5 % CO_2 atmosphere for 5 min. For each assay, three tubes are incubated with 20 µl of vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g for 10 min. The supernatant fluid is aspirated and the pellets dissolved adding 1 ml of solubilizer (Triton X-100 + 50 % ethanol, 1:4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

Evaluation

The percent inhibition at each drug concentration is the mean of three determinations. IC_{50} values are derived from log-probit analysis. IC_{50} values for the standard drugs desipramine and nortriptyline are around 20 nM.

Modifications of the Method

Pacholczyk et al. (1991) described the expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter.

Tejani-Butt (1992) recommended [^{3}H]nisoxetine as radioligand for quantitation of norepinephrine uptake sites.

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Inhibition of [^{3}H]-Dopamine Uptake in Rat Striatal Synaptosomes

Purpose and Rationale

High-affinity, saturable, temperature- and sodium-dependent transport of ^{3}H-dopamine has been observed in various tissue preparations from different brain regions. The area striatum has a high content of dopamine and is suitable for uptake experiments. The ^{3}H-dopamine uptake is inhibited by cocaine, certain phenylethylamines,

and antidepressants like nomifensine and bupropion but not by tricyclic antidepressants. The test can be used to characterize the mode of action of antidepressant drugs.

Procedure

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Corpora striata are prepared, weighed, and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1000 *g* at 0–4 °C for 10 min. The supernatant is decanted and used for the experiments.

Assay

One hundred microliter of tissue suspension are mixed with 900 µl 55.5 nM ^{3}H-dopamine solution in Krebs-Henseleit bicarbonate buffer and 20 µl of drug solution in appropriate concentration (or the vehicle as control). The tubes are incubated at 37 °C under a 95 % O_2/5 % CO_2 atmosphere for 5 min. For each assay, three tubes are incubated with 20 µl of vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 + 50 % ethanol, 1:4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

Evaluation

The percent inhibition at each drug concentration is the mean of three determinations. IC_{50} values are derived from log-probit analyses. IC_{50} values for nomifensine are 460 nM but >20,000 nM for tricyclic antidepressants.

Modifications of the Method

Elsworth et al. (1993) differentiated between cocaine-sensitive and -insensitive dopamine uptake in various brain areas.

Cloning and pharmacological characterization of rat, bovine, and human dopamine transporters have been described (Giros et al. 1991, 1992; Kilty et al. 1991; Shimada et al. 1991; Usdin et al. 1991).

Binding characteristics of the dopamine transporter were studied (Reith et al. 1992; Rothman et al. 1992).

[^{3}H]-3β-(*p*-fluorophenyl) tropan-2β-carboxylic acid methyl ester ([^{3}H]WIN 35,428) is used as ligand for the dopamine transporter (Carroll et al. 1992; Cline et al. 1992).

[^{3}H]GBR12935 (1-[2-(diphenylmethoxy) ethyl]-4-(3-phenylpropyl)piperazine) was used as labeled ligand by Richfield (1991)and Nakachi et al. (1995).

Cocaine receptors are specifically labeled with [^{3}H]WIN 35,428 indicating the role of the dopamine transport system in mediating the behavioral effects and the abuse of cocaine (Madras et al. (1989).

Laruelle et al. (1993) reported **single-photon emission computed tomography** (SPECT) imaging of dopamine and serotonin transporters in nonhuman primates.

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Inhibition of [^{3}H]-Serotonin Uptake

Purpose and Rationale

Selective inhibitors of serotonin uptake (SSRIs) became the most powerful and widely used

antidepressants (Pacher et al. 2001; Fray et al. 2006). These include citalopram (D'Amato et al. 1987; Brøsen and Narajo 2001), fluoxetine, sertraline, paroxetine (Pacher et al. 2001), and escitalopram (Sánchez et al. 2003; Murdoch and Keam 2005).

These antidepressants block the reuptake of 5-HT.^{3}H-5-HT transport in brain has been found to be saturable, sodium and temperature dependent, and to be inhibited by several agents, such as ouabain, tryptamine analogues, and tricyclic antidepressants. Apparently, the 5-HT uptake can be differentiated from catecholamine uptake. Therefore, the test can be used to detect compounds that inhibit serotonin uptake into rat brain synaptosomes and may be potential antidepressants.

Procedure

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Either the whole brain minus cerebellum or the hypothalamus is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1000 g at 0–4 °C for 10 min. The supernatant is decanted and used for further uptake experiments.

Assay

Two hundred microliter of tissue suspension are mixed with 800 µl 62.5 nM ^{3}H-5-HT solution in Krebs-Henseleit bicarbonate buffer and 20 µl of drug solution in the appropriate concentration (or the vehicle as control). The tubes are incubated at 37 °C under 95 % O_2/5 % CO_2 atmosphere for 5 min. For each assay, three tubes are incubated with 20 µl of the vehicle at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g for 10 min. The supernatant is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X100 + 50 % ethanol, 1 + 4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

Evaluation

The percent inhibition of each drug concentration is the mean of three determinations. IC_{50} values are calculated from log-probit analyses. Standard drugs such as chlorimipramine show IC_{50} values in the order of 10 nM for ^{3}H-5-HT uptake in rat synaptosomes from hypothalamus.

Modifications of the Method

Similar methods were used by D'Amato et al. (1987), Weinshank et al. (1992), Cheng et al. (1993), Hatanaka et al. (1996), and Tordera et al. (2002).

Sánchez et al. (2003) and Chen et al. (2005) studied [^{3}H]-5-HT uptake inhibition in a COS-1 cell line stably transfected with h5-HTT.

Hallstrom et al. (1976) studied the platelet uptake of 5-hydroxytryptamine and dopamine in patients with depression.

Cloning of functional serotonin transporters has been described by Blakely et al. (1991) and by Hoffman et al. (1991).The role of serotonin in the mode of action of antidepressant drugs has been discussed by Hyttel and Larsen (1985), Åsberg and Mårtensson (1993), Hyttel (1994), Blier and de Montigny (1997), andKeane and Soubrié (1997).

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Binding to Monoamine Transporters

Purpose and Rationale

There is substantial clinical and experimental evidence that lesions in the serotonergic and noradrenergic systems are responsible for depression and that antidepressant treatment can reverse these alterations (Leonard 2000). Monoamine transporters are principal targets of widely used therapeutic drugs including antidepressants, psychostimulants, and the addictive drug cocaine (Madras et al. 1996; Fleckenstein et al. 1999). The termination of neurotransmission is achieved by rapid uptake of the released neurotransmitter by high-affinity neurotransmitter transporters. Most of these transporters are encoded by a family of genes (Na^+/Cl^- transporters) having a similar membrane topography of 12 transmembrane helices. An evolutionary tree revealed five distinct subfamilies: GABA-transporters, monoamine transporters, amino acid transporters, "orphan" transporters, and bacterial transporters (Nelson 1998).

Tatsumi et al. (1997) described the pharmacological profile of antidepressants at human monoamine transporters.

Procedure

Expression of Human Transporters

The human serotonin transporter cDNA is directionally ligated into the expression vector pRc/CMV and transfected into HEK293 (human embryonic kidney) cells by the Ca^{2+} method. The human dopamine transporter cDNA is directionally ligated into the expression vector pcDNA3 and transfected into HEK293 cells, also by the Ca^{2+} method.

Cell Culture

The cell lines are grown, passaged, and harvested in 150 mm Petri dishes with 17.5 ml of Dulbecco's modified Eagle's medium containing 0.1 mM nonessential amino acid solution, 5 % fetal clone bovine serum product, and 1 U/μl penicillin and streptomycin solution. They are incubated in 10 % CO_2, 90 % air at 37 °C, and 100 % humidity. The selecting antibiotic geneticin sulfate (250 μg/ml) is used continuously for culture of cells expressing the norepinephrine transporter.

Membrane Preparations

For the preparation of the homogenates, the medium is removed by aspiration. The cells are washed with 41 modified Puck's D1 solution (solution 1) (Pfenning and Richelson 1990) and then incubated for 5 min at 37 °C in 10 ml solution 1 and 100 mM EGTA. Afterward cells are removed from the surface by scraping with a rubber spatula, placed in a centrifuge tube, and collected by centrifugation at 110 g for 5 min at 4 °C. The supernatants are decanted. The pellets are resuspended in the respective binding assay buffer by use of a Polytron (Brinkman Instruments, Westbury, NY) for 10 s at setting 6. The mixture is then centrifuged at 35,600 g for 10 min at 4 °C. The pellets are suspended in the same volume of the respective buffer and the centrifugation repeated. The supernatants are decanted and the final pellets suspended in the respective buffer and stored at −80 °C until assayed. The final protein concentration is determined by the Lowry assay using bovine serum albumin as standard.

Radioligand Binding Assays

[^{3}H] Imipramine Binding to Human Serotonin Transporter. Radioligand binding assays are performed by a modification of the method of O'Riordan et al. (1990) with a binding buffer containing 50 mM Tris, 120 mM NaCl, and 5 mM KCl (pH7.4). Compounds to be tested are dissolved in 5 mM HCl and run in duplicate over at least 11 different concentrations against 1 nM [^{3}H]imipramine (specific activity 46.5 Ci/mmol) with 15 μg/tube membrane protein for 30 min at 22 °C. Nonspecific binding is determined in the presence of 1 μM imipramine. With the use of a 48-well Brandel cell harvester, the assay is terminated by rapid filtration through a GF/B filter presoaked with 0.2 % polyethylenimine. The filter strips are rinsed five times with ice-cold 0.9 % NaCl. Finally, each filter is placed in a scintillation vial containing 6.5 ml of Redi-Safe (Beckman Instruments, Fullerton, CA) and counted in a liquid scintillation counter.

[^{3}H] Nisoxetine Binding to Human Norepinephrine Transporter. Radioligand binding assays are performed by a modification of the method of Jayanthi et al. (1993) in binding buffer containing 50 mM Tris, 300 mM NaCl, and 5 mM KCl (pH 7.4). [^{3}H]nisoxetine (specific activity 85.0 Ci/mmol, from Amersham, Arlington Hts., IL) at 0.5 nM is incubated with competing drugs and 25 μg/tube membrane protein for 60 min at 22 °C. Nonspecific binding is determined in the presence of 1 μM nisoxetine. The remainder of the assay is exactly as described above.

[^{3}H] WIN35428 Binding to Human Dopamine Transporter. Radioligand binding assays are performed using a modification of the method of Pristupa et al. (1994) in a binding buffer containing 50 mM Tris and 120 mM NaCl (pH7.4). [^{3}H]WIN35428 (Dupont New England Nuclear, Boston MA, specific activity 83.5 Ci/mmol) at 1 nM is incubated with competing drugs and 30 μg/tube membrane protein for 120 min at 4 °C. Nonspecific binding is determined in the presence of 10 μM WIN35428. The remainder of the assay is exactly as described above.

Evaluation

The data are analyzed using the LIGAND program (Munson and Rodbard 1980) for calculation of K_D values and Hill coefficients.

Modifications of the Method

Using the same methods, Tatsumi et al. (1999) described the pharmacological profile of several neuroleptics at human monoamine transporters.

Gu et al. (1994) constructed stable cell lines expressing transporters for dopamine, norepinephrine, and serotonin using parental LLC-PK1 cells which do not express any of these neurotransmitter transporters.

Meltzer et al. (1997) described inhibitors of monoamine transporters using dopamine and serotonin-transporter assays. Membranes were prepared from coronal slices from caudate-putamen of brain from adult cynomolgus monkeys.

Owens et al. (1997) measured the affinity of several antidepressants and their metabolites for the rat and human serotonin and norepinephrine transporters.

Inazu et al. (1999) characterized dopamine transport in cultured rat astrocytes.

Siebert et al. (2000) used rat neuronal cultures and transfected COS-7 cells to characterize the interaction of haloperidol metabolites with neurotransmitter transporters.

Sato et al. (2000) studied the selective inhibition of monoamine neurotransmitter transporters by synthetic local anesthetics using cloned transporter cDNAs with transient functional expression in COS cells and stable expression in HeLa cells.

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Antagonism of p-Chloramphetamine Toxicity by Inhibitors of Serotonin Uptake

Purpose and Rationale

p-Chloramphetamine causes selective toxicity to serotonin neurons. At 10 mg/kg i.p. p-chloramphetamine (p-CA) causes long-term decreases in 5-HT, 5HIAA, [^{3}H]-5-HT uptake and tryptophan hydroxylase. After acute administration of p-CA, serotonin uptake into synaptosomes isolated from whole brain is reduced for several hours and returns to control values between 1 and 2 days, after which there is a marked long-lasting decrease due to toxic destruction of 5-HT neurones. The initial behavioral effects of p-CA are due to release of dopamine and serotonin. The serotonergic toxicity of p-CA requires active transport into 5-HT neurones where a cytotoxic intermediate compound is formed. Therefore, compounds which block 5-HT uptake will prevent this toxicity. Antagonism of the long-term p-CA-induced reduction of synaptosomal ^{3}H-5-HT in vitro uptake is a highly useful index of a compound's ability to inhibit 5-HT reuptake in vivo.

Procedure

Reagents

Two mg/ml dextrose and 0.30 mg/ml iproniazid phosphate are added to Krebs-Henseleit bicarbonate buffer. The mixture is aerated for 1 h with carbogen.

Serotonin creatinine sulfate (Sigma Chemical Co) as a 0.1 mM stock solution in 0.01 N HCl is used to dilute the specific activity of the radiolabeled 5-HT.

A solution of 62.5 nM ^{3}H-5-HT (5-[1,2-^{3}H (N)]-hydroxytryptamine creatinine sulfate, specific activity 20–30 Ci/mmol, New England Nuclear) is prepared in Krebs-Henseleit bicarbonate buffer.

(d,l)-p-Chloramphetamine (Regis Chemical Co) is dissolved in 0.9 % NaCl at a concentration of 10 mg/ml.

Drug Treatment

Groups of eight male Wistar rats weighing 150–200 g are injected intraperitoneally with saline or molar equivaient doses of the test drug. For initial studies, the dose given is 37.5 μmol/kg (equivalent to 10 mg/kg desipramine base). After 30 min, four rats from each group are injected with saline or with p-CA 10 mg/kg i.p. Three days after treatment, the rats are sacrificed. For multiple dosing, the rats are pretreated with the test drug twice up to four times in 2 h intervals.

Tissue Preparation

The rats are decapitated and the brains rapidly removed. Whole brain minus cerebellum is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvejhem

homogenizer. The homogenate is centrifuged at 1000 g at 0–4 °C for 10 min. The crude synaptosomal supernatant is decanted and used for uptake experiments.

Assay

Eight hundred microliter Krebs-Henseleit bicarbonate buffer +[^{3}H]-5HT

Two hundred microliter tissue suspension

Tubes are incubated at 37 °C under a 95 % O_2/5 % CO_2 atmosphere for 5 min. For each assay, three tubes are incubated at 0 °C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g at 0–4 °C for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 and 50 % ethanol, 1:4, v/v). The tubes are vigorously vortexed, decanted into scintillation vials, and counted in 10 ml of aqueous (Liquiscint) scintillation counting cocktail. Active uptake is the difference between cpm at 37 °C and 0 °C.

Evaluation

The percent protection is calculated according to the following formula:

$$\%\,\text{protection} = \frac{\text{cpm}[(\text{sal}/\text{sal}) - (\text{sal}/\text{pCA})]}{\text{cpm}\left[(\text{sal}/\text{sal} - (\text{sal}/\text{pCA})\right]} - \frac{\text{cpm}[(\text{sal}/\text{sal}) - (\text{drug}/\text{pCA})]}{\text{cpm}[(\text{sal}/\text{sal})] - (\text{sal}/\text{pCA})]}$$

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Receptor Subsensitivity After Treatment with Antidepressants: Simultaneous Determination of the Effect of Chronic Antidepressant Treatment on β-adrenergic and 5-HT_2 Receptor Densities in Rat Cerebral Cortex

Purpose and Rationale

The catecholamine and indolamine systems are thought to be involved in affective disorders such as depression. The effect of antidepressants on biogenic amine reuptake in vitro is immediate, whereas the onset of clinical activity is delayed and parallels more closely the time course of receptor changes measured in animal studies. Therefore, the experiment is designed to determine the in vivo effects of chronic (10 days) treatment with known and potential antidepressants on the β-receptor, as a measurement of noradrenergic interaction, and on the 5-HT_2 receptor as a measurement of serotoninergic interaction in the rat brain. Both receptor densities are measured in cortical tissue from the same animal and compared after treatment with test compounds at doses similar to those of standard drugs.

Procedure

Drug Treatment

Groups of four male Wistar rats, receiving food and water ad libitum are maintained on a 12-h diurnal light cycle and given i.p. injections twice daily for 10 days with saline or molar equivalent doses of the experimental drugs (equivalent to

10 mg/kg imipramine). Twenty-four hours after the last dose, the rats are decapitated and the cerebral cortices split along the mid-sagittal sinuses. One half is used for the (^{3}H)-DAH assay and the other half for the (^{3}H)-spiroperidol assay. This protocol allows the determination of effects of antidepressants on β-receptors and 5-HT_2-receptors in cerebral cortical tissue from the same animal.

Reagents

- (−)-[propyl-1,2,3-^{3}H]Dihydroalprenololol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
- (±)-propranolol HCl is obtained from Ayerst.
- [Benzene-^{3}H]spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear.

Tissue Preparation

^{3}H-Dihydroalprenolol (3H-DHA) Binding. The cerebral cortices are dissected free, weighed, and homogenized in 50 vol of ice-cold 0.05 M Tris buffer, pH 8.0. This homogenate is centrifuged at 40,000 *g* and the supernatant decanted. The pellet is resuspended and again centrifuged at 40,000 *g*. The final pellet is resuspended in 0.05 M Tris buffer, pH8.0. This tissue suspension is then stored on ice until use. The final tissue concentration is 10 mg/mi.

^{3}H-Spiroperidol Binding. The cerebral cortices are dissected, weighed, and homogenized in 50 vol of 0.05 M Tris buffer, pH7.7, and then centrifuged at 40,000 g for 15 min. The supernatant is discarded, the pellet resuspended, and again centrifuged at 40,000 *g*. The final pellet is resuspended in 50 vol of 0.05 M Tris buffer, pH7.7, and stored in an ice bath. The final concentration in the assay is 10 mg/ml.

Assay

^{3}H-DAH Binding

380 µl	H_2O
50 µl	0.5 M Tris buffer pH 8.0
20 µl	Vehicle (for total binding) or 50 µM propranolol (for nonspecific binding)
50 µl	^{3}H-DAH stock solution
500 µl	Tissue suspension

The tissue homogenates are incubated for 15 min at 25 °C with varying concentrations of ^{3}H-DHA (0.25–4.0 nM). With each ligand concentration triplicate samples are incubated with 1 µM propranolol under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 µl of each (^{3}H)-DHA concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed three times with 5 ml of ice-cold 0.05 M Tris buffer, pH8.0. The filters are counted in 10 ml liquid scintillation cocktail.

^{3}H-Spiroperidol Binding

50 µl	0.5 M Tris-physiological salts 380 µl H_2O
20 µl	H_2O (for total binding) or 0.25 mM methysergide (for nonspecific binding)
50 µl	^{3}H-spiroperidol stock solution
500 µl	Tissue suspension

The tissue homogenates are incubated for 10 min at 37 °C with varying concentrations of ^{3}H-spiroperidol (0.1–3.0 nM). With each ligand concentration, triplicate samples are incubated in the presence of 5 µM methysergide under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 µl of each (^{3}H)-spiroperidol concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed three times with 5 ml of ice-cold 0.05 M Tris buffer, pH 7.7. The filters are transferred to scintillation vials and counted in 10 ml of liquid scintillation cocktail.

Evaluation

Specific binding is defined as the difference in CPM in the presence or absence of excess "cold" ligand. The free ligand concentration is the difference between the total added and the specifically bound fraction at each concentration. The equilibrium binding constants (K_d and B_{max}) are determined by Scatchard analyses using least squares regression analysis of the binding data. The Scatchard plot shows "bound/free" versus "bound." The K_d value is the reciprocal of the slope and B_{max} the x-intercept. Significant differences of drug treatment are determined by either Dunnett's or Tukey's test after one-way analysis of variance.

Modifications of the Method

Buckett et al. (1988) found a rapid downregulation of β-adrenoceptors in brains of rats after 3 days of oral treatment with sibutramine.

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Measurement of β-Adrenoreceptor-Stimulated Adenylate Cyclase

Purpose and Rationale

Noradrenaline stimulates the β-adrenoreceptor-linked adenylate cyclase in rat brain. Reduction of this stimulation after treatment with antidepressants is an indicator for receptor downregulation.

Procedure

Groups of male Sprague–Dawley rats are treated intraperitoneally twice daily for 14 days with saline, 10 mg/kg desimipramine or the test compound. The rats are decapitated, frontal cortices removed, and placed into ice-cold Krebs physiological buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 mM $NaHCO_3$, 100 μM $Na_2S_2O_5$, 25 μM EDTA, and 10 mM glucose), pH 7.4. Cortices are cut 350 × 350 μ at right angles, added to 20 ml Krebs physiological buffer in 50 ml conical flasks, and separated by vortex mixing. The slices are incubated at 37 °C for 1 h in a shaking water bath with the buffer replaced by freshly oxygenated medium every 15 min. After this step, 20 μl [^{3}H] adenine is added to each flask. The flasks are capped, and the slices are incubated for an additional 30 min at 37 °C. This medium is then discarded and the slices washed with 20 ml fresh buffer. Most of the medium is removed and 30 μl aliquots of the packed slices pipetted into tubes containing 300 μM 3-isobutyl-1-methylxanthine in 270 μl Krebs physiological buffer. After incubation at 37 °C for 15 min, 30 μl of noradrenaline (final concentration 100 μM) or 30 μl buffer are added to each tube and incubated for an additional 10 min. The reaction is stopped by addition of 0.5 ml 1 M HCl. The tubes are placed in ice for 10 min before addition of 0.5 ml NaOH and then centrifuged at 3000 g for 10 min at 4 °C. The supernatants are transferred to tubes containing [^{14}C]-cyclic AMP (approximately 10,000 cpm) to monitor the recovery of [^{3}H]-cyclic AMP. The radio-labeled cyclic nucleotides are separated by two-stage column chromatography on alumina neutral WN-3 and Dowex AG 50 W-X4 anion exchange resin.

Evaluation

The activity of adenylate cyclase is calculated as the conversion of [^{3}H]-adenine to [^{3}H]-cyclic AMP. After treatment with antidepressants, this conversion rate is not altered without stimulation by noradrenaline but significantly reduced in slices treated with the maximally stimulating concentration of 100 μM noradrenaline.

Critical Assessment of the Method

Determination of β-adrenoreceptor-stimulated adenylate cyclase is another parameter for measurement of downregulation of adrenoreceptors by chronic treatment with antidepressants.

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[^{3}H]Yohimbine Binding to α_2-Adrenoceptors in Rat Cerebral Cortex

Purpose and Rationale

This binding assay is used to investigate the interaction of compounds at central α_2 receptors and may indicate possible modes of action for antidepressant, antihypertensive, and other classes of compounds.

[^{3}H]Yohimbine is a selective antagonist for $\alpha_2$receptors (Starke et al. 1975). The use of an antagonist radioligand avoids the complexity of saturation curves that can be observed with radiolabeled agonist ligands. Furthermore, a [^{3}H]antagonist label for the α_2 receptor permits a better evaluation of $\alpha_{2/}a_1$ selectivity than a [^{3}H]agonist label, since a_1 affinities are measured with a [^{3}H] antagonist ([^{3}H]WB 4101).

Chronic treatment with desipramine has been shown to decrease the binding of [^{3}H]DHA to rat brain cortical β-receptors. Some investigators have reported that coadministration of yohimbine causes β-receptor downregulation to occur after fewer antidepressant treatments (Johnson et al. 1980; Scott and Crews 1983). Therefore, it may be of interest to investigate compounds with yohimbine-like properties as antidepressant candidates themselves or in conjunction with antidepressant candidates.

Procedure

Reagents

1. 20-fold concentrated buffer:
   - 2.36 M NaCl 137.92 g/l
   - 100 mM KCl 7.45 g/l
   - 200 mM glucose 36.03 g/l in 0.5 M Tris, pH 7.4
2. Twofold concentrated buffer:
   - Fold dilution of reagent 1 in deionized water
3. Standard buffer:
   - 20-fold dilution of reagent 1 in deionized water
4. [Methyl-^{3}H] yohimbine (72–86 5.Ci/mmol) is obtained from New England Nuclear:
   - For IC_{50} determinations: [^{3}H] Yohimbine is diluted to 40 nM in deionized H_2O.

- 50 µl of this solution in a 2-ml reaction volume gives a final concentration of 1 nM.

5. L-NE bitartrate is made up to 10 mM in 0.01 N HCl. 20 µl of this solution gives a final concentration of 100 µM in 2 ml of reaction mixture.
6. Test compounds. For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-4} to 10^{-7} M. Six or seven concentrations are used routinely.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cortices are weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle (0.009–0.010 cm clearance). The homogenate is centrifuged at 1000 g for 10 min (approximately 3000 rpm using the Sorvall RC-5 centrifuge with heads SS-34 or SM-24). The supernatant is then recentrifuged at 17,000 g for 20 min (approximately 12,500 rpm) using the Sorvall RC-5 centrifuge and heads SS-34 or SM-24). The pellet (P2) is resuspended in the original volume of 0.32 M sucrose and stored on ice.

Binding Assay

430 µl	H_2O
1000 µl	Twofold concentration buffer (reagent 2)
20 µl	Drug or 10 mM L-NE bitartrate or vehicle
50 µl	^{3}H-yohimbine (reagent 4)
500 µl	Tissue

Tubes are vortexed and incubated at 25 °C for 10 min. Bound [^{3}H]yohimbine is captured via filtration under reduced pressure. The filters are washed three times with 5 ml aliquots of buffer (reagent 3). The filters are then counted in 10 ml of Liquiscint scintillation fluid.

Evaluation

Specific binding of [^{3}H]yohimbine is the difference between total bound (in the presence of vehicle) and nonspecifically bound (in the presence of 100 µML-NE bitartrate). Percent inhibition of specific [^{3}H]yohimbine binding is calculated for each concentration of test drug and IC_{50} values determined by computer-derived log-probit analysis.

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Test for Anticholinergic Properties by [^{3}H]-QNB Binding to Muscarinic Cholinergic Receptors in Rat Brain

Purpose and Rationale

Several tricyclic antidepressants exert considerable anticholinergic effects which limit the therapeutic use in some patients. Amitryptiline has the greatest incidence of these side effects and is the most potent in binding to muscarinic receptors. Desipramine, which exhibits less incidence of atropine like side effects, shows a lower affinity for muscarinic receptors. Since there is no evidence that the anticholinergic effects contribute to the therapeutic efficacy, antidepressant drugs with low anticholinergic effects are desired.

Quinuclidinyl benzilate (QNB) is a specific muscarinic cholinergic antagonist in both peripheral and central tissues. The binding characteristics of ^{3}H-QNB were first described by Yamamura and Snyder (1974), who showed that this ligand was displaced by muscarinic antagonists but not by nicotinic or noncholinergic drugs. The levorotatory isomer being more potent than the racemate is used as the radioactive ligand.

Procedure

Reagents

L-[Benzilic-4,4-^{3}H]-quinuclidinyl benzilate (30–40 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations [^{3}H]-QNB is made up to a concentration of 40 nM and 50 µl added to each tube (yielding a final concentration of 1 nM in the assay).

Atropine sulfate is obtained from Sigma Chemical Co.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

Male Wistar rats are killed by decapitation and their brains rapidly removed. After removal of the cerebellum, each brain is homogenized in 10 volumes of ice-cold 0.32 M sucrose in a Potter-Elvejhem glass homogenizer. The homogenate is then centrifuged at 1000 g for 10 min and the pellet discarded. The resultant supernatant is further dispersed and used for [^{3}H]-QNB binding studies. Specific binding is 20 % of the total added ligand and approximately 95 % of the total bound ligand.

Assay

100 µl	0.5 M Na/K phosphate buffer, pH 7.4 (134 g $Na_2HPO_4 7H_2O$ + 68 g KH_2PO_4)
780 µl	H_2O
50 µl	^{3}H-QNB stock solution
20 µl	Vehicle (for total binding) or 2 mM atropine (for nonspecific binding)
1000 µl	0.05 M Na/K phosphate buffer (1:10 diluted) pH7.4
50 µl	Tissue suspension

Tissue homogenates are incubated for 60 min at 25 °C with 1 nM ^{3}H-QNB and varying drug concentrations. With each binding assay, triplicate samples containing 2 µM atropine sulfate are incubated under identical conditions to determine nonspecific [^{3}H]-QNB binding. After incubation, the samples are cooled and then rapidly filtered through glass filters (Whatman GF/B) under reduced pressure. The filters are washed three times with 5 ml of ice-cold phosphate buffer and then placed in scintillation vials. After the addition of 10 ml of counting cocktail, radioactivity is assayed by liquid scintillation spectrophotometry.

Evaluation

Specific [^{3}H]-QNB binding is determined by the difference between total [^{3}H]-QNB and bound radioactivity in the presence of 2 µM atropine sulfate. Data are converted to percent specific bound [^{3}H]-QNB displaced by test drugs and IC_{50} values obtained from computer-derived log-probit analysis. The inhibition at each drug concentration is the mean of triplicate determinations.

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Monoamine Oxidase Inhibition: Inhibition of Type A and Type B Monoamine Oxidase Activities in Rat Brain Synaptosomes

Purpose and Rationale

The mood-elevating effects of the antituberculosis drug iproniazid have been observed clinically. The mode of action was elucidated to be the inhibition of the enzyme monoamine oxidase. This was followed by wide use of monoamine oxidase inhibitors for the treatment of depression. However, side effects due to interaction with dietary amines have been observed. The biological role of monoamine oxidase is to regulate the levels of endogenous amines (norepinephrine, dopamine, and serotonin) and exogenously administered amines. Based on different substrate and inhibitor specificities two forms of monoamine oxidase (A and B) were described. Dopamine and tyramine are substrates for both types, serotonin and epinephrine are substrates for type A, and β-phenylethylamine and benzylamine are substrates for type B. Iproniazid and tranylcypromine are nonselective inhibitors, clorgyline is a selective inhibitor of type A, and deprenyl and pargyline are selective inhibitors of type B. It has been suggested that treatment with selective blockers of type B results in less detrimental food interactions.

Procedure

Tissue Preparation

Male Wistar rats weighing 150–250 g are sacrificed and the brains rapidly removed. Whole brain minus cerebellum is homogenized in 9 - volumes of ice-cold, phosphate-buffered 0.25 M sucrose, using a Potter-Elvejhem homogenizer. The homogenate is centrifuged at 1000 g for 10 min and the supernatant decanted and recentrifuged at 18,000 g for 20 min. The resulting pellet (P_2) is resuspended in fresh 0.25 M sucrose and recentrifuged at 18,000 g for 20 min. The washed pellet is resuspended in the original volume of 0.25 M sucrose and serves as the tissue source for mitochondrial monoamine oxidase.

Assay

50 µl	0.5 M PO_4 buffer, pH 7.4
450 µl	H_2O
100 µl	H_2O or appropriate drug concentration
200 µl	Tissue suspension

The tubes are preincubated for 15 min at 37 °C and the assay started by adding 100 µl of substrate (^{14}C-5-HT or ^{14}C β-phenylethylamine) at 10 s intervals. The tubes are incubated for 30 min at 37 °C and the reaction stopped by the addition of 0.3 ml of 2 N HCl. Tissue blank values are determined by adding the acid before the substrate. Seven ml of diethylether are added; the tubes are capped and shaken vigorously for 10 min to extract the deaminated metabolites into the organic phase, which is separated from the aqueous phase by centrifugation at 1000 g for 5 min. A 4 ml aliquot of the ether layer is counted in 10 ml of liquid scintillation counting cocktail.

Evaluation

The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values are determined by log-probit analyses.

For example, deprenyl shows IC_{50}-values of 3.9×10^{-6} against MAO A and 3.0×10^{-8} against MAO B.

Modification of the Method

Colzi et al. (1992) measured dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) outflow after a reversible MAO-A inhibitor with a brain microdialysis technique in rats.

Frankhauser et al. (1994) tested the interaction of MAO inhibitors and dietary tyramine by measurement of peak systolic blood pressure in conscious rats. The increase of blood pressure after oral application of tyramine was potentiated by pretreatment with MAO inhibitors. Reversible MAO inhibitors could be differentiated from nonselective, irreversible MAO inhibitors.

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In Vivo Tests

Catalepsy Antagonism

Purpose and Rationale

Observations about **cataleptic behavior in chicken** have first been described more than 300 years ago (Schwenter 1636; Kircher 1646) and again reported about 100 years ago (Czermak 1873; Heubel 1877; Verworn 1898). This phenomenon was used by Vogel and Ther (1963) as a simple method to detect antidepressants besides other central stimulants.

Procedure

Adult white Leghorn chickens are used. The animal is grasped with both hands whereby the left hand pushes the chicken slightly down and the right hand supports the animal from the ventral side. Immediately, the chicken is turned on its back and held with the right hand for 1 min. Usually, cataleptic numbness occurs immediately. The cataleptic state can be sustained by slightly pushing the head of the animal on the table. After 1 min the right hand is carefully withdrawn. The chicken remains in the cataleptic state for several min up to 1 h. The cataleptic rigor is interrupted by noise or fast movements of the observer. Clapping of the hands above the head arouses the chicken which jumps up and runs away. The chicken is always aroused by pulling on the wings. The animals are pretested in order to be sure about the cataleptic behavior of an individual chicken. As already found by previous investigators, the experiment can be repeated several times. Control studies showed that in untreated animals the phenomenon could be elicited six times every 30 min

during a period of 5 days. After the control experiments, the animals are injected i.p. with the test compound or the vehicle. The test is performed four times every 30 min during 2 h.

Evaluation

The test is considered to be positive if the cataleptic rigor does not occur after treatment or is interrupted spontaneously within 1 min at least twice during the 2 h test period. The suppression of the cataleptic phenomenon is the criterion for a positive response. Furthermore, the arousal after hand-clapping or pulling on the wings is recorded in order to register central sedative effects. In order to obtain dose–response curves 12 animals per group are treated with various doses. ED_{50} values are calculated.

Critical Assessment of the Method

The specificity of the method has been tested. Antidepressant agents like imipramine and ethyltryptamine-acetate or other monoamino-oxydase-inhibitors and D-desoxyephedrine show a dose-dependent effect. Moreover, the effects were dependent on time. ED_{50} values decreased on consecutive days after imipramine and monoamino-oxydase inhibitors but not after D-desoxyephedrine. Therefore, the test can be considered as specific for central stimulants allowing the possibility to distinguish between antidepressants and central stimulants of the amphetamine type.

Modifications of the Method

Simiand et al. (2003) and Gabriel et al. (2005) described **tonic immobility in gerbils** as a model for detecting antidepressant-like effects. When grasped by the skin of the nape and lifted into the air, the young of many altricial mammals exhibit an immobility response in which they tuck their limbs against their bodies and remain inert. Such a response can be induced also in adults of some species, such as Mongolian gerbils (De la Cruz and Junquera 1993). To test antidepressant-like effects in the tonic immobility paradigm in gerbils, animals (six to nine per group) were held on a flat surface and were firmly pinched for 15 s at the scruff of the neck using the thumb and the index finger. They were then placed on parallel bars (4 mm in diameter, 28 cm long, spaced 5 cm apart and having a 3-cm difference in height). The front paws were gently placed on the upper bar and the hind paws on the lower bar. The duration of tonic immobility was measured in five successive trials with a 30-s intertribal interval. Each trial ended when an animal started to move or after 90 s of immobility. Experiments were started 30 min after i.p. administration of test drug.

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Despair Swim Test

Purpose and Rationale

Behavioral despair was proposed as a model to test for antidepressant activity by Porsolt et al. (1977, 1978). It was suggested that mice or rats forced to swim in a restricted space from which they cannot escape are induced to a characteristic behavior of immobility. This behavior reflects a state of despair which can be reduced by several agents which are therapeutically effective in human depression.

Procedure

Male Sprague–Dawley rats weighing 160–180 g are used. They are brought to the laboratory at least 1 day before the experiment and are housed separately in Makrolon cages with free access to food and water. Naive rats are individually forced to swim inside a vertical Plexiglas cylinder (height: 40 cm; diameter: 18 cm, containing 15 cm of water maintained at 25 °C). Rats placed in the cylinders for the first time are initially highly active, vigorously swimming in circles, trying to climb the wall, or diving to the bottom. After 2–3 min activity begins to subside and to be interspersed with phases of immobility or floating of increasing length. After 5–6 min immobility reaches a plateau where the rats remain immobile for approximately 80 % of the time. After 15 min in the water the rats are removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages. They are again placed in the cylinder 24 h later and the total duration of immobility measured during a 5 min test. Floating behavior during this 5 min period has been found to be reproducible in different groups of rats. An animal is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position, its nose just above the surface. Test drugs or standard are administered 1 h prior to testing. Since experiments with the standard drug (imipramine) showed that injections 1, 5, and 24 h prior the test gave the most stable results in reducing floating these times are chosen for the experiment.

Evaluation

Duration of immobility is measured in controls and animals treated with various doses of a test drug or standard. Antidepressant drugs, but also stimulants like amphetamine and caffeine, reduce duration of immobility. Dose responses can be evaluated.

Critical Assessment of the Method

The method, also called "forced swim test," has been used and modified by many authors, e.g., Kauppila et al. (1991), van der Heyden et al. (1991).

Advantages of the method are the relative simplicity and the fact that no interaction with other drugs is necessary. Like in other behavioral tests, e.g., the catalepsy test in chickens, not only antidepressants and monoamino-oxydase inhibitors but also central stimulants give positive results.

In a critical review of the forced swimming test, Borsini and Meli (1988) discussed the various modifications and proposed rats to be more suitable than mice for detecting antidepressant activity.

The model is purely behavioral without presuppositions concerning the mechanism of action of potential antidepressants. It is sensitive to a large number of atypical antidepressants otherwise inactive in the more classical tests. The rat version seems to be more selective (fewer false positives) and the mouse version more sensitive (fewer false negatives) (Porsolt et al. 1991).

Natoh et al. (1992) reviewed the theoretical background of the forced swimming test and the various factors which can possibly affect the results.

Modifications of the Method

Wallach and Hedley (1979) reported positive results with antihistamines and Giardina and Ebert (1989) with an ACE inhibitor.

Differentiation is achieved by the simultaneous evaluation of motor activity.

Cervo and Samanin (1987) suggested potential antidepressant activities of a selective serotonin1A agonist based on anti-immobility activity in the forced swimming test in rats without effect on open-field activity.

Nishimura et al. (1988, 1989, 1993) published a modification of the forced swim test using straw suspension in the water tank. The apparatus used was a vertical glass cylinder (height: 40 cm, diameter: 18 cm) equipped with four pieces of straw (length 24 cm, diameter: 0.4 cm) that were suspended from above. The cores of the straws were filled with cotton rope. The straws were painted black from the surface of the water to a height of 10 cm. The apparatus was filled with water to a height of 15 cm and maintained at 25 °C. Thirty minutes after treatment with drugs or saline, the rats were placed in the apparatus without straw suspension and the total duration of immobility measured for a period of 5 min. Immediately thereafter, four pieces of straw were suspended and the total duration of immobility in the following 5-min observation period again measured.

In an effort to enhance the sensitivity of the traditional forced swim test in the rat so that it can be responsive to specific serotonin reuptake inhibitors, Lucki (1997) proposed several simple procedural modifications. These developments include increasing the water depth to 30 cm from the traditional 15–18 cm and using a time sampling technique to rate the predominant behavior over a 5-s interval. Specific components of active behavior can be distinguished: (1) climbing behavior (also known as trashing), which is defined as upward-directed movements of the forepaws along the side of the swim chamber; (2) swimming behavior, the movement (usually horizontal) throughout the swim chamber that also includes crossing into another quadrant; and (3) immobility, which is defined as when no additional activity is observed other than that required to keep the rat's head above the water. As a result of the increase in water depth, there is considerably less immobility than in the traditional test because the animals cannot have contact with the cylinder bottom. The major advantage of this modified test is that it reveals that catecholaminergic agents decrease immobility with a corresponding increase in climbing behavior, whereas 5-HT-related compounds, such as specific serotonin reuptake inhibitors, also decrease immobility but increase swimming behavior.

López-Rubaicava and Lucki (2000) described strain differences in the behavioral effects of antidepressant drugs in the rat forced swim test when the noradrenergic antidepressant, desipramine, and the serotonergic antidepressants fluoxetine and 8-OH-DPAT were tested in Wistar–Kyoto rats and Sprague Dawley rats.

Sun and Alkon (2003, 2004) described the **open-space swimming test** to index antidepressant activity. Rats were placed individually in a round pool with a diameter of 152 cm and a height of 60 cm was filled with 40 cm water at 21 °C. Rats were free to swim (or not to swim) for 15 min and then returned to their home cage. The same procedure was followed 24 h later for 3 days. The swimming/drifting path was recorded with a video tracking system. The measurement is considered to be more objective than the forced swimming test. The procedure induces depressive behavior and impairs learning and memory in the rats.

Galea et al. (2001) found that estradiol alleviates depressive-like symptoms in an animal model of postpartum depression. The effect of hormone withdrawal following hormone-simulated pregnancy on depressive-like behavior in the forced swim test was investigated in female Long-Evans rats.

Using the forced swim test or elevated plus maze, Stoffel and Craft (2004) studied ovarian hormone withdrawal–induced "depression" in female rats.

Gregus et al. (2005) used the forced swim test to study the effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats.

Buckett et al. (1982) described an automated apparatus for behavioral testing of typical and atypical antidepressants in **mice**. A multichannel system can test 10 mice simultaneously. Each mouse is placed in the beam of a Doppler radar head and horn assembly. The moving mouse

causes reflections of a frequency differing from the transmitted signal. Within the Doppler head these reflected waves are mixed with a proportion of transmitted waves to produce a difference signal proportional to the activity of the mouse within the beam. The output of each Doppler head is fed to an amplifier whose gain has been calibrated to compensate for differences in sensitivity between individual heads. The method is claimed to eliminate human error and bias and to allow the testing of large numbers of compounds.

Alpermann et al. (1992) used a slightly modified behavioral despair test in mice. Sixty minutes after administration of the test compounds, the animals are placed in glass cylinders containing water up to a height of 10 cm (water temperature 22–24 °C). From the second minute onward, immobility of each mouse is rated every 30 s. After 10 observations mean values and standard deviations in each treatment group are calculated. Compared with the immobility score of the control group percent reduction can be calculated.

Nomura et al. (1982) published a modification of the despair swim test in mice involving a small water wheel set in a water tank. Mice placed on this apparatus turned the wheel vigorously, but when they abandoned attempts to escape from the water, the wheel stopped turning. The number of rotations of the water wheel were counted.

Hata et al. (1995) studied the behavioral characteristics of SART (specific alterations of rhythm in temperature)-stressed mice in forced swimming tests and evaluated the effects of anxiolytics and antidepressants.

Weiss et al. (1998) reported selective breeding of rats for high and low motor activity in the swim test. Sprague Dawley rats were selectively bred for low motor activity (low struggling time/high floating time), while others were bred for high motor activity (high struggling time/low floating time).

Lucki et al. (2001) investigated the sensitivity to desipramine, a selective norepinephrine reuptake inhibitor, and fluoxetine, a selective serotonin reuptake inhibitor, in seven inbred and four outbred mouse strains. Considerable differences in the results of the forced swim test were found.

Sachdev et al. (2002) used the forced swim model of depression in rats to investigate the antidepressant effect of different frequencies of transcranial magnetic stimulation.

Krahe et al. (2002) tested the effects of rotational side preferences in immobile behavior of normal mice in the forced swimming test.

Buckley et al. (2004) used both the mouse forced swim test and the rat forced swim test to characterize a nicotinic agonist.

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Tail Suspension Test in Mice

Purpose and Rationale

The "tail suspension test" has been described by Steru et al. (1985) as a facile means of evaluating potential antidepressants. The immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. Clinically effective antidepressants reduce the immobility that mice display after active and unsuccessful attempts to escape when suspended by the tail.

Procedure

Male Balb/cJ mice weighing 20–25 g are used preferentially. They are housed in plastic cages for at least 10 days prior to testing in a 12 h light cycle with food and water freely available. Animals are transported from the housing room to the testing area in their own cages and allowed to adapt to the new environment for 1 h before testing. Groups of 10 animals are treated with the test compounds or the vehicle by intraperitoneal injection 30 min prior to testing. For the test the mice are suspended on the edge of a shelf 58 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility is recorded for a period of 5 min. Mice are considered immobile when they hang passively and completely motionless for at least 1 min.

Evaluation

The percentage of animals showing the passive behavior is counted and compared with vehicle-treated controls. Using various doses, ED_{50} values can be calculated.

Critical Assessment of the Method

The tail suspension test has been found to be an easy method to test potential antidepressant compounds. However, it has been reported that several mouse strains are essentially resistant to tail-suspension-induced immobility.

Modifications of the Method

Chermat et al. (1986) adapted the tail suspension test to the rat.

Porsoit et al. (1987)and Stéru et al. (1987) recommended the use of the automated tail suspension test for the primary screening of psychotropic agents. A specially developed computerized device automatically measures the duration of immobility of six mice at one time and at the same time provides a measure of the energy expended by each animal, the power of the movements.

Vaugeois et al. (1996) described a genetic mouse model of helplessness sensitive to imipramine. Lines of mice were selectively bred to diverge in their spontaneous helplessness in the tail-suspension test. By the second generation of selection, only mice of the helpless line were sensitive to the antidepressant imipramine.

Mayorga and Lucki (2001) demonstrated limitations on the use of the C57BL/6 mouse in the tail-suspension test. Under baseline conditions, the majority of C57BL/6 mice climbed up their tails during the 6 min test session. The occurrence of this behavior was not found in other strains of mice.

Liu and Gershenfeld (2001) found genetic differences in the tail-suspension test among 11 inbred strains of mice as far as basal immobility was concerned. Surprisingly, the reduction of immobility by imipramine was independent of basal immobility.

Ripoli et al. (2003) tested antidepressant-like effects in various mice strains in the tail-suspension test and found considerable differences depending on strain and test drug.

Shearman et al. (2003) tested antidepressant-like effects of a cannabinoid CB1 receptor inverse agonist in mice using an automated tail-

suspension apparatus (TSE Systems, Bad Homburg, Germany) with a tail hanger connected to a precision load cell. Mice remained suspended by the tail, at a height of 35 cm from the tabletop, for 6 min. During this time the load cell recorded the mouse's movements and transmitted the information to a computer, which then recorded the rate of immobility within the course of the session, and calculated total duration of immobility.

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Learned Helplessness in Rats

Purpose and Rationale

Animals exposed to inescapable and unavoidable electric shocks in one situation later fail to escape shock in a different situation when escape is possible (Overmier and Seligman 1967; Maier and Seligman 1976). This phenomenon was evaluated as a potential animal model of depression (Sherman et al. 1979; Martin et al. 1986; Christensen and Geoffroy 1991; Tejedor del Real et al. 1991; Weiss and Kilts 1998).

Procedure

Learned helplessness is produced in male Sprague Dawley rats (300 g) by exposure to electric shock (0.7 mA) for 1 h on a schedule of 10 s of shock/min. The apparatus is a 30 × 45 × 30 cm box with a grid floor. At a height of 20 cm above the floor, a platform (7.5 × 7.5 cm) can be inserted through one side wall to allow a jump-up escape response. The platform is not available during training. After the appropriate treatment, the animals are tested for acquisition of a jump-up escape in the same apparatus. At the beginning of a trial, the platform is pushed into the box and a 0.4 mA shock initiated. Shock is terminated in 10 s if the

animal has not escaped onto the platform by this time. If an escape response occurred, the animal is allowed to remain on the platform for the duration of 10 s, then returned to the grid floor. Ten such trials with an intertrial interval of 20 s are given. In a naive control group of rats, this training resulted in 80 % acquiring learned helplessness behavior. Drugs are given before the training and the test period.

Evaluation

A drug is considered to be effective if the learned helplessness is reduced and the number of failures to escape is decreased. Imipramine was found to be active only after repeated applications. A benzodiazepine was effective, whereas chlorpromazine was ineffective.

Critical Assessment of the Method

The "learned helplessness" in the rat can be regarded as an additional measure for antidepressant activity in addition to other tests. The test is time consuming and its specificity questionable. The major drawback of the model is that most of the depression-like symptomatology does not persist beyond 2–3 days following cessation of the uncontrollable shock.

Modifications of the Method

Vaccheri et al. (1984) used an apparatus with a lever to be pressed to interrupt the shock. Giral et al. (1988), Porsolt et al. (1990), and Simiand et al. (1992) used shuttleboxes for escape.

Curzon et al. (1992) described a similar rat model of depression. Rats were tested in an open field after being restrained by taping them to wire grids for 2 h on the preceding day. The reduced activity is antagonized by antidepressant drugs.

A modification of the test incorporating aspects of chronic mild stress paradigm was described by Gambarana et al. (2001) A model of anhedonia is based on the finding that exposure to repeated unavoidable stress prevents the development of an appetitive behavior induced and maintained by a highly palatable food (vanilla sugar) in rats fed ad libitum (Ghiglieri et al. 1997).

The chronic stress procedure involves restraint and novel housing and avoids the problems associated with food deprivation (Reid et al. 1997).

Vollmayr et al. (2001) studied brain-derived neurotrophic factor (BDNF) stress responses in rats bred for learned helplessness in a system provided by TSE Systems, Bad Homburg, Germany. Two strains of rats were bred, one which reacts with congenital helplessness to stress and one which congenitally does not acquire helplessness when stressed. In rats with congenital helplessness, acute immobilization stress does not induce a reduction of BDNF expression in the hippocampus, which is observed in Sprague Dawley rats that do not acquire helplessness.

Vollmayr and Henn (2001) proposed key factors that can be manipulated to enhance both the usability and the reliability of the rat helplessness paradigm.

Rats bred for congenital learned helplessness (King et al. 1993; Shumake et al. 2000) responded less to sucrose but did not show deficits in activity or learning (Vollmayr et al. 2004).

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Muricide Behavior in Rats

Purpose and Rationale

Horovitz et al. (1965) described a selective inhibition of mouse-killing behavior in rats (Karli 1956; Karli et al. 1969) by antidepressants. The test can be used to evaluate antidepressants such as tricyclics and MAO inhibitors.

Procedure

Male Sprague–Dawley rats (300–350 g) are isolated for 6 weeks in individual cages. They have access to food and water ad libitum. One mouse is placed into the rat's cage. About 10–30 % of rats kill the mouse by biting the animal through the cervical cord. Only rats consistently killing mice within 5 min after presentation are used for the test. The mice are removed 15–45 s after they have been killed in order to prevent the rats from eating them. Drugs are injected i.p. to the rats

before the test. Mice are presented 30, 60, and 120 min after drug administration.

Evaluation

Failure to kill a mouse within 5 min is considered inhibition of muricidal behavior. Performing dose–response experiments, the ED_{50} is defined as the dose which inhibits mouse killing in 50 % of the rats.

Modifications of the Method

Injections of 5,7-dihydroxytryptamine into the lateral hypothalamus increased mouse-killing behavior in rats (Vergnes and Kempf 1982).

Molina et al. (1985) considered rats isolated for at least 1 month which do not kill mice in a 30 min test period as spontaneous nonkiller rats. In these animals, mouse-killing behavior could be induced by i.p. injection of 150 mg/kg p-chlorophenylalanine daily for 2 days or by electrolytic lesions in the dorsal and median raphe.

McMillen et al. (1988) studied the effects of housing and muricidal behavior on serotonergic receptors and interactions with novel anxiolytic drugs.

Critical Assessment of the Method

The mouse-killing behavior is inhibited not only by antidepressants but also by central stimulants like damphetamine (Horovitz et al. 1966; Sofia 1969a, b), some antihistamines (Barnett et al. 1969), and some cholinergic drugs (McCarthy 1966; Vogel and Leaf 1972; Wnek and Leaf 1973). Neuroleptics and benzodiazepines are active only in doses which impair motor performance.

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Behavioral Changes After Neonatal Clomipramine Treatment

Purpose and Rationale

Vogel et al. (1988, 1990a)and Hartley et al. (1990) reported that neonatally administered clomipramine produces changes in adult rats that resembles endogenous depression in man, based on earlier observations by Mirmiran et al. (1981) and Rodriguez-Echandia and Broitman (1983). This phenomenon has been studied by several research groups in many respects (Neill et al. 1990; Vogel et al. 1990b, c, d, e, 1996a, b; Velazquez-Moctezuma and Diaz-Ruiz 1992; Yavari et al. 1993; Maudhuit et al. 1995, 1996; Prathiba et al. 1995; 1997; 1998; 1999; Feenstra et al. 1996; Kinney et al. 1997; Bonilla-Jaime et al. 1998; Dwyer and Rosenwasser 1998).

Procedure

Three days after birth, all male pups of Sprague Dawley mother rats are cross-fostered and all female pups killed. Cross-fostering consists of removing all pups from their biological mothers and placing them with another lactating female (the nonbiological foster mother). Each litter is divided into two groups of approximately equal number. Each half of the litter is placed with a different lactating female (the foster mother), and each foster mother receives half the pups from two litters. All pups with each foster mother receive the same treatment (clomipramine or saline), and the two pup groups of each original litter are assigned to different treatments. Thus, each original pup litter contributes equally to experimental and control groups. Each pup is injected subcutaneously between the shoulder blades with 15 mg/kg clomipramine hydrochloride or saline vehicle twice daily on postnatal days 8 through 21. At 1 month of age the pups are weaned and housed as litters until approximately 3 weeks prior testing, at which time they are individually housed.

All testing is conducted in a Coulbourn operant conditioning chamber (Lehigh Valley, PA) placed in a quiet area. The floor rods are connected with a shock generator and a shock scrambler.

Behavioral testing is commenced at 3 months postnatally. Each test pair consists of one clomipramine-treated and one vehicle-treated rat. Prior to testing, all clomipramine- and saline-treated rats are paired by body weight (within 5 g) to diminish size differences that could affect behavioral results. Pairs remain the same throughout the testing.

For evaluation of drugs, clomipramine-treated rats are administered subcutaneously for 4 days twice daily saline, or standard drug (10 mg/kg imipramine), or test drug.

Tests are done daily for 4 days. On the first day, the animals are placed in the chamber for 12 min habituation. On days 2–4, the sessions start with a 2 min habituation period followed by 10 min of shock delivery. Shocks (1.33 mA, 0.5 s duration) are delivered on a variable schedule with a minimum of 5 and a maximum of 10 s shock intervals. This results in a total of 70–80 shocks within the 10-min session.

Two observers score simultaneously. Each watches one rat of the pair. The rats are identified by a red mark on the fur. The behaviors produced are almost totally in response to shock delivery; both animals are almost immobile between the shocks. Both observers are blind to the treatment conditions of the animals.

Offensive and defensive behaviors are scored including the following four behaviors:

1. An upright position which is part of a mutual upright posture in which the dominant rat towers over the submissive rat. The submissive rat rears on its hind legs, with the head positioned at an upward angle, the forepaws extended toward the attacking animal, and the ventral surface of the body continually facing the opponent.

2. In the offensive crouch, the dominant animal turns its flank toward the subordinate; the submissive crouch is characterized by freezing in a motionless crouching position.
3. Mounting behavior is frequently seen and is the same as seen in the male prior to copulating with a female.
4. Leaps in response to the shock which are directed to the other rat are scored as aggressive responses.

Three defensive or submissive behaviors were observed:

1. Defensive upright posture.
2. Submissive crouch.
3. Supine position in submission to the dominant rat. In this system each offensive behavior by one rat is partly defined by a defensive behavior of the other rat and vice versa.

Evaluation

The individual behaviors of the treatment and control groups are listed for offensive and defensive behavior on days 1, 2, and 3 and the total of scores calculated for each group. Analysis of variance is applied to offensive, defensive, difference (offensive minus defensive), and total (offensive plus defensive) behavior scores. Rats treated postnatally with clomipramine have significantly fewer offensive and significantly more defensive behaviors. This effect is ameliorated by treatment with antidepressant drugs.

Critical Assessment of the Test

Not only clomipramine but also other psychotropic drugs induce changes in behavior of adult rats after treatment in neonatal age (Drago et al. 1985; Frank and Heller 1997; Hansen and Mikkelsen 1998); however, clomipramine induces the most pronounced effects (Velazquez-Moctezuma et al. 1993; Vogel and Hagler 1996; Hansen et al. 1997). Specificity of the procedure to evaluate potential antidepressant compounds remains to be established.

Modifications of the Method

Neonatal treatment with clomipramine not only reduces shock-induced aggression but also induces REM sleep abnormalities (Mirmiran et al. 1981; Vogel et al. 1990b, c; Frank and Heller 1997), decreases intracranial self-stimulation (Vogel et al. 1990d), increases activity in open-field tests in adulthood (Hartley et al. 1990; Prathiba et al. 1997), increases immobility in the forced swim test (Velazquez-Moctezuma and Diaz-Ruiz 1992), reduces adult male rat sexual behavior (Neill et al. 1990; Velazquez-Moctezuma et al. 1993; Vogel et al. 1996; Bonilla-Jaime et al. 1998,), and influences the hypothalamic-pituitary-adrenal axis in adult rats (Feenstra et al. 1996; Prathiba et al. 1998, 1999). Vázquez-Palacios et al. (2005) studied antidepressant effects of nicotine and fluoxetine in the animal model of depression induced by neonatal treatment with clomipramine.

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Chronic Stress Model of Depression

Purpose and Rationale

The chronic mild stress paradigm was developed in order to simulate in animals the symptom of anhedonia, a major feature of depression (Willner et al. 1987, 1992; Forbes et al. 1996; Willner 1997; Willner and Mitchell 2002; Grippo et al. 2003; Konkle et al. 2003; Pijlman et al. 2003; Strekalova et al. 2004). The model has been used to test compounds with antidepressant activity (Moreau et al. 1996; Kopp et al. 1999; Papp and Wieronska 2000; Harkin et al. 2002; Ducottet et al. 2003; Gabriel et al. 2005).

The stress regime was modified by several authors.

The stress regimen used by Moreau et al. (1996) consisted of exposing male Wistar rats each week to a variety of unpredictable, mild stressors such as repeated 1-h periods of confinement to small cages with bells ringing every 10 min, one period of overnight illumination, one overnight period of food and water deprivation immediately followed by 2 h of access to restricted food, one overnight period of water deprivation followed by 1 h exposure to an empty bottle, and one overnight period of group housing in a damp cage.

Grippo et al. (2003) exposed male Sprague Dawley rats to the following stressors in random order: continuous overnight illumination, 40° cage tilt along the vertical axis, paired housing, soiled cage, exposure to an empty water bottle immediately following a period of acute water deprivation, stroboscopic illumination, and white noise.

Ducottet et al. (2003) studied the effects of a selective nonpeptide corticotropin-releasing factor receptor 1 antagonist in a mild stress model of depression in **mice**. The method is based on a regimen published by Kopp et al. (1999) and by Griebel et al. (2002a, b).

Procedure

Male BALB/cByJIco mice, aged 9 weeks at the beginning of chronic mild stress experiments, were housed for 1 week individually in small cages with food and water ad libitum at a 12′12 h light/dark cycle and 21 °C. The chronic mild stress procedure consists of restraint in a small tube for 1 h, forced bath in water at 32 °C for 30 min, water and food deprivation for 15 h, paired housing in damped sawdust for 18 h, food restriction for 3 h, access to an empty water bottle for 2 h, and inversion of the light/dark cycle. Over 3 weeks, four to six of these procedures were applied every day in random order. Physical state was controlled weekly using a scale from 1 to 3 using piloerection, dirty fur, and body weight as symptoms. At the end of the experiment, the mice were subjected to a light/dark test.

Treatment started at week 5, while the stress regimen was continuing. Mice were divided into three groups ($n = 20$), each subjected to a 5-week period of chronic mild stress before being administered during a 4-week period with one of the following treatments: vehicle, daily i.p. injections of 10 mg/kg fluoxetine as standard, or test drug.

Evaluation

Data were analyzed using nonparametric exact analyses including the Monte Carlo correction. Data of physical state were analyzed with a Friedman test, followed by a Wilcoxon signed ranks test comparing initial week with chronic mild stress weeks and treatment weeks. Data of body

weight were treated with a Kruskal-Wallis test for each week followed by a permutation test for two independent samples. The light/dark test was submitted to a Kruskal-Wallis test followed by an a posteriori permutation test.

Modifications of the Method

Since the incidence of clinical depression is significantly higher in women than in men, Konkie et al. (2003) compared the effects of 6 weeks of chronic mild stress administration among female and male rats of Sprague Dawley and of Long–Evans strains.

Matthews and Robbins (2003) reported that a specific periodic neonatal maternal separation procedure leads to a robust constellation of behavioral changes in the adult rat that resembles core aspects of anhedonia in men.

Sammut et al. (2001, 2002) studied antidepressant reversal of interferon-a-induced anhedonia utilizing a three-bottle test (Muscat et al. 1991). A drinking and feeding monitoring system (TSE, Systems Bad Homburg, Germany) was used. In this test, three concentrations of sucrose (1 %, 8 %, and 32 %) are simultaneously presented to each individually caged rat. The three-bottle test allows demonstration of the monotonic relationship between intake and hedonic value as is characteristic of human studies.

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Novelty-Induced Hypophagia Test

Purpose and Rationale

The onset of the therapeutic response to antidepressant treatment exhibits a characteristic delay. While acute antidepressant treatment increases synaptic monoamines within minutes to hours, weeks of sustained treatment are required to induce therapeutic effects (Blier 2003). Few models have been developed in which chronic, but not acute, antidepressant treatment alters behavior (Brodnoff et al. 1988, 1989; Borsini et al. 2002; Cyran et al. 2002; Cyran and Mombereau 2004). Dulawa et al. (2004) and Dulawa and Hen (2005) used the novelty-induced hypophagia test to study chronic antidepressant effects in mice.

Procedure

For chronic testing, male Balb/cL mice were given various doses of test compound in the drinking water. On day 23 of treatment, mice were singly housed. They were trained to drink sweetened condensed milk for three consecutive days presented for 30 min each day in 10-ml serological pipettes with sippers attached with parafilm. Home cage testing occurred on day 28 when mice were briefly removed from their cages to position pipettes containing milk, and testing began when mice were returned to their home cages. The latency to drink and the volume consumed were recorded every 5 min for 30 min. Home cage testing occurred under dim lighting. Novel cage testing occurred on day 29, when mice were placed into new clean cages with the same dimensions but without shavings, with pipettes containing the milk positioned. Novel cage testing occurred under bright lighting with white paper under cages to enhance aversiveness. Again, the latency to drink and the volume consumed were recorded every 5 min for 30 min. For latency scores, a maximum cutoff of 600 s was used.

Evaluation

Two-factor ANOVA with the home/novel cage condition as a within-subjects factor was applied to latency values. For consumption data, a three-factor ANOVA with block (5-min intervals) and the home/novel cage condition as within-subject factors and drug as a between-subject factor was used to assess whether the novel cage was sufficiently anxiogenic to reduce consumption.

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Reduction of Submissive Behavior

Purpose and Rationale

Pairs of rats competing for food develop a dominant/submissive behavior, which can be measured in a competition test as the relative success of two food-restricted rats to gain access to a sweetened milk supply. Submissive behavior for one subject can be objectively measured as the amount of time spent at the feeder relative to that by the paired dominant animal. Malatynska and Kostowski (1984), Malatynska et al. (1995, 2002), and Knapp et al. (2002) described reduction of submissive behavior in rats as a test for antidepressant drug activity.

Procedure

Drugs were given by i.p. injection once a day during the treatment period (including weekends). On weekdays, drugs were administered 1 h before behavioral testing. The testing apparatus and the reduction of submissive behavior model procedure are described by Malatynska et al. (2002).

The apparatus was constructed at OmniTech (Columbus, OH) according to a design by Malatynska and Kostowski (1984). The transparent Plexiglas apparatus consisted of two identical chambers (24 × 17 × 14 cm) connected by a 4.5 × 4.5 × 52 cm passage. In the middle of the passage, a 10-ml glass beaker was placed into a hole cut in the floor of the passage. Prior to behavioral testing, the beaker was filled with sweetened milk (10 g sucrose/cup) through a hinged door in the ceiling of the passage.

The animals were randomly assigned to pairs prior to testing. They were group housed four to a cage between testing sessions so that paired animals were always separated. Behavioral testing was performed once a day for a 5-min period on weekdays. During the testing period, an animal received one point when it was observed drinking milk during each 5-s interval of the 5-min period. Four different observers contributed to scoring different animal pairs, and all were blinded to the treatment animals received. The design of the apparatus permits only one animal to drink at a time, but it is possible for both animals to have consumed milk during an interval. At the end of the 5-min period, the animals were returned to their home cages and given free access to food for 1 h. The animals were also given free access to food from the Friday afternoon following testing to the Sunday afternoon when they were once again food deprived. The animals showed normal weight gain during the course of the study.

Evaluation

Each animal of the pair was scored on each testing day for 2 weeks. Week 2 data for the two animals of a pair were tested for significant differences using the two-tailed *t*-test assuming unequal variance. The member of a pair having a significantly lower drinking score ($p < 0.05$) was defined as "submissive" and the partner as "dominant." Any

pairs not showing significant dominant-submissive relationship were dropped from the study. Submissive animals were treated with drugs or vehicle and dominant animals with vehicle for the next 3–4 weeks.

The data used for subsequent analyses are referred to as dominance level values. They are calculated as the difference in daily drinking scores between paired rats. The daily dominance level values were averaged over each week starting with the second week of testing for each pair selected. The *n* used for statistical purposes in the study was the averaged weekly dominance level value for a single pair of animals. The effect of treatment on dominance level values for animal pairs within a treatment group was tested for statistical significance by comparing dominance level values during the second week of testing (control period) to values measured during subsequent weeks of testing (treatment period) by analysis of variance (ANOVA) followed by post hoc unpaired *t*-tests for significance between control and treatment data. A *p*-value of less than 0.05 was used as the cutoff for significance. This approach gives information for the time of effect onset and effect for a selected period. Effect onset is defined as the period (weeks) at which the drug-induced reduction of the dominance level becomes significant relative to the control value measured after the second week before treatment is initiated. The relative effect of two treatments is determined for the same treatment period. This is done for the third week of treatment after all drugs have passed the onset of activity.

Modifications of the Method

Pinhasov et al. (2005) used automatic scoring by a multiple-subject video-tracking system for antidepressant activity testing of the reduction of submission behavior.

Leo et al. (2005) applied nuclear magnetic resonance-based metabolomics to the dominant-submissive rat behavioral model. Principal component analysis revealed a metabolite from milk sugar, galactose, as a discriminating factor between rats classified as dominant and those classified as submissive.

Malatynska et al. (2005) described submissive behavior in **mice** as a test for antidepressant drug activity.

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Animal Models of Bipolar Disorder

Purpose and Rationale

Bipolar disorder is a psychiatric condition characterized by episodes of mania, depression, and underlying mood instability. The disease is

distinguished by its cyclicity. Several attempts have been made to induce cyclic symptoms in animals. Preclinical studies with animal models of mania and depression have been developed to evaluate the potential efficacy of new psychotropic drugs. The main problem is to find a model that mimics mood cyclicity, which is a hallmark of bipolar disorder (Machado-Vieira et al. 2004). Antelman et al. (1995, 1998), Caggiula et al. (1998a, b), and Kucinski et al. (1999) reported that repeated exposure to cocaine or other stressors can induce an oscillation or cycling in various physiological systems.

Cao and Peng (1993) and Arban et al. (2005) described a rodent model of mania, in which hyperactivity is induced by the combination of D-amphetamine and chlordiazepoxide.

Procedure

Male CDI mice weighing 22–26 g were group housed at 21 ± 2 °C with 45–70 % humidity on a 12/12 h light/dark cycle. Test drugs were administered orally 60 min before the test. D-amphetamine sulfate (1.25 mg/kg i.p.) and chlordiazepoxide (6.25 mg/kg i.p.) were injected 30 min before the session. Locomotor activity was recorded using a Digiscan Analyzer (Model RXYZCM-8, Omnitech). Animals were individually placed in Plexiglas cages equipped with 48 photocells, and the total distance traveled (cm) by each mouse over a 30-min period was determined.

Evaluation

Statistical analysis was carried out using an ANOVA followed by Dunnett's test; results are expressed as mean ± SEM of the total distance traveled (in cm), with group size ranging from 9 to 12 mice.

Modifications of the Method

Petty and Sherman (1981) proposed hyperactivity in rats induced by intraventricular injection of 6-hydroxydopamine as a pharmacological model of mania.

Gould et al. (2001) reported differential sensitivity to lithium's reversal of amphetamine-induced open-field activity in two inbred strains of mice.

Gessa et al. (1995) studied sleep deprivation in rats by the platform method as a possible animal model of mania.

Gambarana et al. (2000) found that long-term lithium administration abolishes the resistance to stress in rats sensitized to morphine and discussed this as a model of mania.

Shaldubina et al. (2002) induced a biphasic locomotor response starting with inhibition and followed by excitation by quinpirole, a D2/D3 agonist, and recommended this as a model of bipolar disorder.

D'Aquila et al. (2004) treated rats for 4 weeks with imipramine. After a withdrawal time of 40 days, the animals showed a depressive-like behavior indicated by an increase of immobility in the forced swimming test.

El Mallak et al. (1995, 2003) and Decker et al. (2000) administered intracerebroventricularly 5 µl of ouabain (10^{-3} M) to rats, which induced hyper- and hypoactivity, and recommended this as a model of bipolar illness.

Wei et al. (2004) generated transgenic mice overexpressing the glucocorticoid receptor specifically in the forebrain. These mice display a significantly increased emotional lability and depressive-like behavior relative to wild type.

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Animal Models of Obsessive-Compulsive Disorder

Purpose and Rationale

Obsessive-compulsive disorder (OCD) is characterized by recurrent intrusive thoughts, feelings, or ideas (obsessions) and ritualistic actions (compulsions) which cause significant distress and dysfunction. The disease has gained increasing attention (Stein 2000, 2002). The relevance of ethological (naturally occurring) animal models (dogs: Stein et al. 1998; Overall 2000; birds: Woods-Kettelberger et al. 1997; horses: Nurnberg et al. 1997) has been discussed. Clinical studies and pharmacological experiments, mostly performed in rats (Joel et al. 2005; Tsaltas et al. 2005; Joel 2006), indicate the involvement of the serotoninergic system.

Deficits in spontaneous alterations produced by 8-OH-DPAT in rats were used as animal model of obsessive-compulsive behavior by Yadin et al. (1991), Fernández-Guasti

et al. (2003), van Kuyck et al. (2003), Ulloa et al. (2004a, b), and Agrati et al. (2005).

Procedure

Apparatus

The testing apparatus for spontaneous alterations was a Plexiglas T maze with goal boxes characterized by distinctive cues. All arms (including the main arm and the two goal boxes) measured 50 × 10 cm. Guillotine doors separated the main arm and the goal boxes from the main body of the maze. Small plastic cups were placed in the corners of both goal boxes. The maze was covered with clear Plexiglas lids.

Experimental Procedure

Wistar rats weighing 250–300 g were exposed to three training sessions. On the first training day, animals were habituated to the maze for 20 min during which time they were allowed to explore the entire area. Thereafter, they were exposed to chocolate milk in their home cages, in order to acquaint them to the novel stimulus. On the second training day, animals were confined for 5 min to each goal arm, where chocolate milk was available. On the third training day, animals were placed in the main arm, the guillotine doors were lifted, and rats were allowed to choose between the two goal arms, each one baited with chocolate milk. When the animal placed all four paws in a goal arm it was confined to it for 30 s and the entry considered as a choice. Thereafter, the animals were removed and placed in a holding cage for 10 s. This procedure was repeated for a total nine runs.

Drug treatment (clomipramine or test drugs) was started on three consecutive days before the experimental sessions. One the day before, the animals were deprived of food for 24 h.

Thereafter, they were injected i.p. with 1 mg/kg of the selective 5-HT_{1A} agonist 8-OH-DPAT and 15 min later placed in the main arm and allowed to choose between the two goal arms. The procedure was repeated up to seven runs or until they alternated their election. The choice made by the animal (right or left) was recorded. The number of repetitive choices until an alternation occurs indicates compulsive-like behavior. Additionally, the animals that chose the same alley in three consecutive occasions were defined as highly perseverative.

Evaluation

The number of consecutive entries to the same alley of the T-maze is presented as the median and the number of highly perseverative per group expressed as a percentage. Data were analyzed by means of nonparametric tests.

Modifications of the Method

Joei and Avisar (2001) proposed excessive lever pressing following post-training signal attenuation in rats: a possible animal model of obsessive-compulsive disorder.

Szechtman et al. (2001) discussed compulsive checking behavior of quinpirole-sensitized rats as an animal model of obsessive-compulsive disorder.

Berridge et al. (2005) proposed sequential superstereotypy of an instinctive fixed action pattern in hyperdopaminergic mutant **mice** as a model of obsessive-convulsive disorder.

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Antidepressant-Like Activity in Differential-Reinforcement of Low Rate 72-Second Schedule

Purpose and Rationale

The differential-reinforcement of low-rate (DRL) 72-s schedule has been recommended for evaluation of antidepressant drugs (O'Donnell and Seiden 1983).

Procedure

Male Sprague Dawley rats weighing 350–450 g are housed in suspended wire cages in rooms maintained at 21–23 °C and 30–40 % relative humidity having free access to laboratory chow except during experimental sessions. Water is provided after each daily session in order to maintain body weights.

Apparatus

Model C Gerbrand operant-conditioning chambers (O'Donnell and Seiden 1983) or Coulbourn operant chambers (Pollard and Howard 1986) are used.

A lever that operates a microswitch is mounted on one wall 3 cm from the side, 2.5 cm above a grid floor, and 6.5 cm from an access port for a dipper that provides 0.025 ml of water. A houselight is mounted on the opposite wall. A downward force equivalent to approximately 15 g operates the lever, constituting a response. When the schedule requirements are met, the dipper is lifted from a water trough to an opening in the floor of the access port for 4 s, constituting a reinforcer.

Rats are water deprived for 22 h before each session. Each rat is initially trained under an alternative fixed-ratio 1, fixed-time 1-min schedule for water reinforcement. Thus, each response is reinforced, and reinforcement is also provided every minute if no responding occurs. All rats are then placed under a DRL (differential-reinforcement-of -low-rate) 18 s schedule for 3 weeks. The schedule requirement is then increased to 72 s (DRL 72-s). When performances on the DRL 72-s schedule are stabilized (approximately 6 weeks), drug treatments are initiated.

Each group of 6–10 rats is subjected to a dose–response determination for one ore more drugs. Drugs are administered 1 h before testing by intraperitoneal injection.

Evaluation

Data are expressed as percentages of noninjected controls. The control values are the response and reinforcement rates on those days immediately preceding the test day. The number of responses and reinforcers per session at each drug dose are tested for statistically significant differences from control values with paired *t*-tests using a two-tailed criterion of statistical significance.

Critical Assessment of the Test

The method is rather time consuming. Moreover, the specificity as screening method for antidepressants has been challenged (Pollard and Howard 1986).

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Tests for Antidepressant Activity Based on the Mechanism of Action

Potentiation of Norepinephrine Toxicity

Purpose and Rationale

Antidepressants block the reuptake of biogenic amines into nervous tissue. In this way, the toxic effects of norepinephrine are potentiated.

Procedure

Male NMRI mice (22–25 g) are randomly assigned to test groups of 10 subjects. The test drug, the standard, or the vehicle are given orally 1 h prior to the s.c. injection of the sublethal dose

of 3 mg/kg noradrenaline. The groups of 10 mice are placed into plastic cages with free access to food and water.

Evaluation

The mortality rate is assessed 48 h post dosing. ED_{50}, or dose which causes death of 50 % of the treated subjects, is calculated by means of linear regression analysis.

Critical Assessment of the Method

Several antidepressants block not only the uptake of noradrenaline but also of dopamine and of serotonin. By definition, this test can only measure the noradrenaline uptake inhibition.

One of the earliest observations on the pharmacoiogy of antidepressants was by Sigg (1959), who showed that imipramine, as distinguished from other tricyclic substances known at this time, produced a profound potentiation of the cardiovascular effects of exogenously administered catecholamines in animals.

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Compulsive Gnawing in Mice

Purpose and Rationale

In man and in other species, like dogs, apomorphine induces emesis. Treatment of rodents with apomorphine causes compulsive gnawing instead of vomiting. The compulsive gnawing in mice induced by apomorphine is due to dopaminergic stimulation. Centrally acting anticholinergics shift the balance between acetylcholine and dopamine resulting in an enhancement of the apomorphine effect. Therefore, many compounds with psychotropic activity are known to have an apomorphine-synergistic effect. This enhancement is also found after administration of tricyclic antidepressants (Ther and Schramm 1962).

Procedure

NMRI mice with a body weight between 18 and 20 g are injected s.c. with 10 mg/kg apomorphine. At the same time they are treated i.p. or s.c. with the test drug or the vehicle. For testing oral activity the animals are treated 30 min prior to apomorphine injection. Immediately after apomorphine injection six mice are placed into a cage 45 × 45 × 20 cm with a wired lid. The bottom of the cage is covered with corrugated paper, the corrugation facing upward. The mice start to bite into the paper causing fine holes or tear the paper. This behavior is enhanced by antidepressants. The mice remain 1 h in the cage.

Evaluation

The number of bites into the corrugated paper is evaluated by placing a template upon the paper. The template has 10 rectangle windows divided into 10 areas of the same size. In a total of 100 areas the number of bites is checked. In this way percentage of damaged paper is calculated. Ten mg/kg apomorphine does not increase the biting rate of 5–10 % which also occurs in normal animals. Likewise, atropine in doses of 40 and 80 mg/kg s.c. alone does not increase the biting behavior. In contrast, the combination of apomorphine and atropine greatly enhances the occurrence of gnawing. The same is true for antidepressants. For example, a dose of 25 mg/kg imipramine increases the damaged area by 40–70 %. Percent gnawing of the test compound is compared with that of the standard.

Critical Assessment of the Test

Not only antidepressants but also centrally acting anticholinergics and antihistaminics are active in this test. However, the test has the advantage of simplicity without any pretraining of the animals.

Modifications of the Method

DeFeo et al. (1983) reported on possible dopaminergic involvement in biting compulsion of mice induced by large doses of clonidine.

Stereotyped gnawing in mice is induced by methylphenidate (Pedersen and Christensen 1972). The phenomenon is potentiated by various drugs, such as benzodiazepines (Nielsen et al. 1991).

Stereotyped behavior in **guinea pigs** induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

Molander and Randrup (1974) investigated the mechanisms by which L-DOPA induces gnawing in mice.

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Apomorphine-Induced Hypothermia in Mice

Purpose and Rationale

Apomorphine induces hypothermia in mice which can be prevented by antidepressants.

Procedure

Male NMRI mice (20–22 g) are used and randomly assigned to test groups of six subjects. One hour after oral administration of the test compounds or the vehicle a dose of 16 mg/kg apomorphine is injected s.c. The rectal temperature of each mouse is measured by an electronic thermometer immediately prior to apomorphine administration and 10, 20, and 30 min later. During the entire experiment, subjects are housed in groups of three in glass jars at room temperature.

Evaluation

A time curve is constructed by plotting the temperature (mean of each group) against time in min. The AUC is calculated for all groups and converted into percent inhibition of apomorphine-induced hypothermia in the control group. An ED_{50} can be calculated by linear regression analysis.

Critical Assessment of the Method

Antagonism against apomorphine-induced hypothermia can be regarded as a hint for antidepressant activity. Compounds with a marked noradrenergic or dopaminergic component are active against apomorphine-induced hypothermia but not antidepressants acting mainly through serotoninergic mechanisms.

Modifications of the Method

Cox and Lee (1981) induced hypothermia in rats by intrahypothalamic injection of 5-hydroxytryptamine and recommended this as a model for the quantitative study of 5-hydroxytryptamine receptors.

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Tetrabenazine Antagonism in Mice

Purpose and Rationale

Tetrabenazine (TBZ) induces a depletion of biogenic amines (e.g., noradrenaline, dopamine, serotonin) without affecting their de novo synthesis. TBZ depletes noradrenaline from nerve terminals and prolongs reuptake into the granula. Noradrenaline is degraded by monoaminooxydase. Antidepressants inhibit the reuptake of noradrenaline into the nerve terminals and increase thereby the noradrenaline concentration at the receptor site. In this way, the effect of TBZ is antagonized. Therefore, both MAO inhibitors and tricyclic antidepressants are known to prevent or to antagonize these effects. The prevention of TBZ-induced ptosis and catalepsy can be used for evaluation of antidepressants.

Procedure

Groups of 5–10 male NMRI mice (20–22 g) are used. Sixty min after oral or 30 min after i.p administration of the test compound or the vehicle 40 mg/kg i.p. TBZ are injected. The animals are placed into individual cages. The test is started 30 min after TBZ administration and repeated every 30 min up to 2 h. Catalepsy and ptosis are used as criteria. A stair is formed with two cork stoppers having two steps of 3 cm height. The animals are placed head downward with their hind legs upon the top cork. As long as TBZ exerts its cataleptic effect the animals remain in this catatonic state. If the cataleptic effect is not antagonized after a limit of 60 s the animals are placed into a normal position.

Evaluation

Thirty days after replacement the degree of ptosis is scored: eyes closed = 4, eyes 3/4 closed = 3, eyes 1/2 closed = 2, eyes 1/4 closed = 1, eyes open = 0 (Rubin et al. 1957). Similarly, the cataleptic effect is scored according to the duration of catalepsy. Catalepsy more than 60 s = 5, between 30 and 60 s = 4, between 10 and 30 s = 3, between 5 and 10 s = 1, less than 5 s = 0. The scores of the TBZ controls are taken as 100 % and the percentage calculated for the treated animals. Imipramine was found to be effective at a dose of 10 mg/kg s.c. or 20 mg/kg orally.

Critical Assessment of the Method

The TBZ antagonism has been found to be a simple and reliable test for evaluation of classical antidepressants.

Modifications of the Method

Ptosis in mice can be induced in a similar way by treatment with reserpine. Mice receive a single oral dose of the test compound followed by subcutaneous administration of 5 mg/kg reserpine 30 min later. Ptosis is observed for the next 1 or 2 h and scored in a similar way as in the TBZ test.

TBZ ptosis can also be elicited in rats. The test procedure and the evaluation is the same as in mice. Tetrabenazine-induced hyperthermia in mice has been proposed as test for antidepressant activity by Gylys et al. (1963).

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Reserpine-Induced Hypothermia

Purpose and Rationale

Depletion of biogenic amines (noradrenaline, 5-hydroxytryptamine, dopamine) in the brain induces not only catalepsy and ptosis but also hypothermia in rodents. The decrease of body temperature induced by reserpine is antagonized by antidepressants, MAO inhibitors, and central stimulants. The subcutaneous administration of 2 mg/kg reserpine leads to a decrease of core temperature in mice to 20–23 °C after 18 h. The fall in temperature can be antagonized by antidepressants but also by amphetamine-like drugs. However, the time course is different: tricyclic antidepressants have a slow onset of action and a long-lasting effect, whereas amphetamine-like drugs have a quick onset of action and a short-lasting effect.

Procedure

Groups of five male NMRI mice (19–21 g body weight) are used. On the day before testing, they are dosed with 2 mg/kg reserpine s.c. They are housed in a climate-controlled animal colony and have free access to food and water. Eighteen hours after reserpine administration, the animals are placed into individual cages. The initial rectal temperature is determined by insertion of an electronic thermometer (e.g., Ellab T-3) to a constant depth of 2 cm. Following administration of the test compound (either i.p. or p.o.), the rectal temperature is measured again at 60 min intervals for 7 h.

Evaluation

Rectal temperature is recorded every hour. The difference in temperature from vehicle controls is calculated for each time and the maximal difference scored. The differences are then statistically compared using the *t*-test.

Modifications of the Method

The time course and the administration route can be changed. Male mice are treated with the test drug or the standard 1 h prior to intravenous injection of 2 mg/kg reserpine. Rectal temperature is measured by a rectal thermometer prior and every 60 min up to 6 h. The animals are kept in groups of three in glass jars at a controlled temperature of 20–21 °C. Using a computer program the area under the curve is calculated by plotting the temperature (mean of each group) before and the decrease after reserpine against time in hours. Areas of treated groups are converted to percentage of controls.

Colpaert et al. (1975) and Niemegeers (1975) described the antagonism of antidepressants and other drugs against Ro-4–1284, a benzoquinolizine derivative which by itself exhibits reserpine-like activities.

Critical Assessment of the Method

The test has been proven as a simple and reliable method to detect antidepressant activity. However, the reversal of hypothermia is not specific for antidepressants. The fall in body temperature can also be antagonized by amphetamines and some antipsychotic agents (chlorpromazine). The different time course of antidepressants (slow onset of action, long-lasting effect) and amphetamine-like drugs (quick onset of action,

short-lasting effect) allows differentiation between the two groups of drugs.

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5-Hydroxytryptophan Potentiation in Mice

Purpose and Rationale

According to the monoamine hypothesis of depression compounds exert antidepressant activity because they are capable of enhancing central noradrenergic and/or serotoninergic functions. Several antidepressant agents potentiate serotonin effects by a block of the reuptake of serotonin. DL-5-Hydroxytryptophan is used as the precursor of serotonin. Enzymatic breakdown is inhibited by the MAO inhibitor pargyline. In mice the characteristic symptom of head twitches is observed.

Procedure

Groups of six male mice (18–30 g) are used. They are treated i.p with the test drug or the vehicle. Thirty minutes later, the mice receive 75 mg/kg pargyline HCl s.c. Ninety minutes after pargyline the animals are injected with 10 mg/kg DL-5-hydroxytryptophan (5-HTP) i.v.

Evaluation

Animals positively influenced show a characteristic behavior of head twitches. An animal is considered to be positive if it shows head twitches 15 min after 5-HTP injection. Enhancement is observed after treatment with serotonin uptake blockers relative to animals pretreated with pargyline only.

Critical Assessment of the Method

The test can be considered as additional evidence for antidepressant activity based on uptake inhibition.

Modification of the Method

The head twitch in mice can also be elicited without a MAO inhibitor by using higher doses (200 mg/kg) of DL-5-hydroxytryptophan.

Moser and Redfern (1988) studied the effects of four benzodiazepines on the head twitch response induced in mice by several 5-HT receptor agonists.

Meert et al. (1988) studied partial and complete blockade of 5-hydroxytryptophan (5-HTP)-induced head twitches in the *rat* by serotonin S_2 antagonists.

Niemegeers et al. (1983) and Awouters et al. (1988) used *mescaline*-induced head twitches in the rat as an in vivo method to evaluate serotonin S_2 antagonists. Head twitches were counted for 15 min after intravenous injection of 20 mg/kg mescaline. Twitch counts of less than 2 were considered as inhibition and of less than 2 as blockade of the mescaline response.

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5-Hydroxytryptophan Potentiation in Rats

Purpose and Rationale

The inhibition of serotonin reuptake by some antidepressants can be tested in vivo in rats by administration of the precursor 5-hydroxytryptophan and inhibition of its breakdown by the MAO inhibitor pargyline. In contrast to mice exhibiting head twitches, rats show other symptoms such as continuous forelimb clonus.

Procedure

Groups of six male Wistar rats weighing 150–200 g are used. Four hours prior to testing pargyline HCl is injected s.c. at a dose of 75 mg/kg. Thirty minutes before i.p. injection of 1 mg/kg L-5-hydroxytryptophan, test compounds

or standards are administered intraperitoneally. Fifteen minutes after the 5-hydroxytryptophan injection, the animals are observed for fifteen minutes. An animal is considered to be positive if is exhibits continuous forelimb clonus.

Evaluation

Enhancement is expressed as normalized percent increase relative to the vehicle control. Using various doses, ED_{50} values with 95 % confidence limits can be determined by probit analysis.

Modifications of the Method

Hailberg et al. (1985) developed a registration device based on accelerometry in order to accomplish an objective quantification of tremors in conscious unrestrained rats. Tremor intensity was continuously recorded by a small piecoresistive accelerometer mounted on the back of the freely moving rat. The accelerometer was connected to a Grass polygraph. The integrated signals were further analyzed by a desktop computer.

The behavior in rats induced by 10 mg/kg L-5-hydroxytryptophan i.p. can be antagonized by compounds having 5-HT_2 antagonist properties (Colpaert and Janssen 1983).

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Yohimbine Toxicity Enhancement

Purpose and Rationale

Yohimbine occupies central α_2 receptors and prevents noradrenaline from binding to these receptors. Compounds with antidepressant properties are known to inhibit physiological inactivation of noradrenaline and other biogenic amines by blocking the reuptake at nerve terminals. Administration of a test compound with antidepressant properties leads to an increase in noradrenaline concentration. Following the simultaneous administration of yohimbine and antidepressants the animals die of noradrenaline poisoning.

Procedure

Groups of ten male NMRI mice (25–28 g) are used. Mice are placed in plastic cages and receive the test compound or the vehicle by oral or i.p. administration. Thirty minutes later, a dose of 25 mg/kg yohimbine (a sublethal dose) is given s.c.

Evaluation

Mortality rate is assessed 1, 2, 3, 4, 5, and 24 h after dosing. Lethality in the control group (Yohimbine only) is less than 10 %, whereas 10 mg/kg desipramine-HCl causes death in about 90 %. Using various doses ED_{50} values can be calculated.

Critical Assessment of the Method

The test has been proven as a simple method to detect antidepressants with monoamine uptake-inhibiting properties.

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Tryptamine Seizure Potentiation in Rats

Purpose and Rationale

Monoamine oxidase (MAO) inhibitors like iproniazid enhance seizures in rats caused by an intravenous infusion of tryptamine HCl. This procedure can be used to elucidate the in vivo MAO inhibiting properties of compounds.

Procedure

Groups of five male Wistar rats weighing 150–200 g are used. Test compounds, standard, or vehicle controls are administered intraperitoneally 0.5, 1, 2, and 4 h prior testing. At the time of testing 5 mg/kg tryptamine HCl freshly dissolved in saline are injected intravenously. Immediately after tryptamine HCl administration, the animals are observed individually for 3 min for the appearance of clonic "pedaling" movements of the forepaws which is considered a positive response. Frequently, these clonic seizures are preceded by a kyphotic curvature of the spine, but this sign does not constitute a positive response.

In addition to the vehicle control group, a series of five positive control animals receiving tranylcypromine at 5 mg/kg i.p. with a 0.5 h pretreatment time are subjected to the test in order to check the effectiveness of the tryptamine HCl solution which is relatively unstable. A 100 % response is expected. Fresh tryptamine HCl solution should be prepared hourly as needed.

Evaluation

The normalized percent potentiation is calculated as follows:

$$100 \times \left[\frac{\%\,\text{animals potent. in durg group}}{1 - \%\,\text{animals potent. in vehicle group}} - \frac{\%\,\text{animals potent. in vehicle}}{1 - \%\,\text{animalspotent. in vehicle group}} \right]$$

A dose–response is obtained in the same manner at the peak time of drug effect except that a group size of 10 animals is used and four different doses are tested in addition to the vehicle and the tranylcypromine groups.

An ED_{50} is calculated using probit analysis.

Modifications of the Method

Graham-Smith (1971) described the inhibitory effect of chlorpromazine on the syndrome of hyperactivity produced by L-tryptophan or 5-methoxy-N, N dimethyltryptamine in rats treated with a monoamine oxidase inhibitor.

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Serotonin Syndrome in Rats

Purpose and Rationale

Compounds which stimulate serotonin receptors cause a series of behavioral changes in rats which is called the serotonin syndrome (Jacobs 1976; Green and Heal 1985; Tricklebank 1985) such as head weaving, increased locomotion, forepaw treading, flat posture, and lower lip retraction. With increasing knowledge about the subtypes of serotonin receptors these symptoms were defined to be associated with 5-HT_{1A} receptors and their specific agonists (Smith and Peroutka (1986), Blanchard et al. 1993; Yu and Lewander 1997).

Procedure

Time and dose dependence of **forepaw treading**, which is scored at 15, 30, 45, and 60 min according to a three-point scale (0 = absent, 1 = periodic and weak, 2 = continuous) according to Arnt and Hyttel (1989) and Porsolt et al. (1992), was measured by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Berendsen and Broekkamp (1990), Schoeffter et al. (1993), Andersson and Larsson (1994), Forster et al. (1995), Kofman and Levin (1995), Lu and Nagayama (1996), O'Connell and Curzon (1996), Gaggi et al. (1997), Kleven et al. (1997), and Yu and Lewander (1997); of **flat body posture** by Deakin and Green (1978), Goodwin and Green (1985), Goodwin et al. (1986), Blanchard et al. (1993), Schoeffter et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), Kofman and Levin (1995), O'Connell and Curzon (1996), Gaggi et al. (1997), Kleven et al. (1997), Woiff et al. (1997), and Yu and Lewander (1997); of **hind limb abduction** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), and Kofman and Levin (1995); of **increased motility** by Tricklebank (1985), Forster et al. (1995), Gaggi et al. (1997), andO'Neill and Parameswaran (1997); of **decreased body temperature** by Martin et al. (1992), Schoeffter et al. (1993), Simiand et al. (1993), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), O'Connell and Curzon (1996), Bagdy and To (1997), Wolff et al. (1997), and Yu and Lewander (1997); of **head twitches** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Meert et al. (1988), Berendsen and Broekkamp (1990), Kofman and Levin (1995), Gaggi et al. (1997), and Kleven et al. (1997); and of **lower lip retraction**, which is scored according to Berendsen et al. (1989) after 15, 30, and 45 min as follows: 0 = lower incisors not or hardly visible (not different from nontreated animals), 0.5 = partly visible, 1 = completely visible, by Smith and Peroutka (1986), Berendsen et al. (1994, 1997), Porsolt et al. (1992), Blanchard et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Moore et al. (1993), De Boer et al. (1995), Berendsen et al. (1996), Bagdy and To (1997), Groenink et al. (1997), Kleven et al. (1997), and Wolff et al. (1997).

Evaluation

Scores of each symptom are registered for each test animal. Average values of treated animals are compared with controls treated with vehicle alone. Dose–response and time–response curves are evaluated.

Critical Assessment of the Test

Among the many symptoms of the serotonin syndrome in rats, forepaw treading, flat body posture, and lower lip retraction were used by most authors to characterize agonists and antagonists of 5-HT_{1A} receptors.

Modifications of the Method

Truison et al. (1976) used the serotonin syndrome to test the supersensitivity after destruction of central serotonergic nerve terminals by intracerebral injection of 5,7-dihydroxytryptamine.

Blanchard et al. (1997) described the symptoms of selective activation of 5-HT_{1A} receptors in **mice**.

Locomotor activity of **guinea pigs** was increased in a dose-dependent manner by 5-HT_{1A} receptor agonists (Evenden 1994).

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Hypermotility in Olfactory Bulbectomized Rats

Purpose and Rationale

Bilateral olfactory bulbectomy in the rat is associated with changes in exploratory behavior that are reversed by chronic but not acute treatment with antidepressant drugs (Cairncross et al. 1978, 1979; Leonard and Tuite 1981; Janscár and Leonard 1984). The olfactory bulbectomized rat is used as an animal model of depression. Several behavioral changes have been observed following bilateral olfactory bulbectomy: hyperactivity in a closed arena, such as the open field; enhanced nocturnal activity; deficits in memory as shown by passive avoidance behavior and in the Morris water maze and the radial maze; increased open-arm entries in the elevated-plus maze; and changes in food-motivated and conditioned taste aversion behavior. Alteration in the noradrenergic, serotonergic, cholinergic, GABAergic, and glutamatergic neurotransmitter systems are also associated with olfactory bulbectomy (Wren et al. 1977; Van Riezen and Leonard 1990; Kelly et al. 1997).

Procedure

Male Sprague Dawley rats are anesthetized with intraperitoneal injection of 2.5 % tribromoethanol solution (Cairncross et al. 1977). Following exposure of the skull, a burr hole is drilled at points 7 mm anterior to the bregma and 2 mm either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs are removed by suction and the burr holes filled with hemostatic sponge. Tetramycin powder is then applied to the wound and the skin closed by surgical clips. Sham-operated animals are treated in the same way, but although the dura above the bulbs is cut, the bulbs are left intact. The animals are allowed to recover for 14 days after surgery.

For drug evaluation the animals are treated subcutaneously with the test drug or the standard or the vehicle once daily at 09:00 for 14 days. In each experiment sham-operated controls treated with test drug, standard, and vehicle are included.

The behavior of the animals is tested from the 12th day onward. The rats are placed singly in the center of an open-field apparatus. Ambulation (no. of squares crossed), rearing (forepaws raised from the floor), grooming, and defecation (no. of fecal boli) scores are recorded for a 3 min period of observation.

Evaluation

The results are analyzed statistically using Student's t-test (two-tail test), the significance being set at $P < 0.05$.

Critical Assessment of the Method

The bulbectomized rat model has been shown to be highly selective for both typical and atypical antidepressants; however, the procedure is quite time consuming.

Modifications of the Method

Various authors used this model to demonstrate antidepressant-like activity such as Briley et al. (1996) for milnacipran, a serotonin and noradrenaline uptake inhibitor, Hancock et al. (1995) for A-80426, an α_2 adrenoceptor antagonist with serotonin uptake blocking activity, McNamara et al. (1995) for the centrally active serotonin agonist 8-hydroxy-2-(di-npropylamino) tetralin (8-OH-DPAT), Redmont et al. (1997) for dizocilpine (MK-801), Song and Leonard (1994) for the serotonin reuptake inhibitors fluvoxamine and sertraline, Song et al. (1996a) for centrally administered neuropeptide Y, and Song et al. (1996b) for the H1 receptor antagonist terfenadine.

Kelly and Leonard (1994, 1995) studied the effects of potential antidepressants, such as selective serotonin reuptake inhibitors, in olfactory bulbectomized rats. Kelly et al. (1997) anesthetized male Sprague Dawley rats weighing 230–280 g with 375 mg/kg i.p. chloral hydrate. The surgical procedure involves drilling two burr holes (2 mm diameter, 8 mm anterior to bregma) either side, 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The olfactory bulbs can be visualized through these burr holes and can be aspirated by means of a blunt hypodermic needle attached to a water pump, taking care not to cause damage to the frontal cortex. Prevention of blood loss from the burr holes is

achieved by filling them with hemostatic sponge. Antibiotic powder is applied to the wound, prior to closing with 7.5-mm surgical clips. A period of 2 weeks allows for the recovery from the surgical procedure and is optimal for the development of the bulbectomy syndrome.

Potential antidepressant drugs are preferably administered for a period of 2 weeks. Various procedures can be used to test the drug's effect in olfactory buibectomized rats, such as open field (Cryan et al. 1999; Slotkin et al. 1999), home cage activity (Redmont et al. 1997), forced swim test (Kelly and Leonard 1999), elevated plus maze (McGrath and Norman 1999), passive avoidance (Martin et al. 1998; Nowak et al. 2003), and olfactory bulbectomy–induced hyperemotionality (Chaki et al. 2004).

Redmont et al. (1995) studied the effect of chronic antidepressant administration on the conditioned taste aversion to 8-OHDPAT in the olfactory bulbectomized rat.

Kelly et al. (1997) gave an update of the olfactory bulbectomized rat as a model of depression. Tricyclic antidepressants (amitryptiline, desimipramine), atypical agents (mianserin), selective serotonin reuptake inhibitors (paroxetine, sertraline, fluvoxamine), reversible inhibitors of monoamine oxidase A (moclobemide), as well as putative antidepressants, such as 5-HT_{1A} agonists (zalospirone, ipsapirone), noncompetitive NMDA antagonists (MK-801), and triazolobenzazepines (alprazolam, adinazolam), have demonstrated antidepressant-like activity in this model.

Zhou et al. (1998) found serotoninergic hyperinnervation of the frontal cortex in the bulbectomized rat.

Increases in the concentrations of the neuropeptides corticotropin-releasing factor, thyrotropin-releasing factor, somatostatin (Bissette 2001), and neuropeptide Y (Holmes et al. 1998), which may play a role in mediating the antidepressant-sensitive behaviors, have been demonstrated.

Comparing the behavioral and biochemical effects of bulbectomy in young versus aged rats, Slotkin et al. (1999) suggested that this test might provide a useful animal model with which to test therapeutic intervention for geriatric depression.

Ho et al. (2000) demonstrated increased striatal glutamate release during novelty exposure-induced hyperactivity in olfactory bulbectomized rats that may have a modulatory role in the antidepressant-sensitive response.

Wrynn et al. (2000) performed an in vivo magnetic resonance imaging study in olfactory bulbectomized rats and demonstrated alterations in signal intensities in cortical, hippocampal, caudate, and amygdaloid regions.

Holmes et al. (1998) studied the effects of olfactory bulbectomy on neuropeptide gene expression in the rat olfactory system.

Bilkei-Gorzo et al. (2002) and Zueger et al. (2005) tested alterations in locomotor activity and exploratory behavior after olfactory bulbectomy in **mice** (using systems provided by TSE Systems, Bad Homburg, Germany).

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Sexual Behavior in Male Rats

Purpose and Rationale

Sexual behavior in male rats is stimulated by 5-HT_{1A} receptor agonists (Ahlenius et al. 1981; Gorzalka et al. 1990; Foreman et al. 1993, 1994, 1995; Andersson and Larsson 1994b; Tallentire et al. 1996; Ahlenius and Larsson 1997; Fernández-Guasti and Rodriguez-Manzo 1997) and inhibited by serotonin receptor antagonists (Mendelson and Gorzalka 1981) and by 5-HT_{1B} receptor antagonists (Fernández-Guasti et al. 1989). The test procedure was described in detail by Arnone et al. (1995).

Procedure

Adult male and ovarectomized female Sprague Dawley rats are housed in sex-separated rooms at 21 ± 1 °C in a reversed light–dark cycle with free access to food and water.

Mating Behavior

Male rats of two levels of sexual performance are selected for drug testing: sexually naive and sexually experienced. The latter are given four mating pretests, twice a week; only sexually active males that achieve at least two ejaculations per test are included in the experiments.

The mating tests are performed between 12:30 and 17:00, during the dark phase of the lighting cycle. Drug is administered orally 1 h before the test to the male rat. The animal is allowed to adapt to the test area (60 cm diameter, 50 cm high) illuminated with a dim red light. Each behavioral test starts with the introduction of a stimulus female brought into sexual receptivity by s.c. treatment with estradiol benzoate (10 μg/rat), followed 48 h later by progesterone (500 μg/rat), 4–5 h before testing. The tests end either 20 min later or after the first postejaculatory mount (or 2 h later for sexual satiation).

The following behavioral parameters are recorded:

- **Mount and intromission latencies**: time interval from the first introduction of the female to the first mount or intromission, respectively. Mounts are accompanied by an average of

three or four brief shallow thrusts, while the intromission, which succeeds this event, is marked by a single deep thrust indicative of penile insertion.
- **Mount frequency**: total number of pre-ejaculatory mounts with and without intromission.
- **Intromission frequency**: total number of pre-ejaculatory intromissions.
- **Ejaculatory latency**: time interval from the first intromission to ejaculation. A total of 1200 s is scored for the latencies of rats failing to mount, intromit, or ejaculate.

Sexual Satiation

The mating pattern of the male rat consists of repeated mounts and intromissions, culminating in an ejaculation. The ejaculation is followed by a period of 4–5 min during which time the male remains refractory to sexual stimulation by the female. The sexual activity is thereafter resumed with a new series of mounts and intromissions followed by ejaculation. If uninterrupted, the rat may achieve five to six ejaculations before sexual satiation sets in. Sexually experienced male rats are allowed to copulate with a receptive female for 2 h. The behaviors are recorded on a videotape. The criterion for satiation is that the male fails to mount within 20 min post ejaculation. The latency to satiation and the number of ejaculations are recorded.

Finally, for each ejaculatory series, the following parameters are recorded:

- **Copulation duration**: time interval between the first mount and the ejaculation
- **Postejaculatory interval**: time interval from the ejaculation to the first mount of the next series

Penile Erections

One hour before the experiment, male rats are allowed to adapt to the quiet observation room. One hour after oral drug administration, rats are placed in individual plastic cages (10.5 × 24 × 16 cm). Series of nine rats comprising, at random, control and drug-treated animals are observed simultaneously for 45 min and the number of penile erections counted. Penile erection is defined as a period of pelvic thrusting followed by an upright position with genital grooming and the display of the engorged penis. Animals are used only once.

Evaluation

Data are analyzed using nonparametric statistics. The Fisher test is used for percentage responding. For quantified behavioral parameters, the Kruskal-Wallis test followed by the Mann–Whitney *U*-test corrected by Holm's method is used for comparisons versus the control group. The Mann–Whitney *U*-test is used for comparison of a single treated group versus its own control group.

Modifications of the Method

Barr et al. (1999) subjected male rats to a 4-day escalating dose schedule of D-amphetamine. Twelve hours after the final dose the rats were tested for sexual behavior. Withdrawal from the amphetamine was associated with decrements in several motivational components of sexual behavior. The procedure was recommended as a rodent model of depression.

Pomerantz et al. (1993) studied the influence of 5-HT receptor agonists on male sexual behavior of **rhesus monkeys**.

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Genetic Models of Depression

Flinders Sensitive Line of Rats

The **Flinders Sensitive Line of rats** was established by genetically selecting (breeding) Sprague Dawley rats for a behavioral trait, supersensitivity to cholinergic agents (Overstreet and Russell 1982; Overstreet 1986; Overstreet et al. 1986, 1990, 1992; Daws and Overstreet 1999). The name Flinders refers to University of Flinders, Australia, where the line was first selected. The usefulness of the Flinders Sensitive Line of rats as an animal model for depression is evident because it exhibits behavioral features characteristic of depression in humans and responds to chronic, but not acute, treatment with antidepressants (Overstreet 1993; Overstreet et al. 1995, 2004; Yadid et al. 2000; Overstreet and Griebel 2004; Dremencov et al. 2005).

Vasquez et al. (2000) tested the effects of electroconvulsive stimuli and D-amphetamine on neuropeptide Y concentrations in brain and on locomotion in the Flinders Sensitive Line rat.

Ferreira-Nuño et al. (2002) studied masculine sexual behavior features in Flinders sensitive and resistant line rats.

Shayit et al. (2003) demonstrated 5-HT_{1A} receptor subsensitivity in infancy and supersensitivity in adulthood in the Flinders Sensitive Line rat.

Dremencov et al. (2004) showed in the Flinders Sensitive Line of rats that the serotonin–dopamine interaction is critical for the fast-onset action of antidepressant treatment. Seven-day treatment with nefazodone (a putative fast-onset antidepressant) but not with desipramine (a classical antidepressant) normalized immobility time in the swim test in Flinders

Sensitive Line rats. Serotonin-induced dopamine release but not basal dopamine levels correlated with the improvement of depressive-like behavior.

Lavi-Avnon et al. (2005) showed an abnormal pattern of maternal behavior in the Flinders Sensitive Line of rats.

Maayan et al. (2005) demonstrated the involvement of dehydroepiandrosterone and its sulfate ester in blocking the therapeutic effect of electroconvulsive shocks in the Flinders Sensitive Line of rats.

King and Edwards (1999), King et al. (2001), and Shumake et al. (2003) bred **congenital learned helpless rats**, which exhibit physiological symptoms of analgesia, cognitive deficits, and hyporesponsivity of the hypothalamic–pituitary–adrenal axis similar to those observed in human subjects with post-traumatic stress disorder.

Will et al. (2003) described **selectively bred Wistar-Kyoto rats** as an animal model of depression and hyper-responsiveness to antidepressants. Based on the significant decrease in investigative behavior of male rats toward a female intruder, reflecting the presence of anhedonia, Paré (2000) concluded that the Wistar-Kyoto rat strain represents a useful animal model of depressive behavior.

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Genetically Altered Mice as Models of Depression

Cryan et al. (2002) published a survey on the use of genetically altered mice to assess antidepressant-related phenotypes. Cyran and Mombereau (2004) discussed the utility of models for studying depression-related behavior in genetically modified mice.

Cases et al. (1995) found aggressive behavior and altered amounts of brain serotonin and epinephrine in mice lacking **MAO_A**. Pup behavioral alterations including trembling, difficulties in righting, and fearfulness were reversed by the serotonin inhibitor parachlorophenylalanine. Adults manifested a distinct behavioral syndrome, including enhanced aggression in males.

Lijam et al. (1997) created mice deficient in *Dvll*, one of the three mouse homologues of the *Drosophila* polarity gene *Dishevelled*, by gene targeting. These mice exhibited reduced social interaction and sensorimotor gating abnormalities.

Grimsby et al. (1997) reported an increased stress response and increased levels of α-phenylethylamine in **MAO_B**-deficient mice. In addition, mutant mice were resistant to the neurodegenerative effects of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a toxin that induces a condition reminiscent of Parkinson's disease.

Sallinen et al. (1999) reported that general alteration of the **α_2-adrenoceptor subtype c** in mice affects the development of behavioral despair and stress-induced increases in plasma corticosterone levels. α_{2c}-Adrenoceptor overexpression increased and the lack of α_{2c}-adrenoceptors decreased the immobility of mice in the forced swimming test.

Cyran et al. (2001) used **dopamine-beta-hydroxylase-deficient** mice to determine the role of norepinephrine in the mechanisms of action of antidepressant drugs. The dopamine-beta-hydroxylase-deficient mice failed to demonstrate antidepressant-like behavioral effects following the administration of several classes of antidepressants.

Mayorga et al. (2001) studied antidepressant-like behavioral effects in **5-hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1B}$receptor mutant mice**. The results suggested that 5-HT_{1A} and 5-HT_{1B} receptors have different roles in the response to antidepressant drugs in the tail suspension test.

Froger et al. (2001) found that 5-hydroxytryptamine$_{1A}$ autoreceptor adaptive changes in **substance P (neurokinin 1) receptor**

knockout mice mimic antidepressant-induced desensitization. The constitutive lack of NK_1 receptors appeared to be associated with a downregulation/functional desensitization of $5\text{-}HT_{1A}$ autoreceptors resembling that induced by chronic treatment with antidepressants.

Jaber et al. (1997) inactivated the expression of the **dopamine transporter** in mice. The authors claimed that these genetically altered mice offer a unique model to test the specificity and selectivity of dopamine-transporter-acting drugs and provide new concepts related to clinical conditions such as Parkinson's disease, schizophrenia, and drug addiction.

Xu et al. (2000) reported that mice lacking the **norepinephrine transporter** are supersensitive to psychostimulants. Disruption of the norepinephrine transporter (NET) gene prolonged the clearance of norepinephrine and elevated extracellular levels of this catecholamine. In a classical test for antidepressant drugs, the NET-gene-deficient mice behaved like antidepressant-treated wild-type mice.

Holmes et al. (2001) characterized the **dopamine receptor D_5null mutant** of mice in behavioral tests.

Based on pharmacology of adenosine A_{2A} receptor antagonists and results with **A_{2A} receptor knockout mice**, El Yacoubi et al. (2001) claimed that adenosine *A*2A receptor antagonists are potential depressants.

MacQueen et al. (2001) investigated the performance of heterozygous **brain-derived neurotrophic factor knockout mice** on behavioral analogues of anxiety, nociception, and depression.

Conti et al. (2002) reported that cAMP-response-element-binding protein is essential for the upregulation of **brain-derived neurotrophic factor** transcription but not the behavioral or endocrine responses to antidepressant drugs. cAMP-response-element-bindingprotein-deficient mice were used to study the effects of desipramine and fluoxetine in behavioral, endocrine, and molecular analyses.

Stork et al. (2000) studied postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65. Adult **GAD65(−/−) mice** showed a largely abnormal neural activity with frequent paroxysmal discharges and spontaneous seizures. They furthermore displayed increased anxiety-like behavior in a light/dark avoidance test and reduced intermale aggression, as well as reduced forced-swimming-induced immobility indicative of an antidepressant-like behavior.

Svenningsson et al. (2002) studied the involvement of striatal and extrastriatal DARPP-32, a cAMP-regulated phosphoprotein, in biochemical and behavioral effects of fluoxetine using **DARPP-32 knockout mice**.

Calapai et al. (2001) used **interleukin-6 knockout mice** to characterize the antidepressant action of *Hypericum perforatum*.

Porsolt (2000) reviewed the utility of animal models of depression for transgenic research.

El Yacoubi et al. (2003) published the behavioral, neurochemical, and electrophysiological characterization of a genetic mouse model of depression.

Wei et al. (2004) generated mice overexpressing glucocorticoid receptor specifically in forebrain. These mice display a significant increase in anxiety-like and depression-like behaviors relative to wild type.

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Yohimbine Toxicity Enhancement

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Anti-Parkinson Activity

Mary Jeanne Kallman

Contents

General Considerations

A fundamental lesion in Parkinson's disease is a marked deficiency in the dopaminergic innervation of the basal ganglia owing to degeneration of neurons in the substantia nigra. Enhancement of dopaminergic transmission restores motor function at least partially. The decrease in dopaminergic activity in the basal ganglia results in a relative excess of cholinergic influence. Therefore, dopaminergic agonists, such as levodopa, a precursor of dopamine, and cholinergic (muscarinic) antagonists can be combined in the treatment of Parkinson's disease. Parkinson-like syndromes also occur after depletion of central stores by reserpine and after treatment with phenothiazines and other antipsychotic drugs blocking dopamine receptors (Vernier 1964; Marsden et al. 1975; Duvoisin 1976; Hornykiewicz 1975; Miller and Hiley 1975). The pathology of Parkinson's disease is typified by the presence of cytoplasmic inclusions (Lewy bodies). The formation of these proteinaceous inclusions involves the interaction of several proteins, including α-synuclein, synphilin, parkin, and ubiquitin carboxyl-terminal hydrolases (Goldberg and Lansbury 2000; Shimohama et al. 2003; Le and Appel 2004; Meredith et al. 2004; Snyder and Wolozin 2004; von Bohlen und Halbach et al. 2004). Orr et al. (2002) gave a review on inflammatory aspects of Parkinson's disease and highlighted the cell-to-cell interactions and immune regulations critical for neuronal

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homeostasis and survival. Parkinson's disease and related synucleinopathies are considered as a new class of nervous system amyloidoses (Trojanowski and Lee 2002; Dev et al. 2004; Liu et al. 2005).

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In Vitro Methods

Culture of Substantia Nigra

Purpose and Rationale

Although in vivo animal studies have been used to examine the effects of a number of Parkinson-inducing compounds, there is little information on reliable in vitro methodologies that can recapitulate the previously observed in vivo results. Cardozo (1993) and Smeyne and Smeyne (2002) described details of a method for generating mixed and chimeric neuron/glial cultures of postnatal substantia nigra (SN), independent of other monoaminergic nuclei in the ventral midbrain. Since many toxins do not affect regions of the midbrain except the SN, use of whole ventral midbrain from embryos can dilute any measurement of cell death. By specifically culturing ventrolateral midbrain containing the SN, one can more directly target the effects of dopaminergic toxins. In addition, this method can be used to test potential therapies for amelioration of Parkinson's disease.

Procedures

Generation of SN Cultures

1. Postnatal day 2–5 C57Bl/6 mice and/or Swiss-Webster (SWR) mice (Harlan) are placed on ice for 2–3 min to achieve an appropriate plane of anesthesia.
2. Animals are quickly decapitated and ≈8–10 brains from P2–P5 C57BL/6 or SWR matings are removed and placed in a freshly prepared dissociation media (= DM containing 12.8 g sodium sulfate, 5.2 g potassium sulfate, 0.036 g calcium chloride $2H_2O$, 1.18 g magnesium chloride $6H_2O$, 1.8 g glucose anhydrous, 0.238 g HEPES in 1 l deionized water, adjusted to pH 7.4 with NaOH).
3. Brains are placed on their ventral surface and a slice of midbrain rostral to the cerebellum and caudal to the hippocampus is isolated. This removed brain slab is placed flat in DM and either the entire midbrain or the ventrolateral midbrain containing the SN is dissected and minced into small pieces.
4. The minced SN or midbrain is then incubated in papain and DNAase (Dissociation Kit, Worthington Biochemical, Freehold, N.J., USA, follow kit instructions) for 30 min at 37 °C. A second incubation with fresh papain solution (30 min, 37 °C) is followed by three rinses in DM and one rinse in plating media (PM).
5. Tissue is triturated in 5 ml PM and the cell suspension is added to 2 ml (1 ml of BSA stock and 1 ml ovalbumin stock).
6. The cell suspension is spun 1400 rev/min for 8 min and the pellet is then resuspended in 1.0 ml plating media containing 2 % rat serum.
7. Once resuspended, cells are counted using trypan blue (0.4 %) to determine cell viability.
8. Cells are adjusted to 1.2×10^6 cells/ml and plated at ≈200,000 cells/cm^2 in Lab-Tek 4-well Permanox™ chamber slides that were previously coated with laminin (200 mg/ml, Collaborative Biomedical Products) and poly-D-lysine (200 mg/ml, Collaborative Biomedical Products) 1:1 (v:v) and rinsed once with deionized water. Cells are maintained in an incubator at 37 °C, 5 % CO_2, and fed 2–3 times per week with feeding media complete with 2 % rat serum (RS) by exchanging approximately one-fifth of the media (100 μl).

Chimeric Neuron/Glial Cultures

1. SNpc cells plated on pre-plated SNpc glia are produced using a variation of the previously described methods. SNpc glial feeder layers from P2–P5 C57BL/6 or SWR mice are produced using the above-described method, but cells are plated at 20,000–50,000 cells/cm^2 and fed with plating media containing 2 % rat serum and 8 % fetal bovine serum to promote glial proliferation and neuronal death.
2. For astrocyte cultures, this is the final step.
3. To generate mixed neuron/glial cultures, 3–4 weeks after the initial SNpc cells are plated, the glial feeder layers are rinsed once with plating media without serum, and 0.5 ml of plating media with 2 % rat serum (RS) is added.
4. SNpc cells from C57BL/6 or SWR mice are isolated (as above) and plated at 250,000 cells/well on the previously generated glial feeding layers.
5. Twenty-four hours after plating of neurons onto the glial feeder layers, the cultures are fed with feeding media complete with 2 % RS by an exchange of approximately one-fifth of the medium (100 μl) and cytosine β-D-arabinofuranoside (Ara-C, 2 μM) to prevent glial proliferation of the freshly plated cells. Thereafter, Ara-C (10 μM, final concentration) is added at each feeding.

MPTP Treatment

1. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is added to cultures 7–10 days after the neurons are plated onto glia. The MPTP is prepared by dissolving MPTP in feeding media for a 1-mM stock, then diluting to a 1-μM stock with feeding media complete with 2 % rat serum, and adding this directly to the cultures. The final concentration of the MPTP in media is 50 nM. Two feedings of MPTP, in 2 days, are necessary to achieve the desired toxic effect.

2. Seven days after MPTP is added, the cultures are rinsed 3 × with TBS, fixed in 4 % buffered paraformaldehyde for 10 min, and rinsed 3 × with TBS.

Evaluation

Identification of SN Cells

1. To determine the number of SN cells, cultures are immunostained for expression of tyrosine hydroxylase. First, endogenous peroxidase activity is quenched by rinsing with 0.3 % hydrogen peroxide in methanol in 1 × TBS for 2 × 15 min. Cells are permeabilized with 0.1 % Triton X-100, 5 % goat serum in TBS for 2 × 15 min. Cultures are covered in 400 µl of a polyclonal antibody directed against tyrosine hydroxylase (Eugene Tech International, Ramsey, N.J., USA, or Pel-Freeze, Burlingame, Calif., USA; each 1:500) and incubated in this solution overnight at 4 °C. The next day, cultures are rinsed three times with TBS followed by application of secondary antibody (goat α-rabbit) and amplification with Avidin–Biotin (ABC Elite Peroxidase kit, Vector Labs, Burlingame, Calif., USA). Final visualization of the immunopositive neurons is made using diaminobenzidine (DAB kit, Vector Labs) as a chromagen.
2. All TH-positive cells having the previously described characteristics of SNpc neurons (TH-positive cytoplasm surrounding a pale unstained nucleus, Hamre et al. 1999) from each culture are counted at a magnification of 200×. Note: to control for variability between cultures, all MPTP treatments are performed on matched cultures present on one single slide. Once cells are counted in each well, the number of TH-positive cells in the MPTP-treated cultures from each genotype is compared to the adjacent non-treated culture. Since an identical number of cells generated from the same brains was plated on a single slide, these cultures are directly compared to determine the percentage cell loss following MPTP. The percentage cell loss data following MPTP are then pooled from all C57Bl/6, SWR or mixed cultures.

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Inhibition of Apoptosis in Neuroblastoma SH-SY5Y Cells

Purpose and Rationale

A dopamine-derived neurotoxin 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline [*N*-methyl(*R*)salsolinol] was found to cause parkinsonism in rats and to deplete selectively dopamine neurons in the substantia nigra after infusion in the striatum. This isoquinoline occurs enantio-specifically in the nigra-striatum of human brains. The biosynthesis from dopamine is catalyzed by two enzymes, (*R*)salsolinol synthase and (*R*)salsolinol *N*-methyltransferase. The isoquinoline increases in the cerebrospinal fluid from parkinsonian patients, and the increase is ascribed to high activity of its synthesizing neutral (*R*)salsolinol *N*-methyltransferase, as shown by analyses in lymphocytes. The cell death caused by this neurotoxin in dopaminergic human neuroblastoma SH-SY5Y cells proved to be apoptotic. Apoptosis by this neurotoxin is mediated by intracellular sequential process, loss of mitochondrial membrane potential, activation of caspases, and DNA fragmentation (Maruyama et al. 1997; Naoi et al. 1996, 1997, 2000, 2002).

Neuroprotection to halt progressive cell death of neurons has been proposed as a future therapy for neurodegenerative disorders. In these disorders, such as Parkinson's disease and Alzheimer's

disease, apoptosis contributes to neuronal death in most cases (Tatton 2000). The well-regulated and relatively slow apoptotic process was proposed as a target of neuroprotection (Thompson 1995; Naoi and Maruyama 2001). Apoptosis is induced in neurons by various insults: oxidative stress, metabolic compromise, excitotoxicity, and neurotoxins. Apoptotic signaling is a multi-step pathway induced by opening a mitochondrial megachannel called permeability transition (PT) pore, followed by decline in membrane potential, ΔΨm, release of apoptosis-inducing factors, activation of caspases, and fragmentation of nuclear DNA. Mitochondrial PT pore is regulated by Bcl-2 protein family, preventively by Bcl-2 and Bcl-xL, and in a promotive way by BAX and BAD. Several papers describe the neuroprotective-antiapoptotic action of the anti-Parkinson drug rasagiline (Akao et al. 2002a, b; Youdim et al. 2003; Maruyama et al. 2002, 2004b).

Maruyama et al. (2004a) described the neuroprotective function of R-(–)-1-(benzofuran-2-yl)-2-propylaminopentane, [R-(–)-BPAP], against apoptosis induced by α-methyl (R) salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells.

Procedure

SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5 % fetal calf serum in an atmosphere of 95 % air /5 % CO_2.

Assessment of Apoptosis Induced by NM(R) Sal and the Protection by BPAP Derivatives

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with FACScan and CellQuest software (Becton Dickinson, San Jose, Calif., USA). Cells cultured in a 6-well poly-l-lysine-coated culture flask were incubated with or without 1 μM to 1 nM (–)-BPAP analogs at 37 °C for 30 min and then for 24 h with 250 μM AM(R)Sal in Cosmedium-001 culture medium supplemented with fetal calf serum. The cells were treated with trypsin, gathered, and washed with the culture medium and twice with phosphate-buffered saline (PBS). The cells were incubated with 100 nM test drugs solution in an icebath for 30 min, washed and suspended in PBS, and then subjected to FACS analysis.

Measurement of Changes in Δ Ψ m

Decline in Δ Ψ m induced by *N*M(*R*)Sal was quantified by measuring the reduction of Rhodamine 123 fluorescence preloaded in the cells (Patorino et al. 1996), as reported by Akao et al. (2002b). To examine the effects of (–)-BPAP analogs, the cells cultured in 6-well poly-L-lysine-coated tissue culture flasks were stained with 5 μM Rhodamine 123 in DMEM for 30 min at 37 °C. After being washed twice with PBS, the cells were suspended in DMEM and incubated with 1 μM to 1 nM BPAP derivatives for 30 min and then with 250 μM *N*M(*R*)Sal for 1 h. After being washed and gathered by treatment with trypsin, the cells were suspended in PBS and the fluorescence at 535 nm was measured with excitation at 505 nm in a Shimadzu spectrofluorophotometer, RF-5000 (Kyoto, Japan).

Measurement of Bcl-2 mRNA Level in the Cells Treated with BPAP Derivatives

SH-SY5Y cells were cultured in the presence of various concentrations (100 nM to 10 μM) of (–) BPAP analogs for 24 h, and mRNA levels of Bcl-2 were quantitatively assessed by RT-PCR (Akao et al. 2002a, b). The cells were gathered and washed with PBS, and the total RNA was extracted by the phenol/guanidinium thiocyanate method. cDNA was generated by reverse transcription of 2 μg of the total RNA, and the cDNA fragments were amplified using the PCR primers. The linearity of the relationship of the amount of PCR product to the time of PCR amplification was confirmed under the conditions used in this study. PCR products were analyzed by electrophoresis on 3 % agarose gels, and α-actin was used as an internal standard. The amounts of mRNA were quantified using NIH imaging software (version 1.62, developed at the US National Institutes of Health).

Measurement of Bcl-2 Levels in the Cells Treated with (–)-BPAP Derivatives

SH-SY5Y cells were treated with 1 to 1 μM (–)-BPAP analogs for 24 h, and the cells were gathered, washed with PBS, and suspended in RIPA buffer (10 mM Tris–HCl buffer, pH 7.5, containing 1 % NP-40, 0.1 % sodium deoxycholate, 0.1 % sodium dodecylsulfate, 150 mM NaCl and 1 mM EDTA 2Na). The lyzed protein (5 μg) was separated by SDS-PAGE using a 10–20 % gradient polyacrylamide gel (Bio-Rad, Hercules, Calif., USA) and electroblotted onto PVDF membranes (DuPont, Boston, Mass., USA). After blockage with 5 % nonfat milk in PBS containing 0.1 % Tween 20, the membrane was incubated overnight at 4 °C with antihuman Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or anti-β-actin antibody as control (Sigma, St. Louis, Mo., USA). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, Wis., USA) at room temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, Mass., USA) and quantified by computer-assisted image analysis with the NIH imaging software.

Evaluation

Experiments were repeated four to eight times, and the results were expressed as the mean and SD. Differences were statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's *F*-test. A p value less than 0.05 was considered to be statistically significant.

Modifications of the Method

Maruyama et al. (2000) found that dopaminergic neurotoxins, 6,7-dihydroxy-1-(3′, 4′-dihydroxybenzyl)-isoquinolines, cause different cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines.

Maruyama et al. (2003) described the antiapoptotic action of an anti-Alzheimer drug, TV3326 [(N-propargyl)-(3R)-aminoindan-5-yl)-ethyl methyl carbamate], a novel cholinesterase–monoamine oxidase inhibitor.

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In Vivo Methods

Tremorine and Oxotremorine Antagonism

Purpose and Rationale

The muscarinic agonists tremorine and oxotremorine induce parkinsonism-like signs such as tremor, ataxia, spasticity, salivation, lacrimation, and hypothermia. These signs are antagonized by anticholinergic drugs.

Procedure

Groups of six to ten male NMRI mice weighing 18–22 g are used. They are dosed orally with the test compound or the standard (5 mg/kg benzatropine mesilate) 1 h prior to the administration of 0.5 mg/kg oxotremorine s.c. Rectal temperature is measured before administration of the compound (basal value) and 1, 2, and 3 h after oxotremorine injection. Tremor is scored after oxotremorine dosage in 10-s observation periods every 15 min for 1 h.

Tremor	Score
Absent	0
Slight	1
Medium	2
Severe	3

Salivation and lacrimation are scored 15 and 30 min after oxotremorine injection

Absent	0
Slight	1
Medium	2
Severe	3

Evaluation

Hypothermia

The differences of body temperature after 1, 2, and 3 h versus basal values are summarized for each animal in the control group and the test groups. The average values are compared statistically.

Tremor

The scores for all animals in each group at the three observation periods are summarized. The numbers in the treated groups are expressed as percentage of the number of the control group.

Salivation and Lacrimation

The scores for both symptoms for all animals in each group are summarized at the two observation periods. The numbers in the treated groups are expressed as a percentage of the number of the control group.

Critical Assessment of the Method

The oxotremorine antagonism has been proven to be a reliable method for testing central anticholinergic activity. The overt isomorphism between the animal models and the symptoms of Parkinson's disease recommend this test for screening of anti-Parkinson drugs (Cho et al. 1962; Vernier 1964; Everett 1964; Turner 1965; Bebbington et al. 1996; Frances et al. 1980; Ringdahl and Jenden 1983). However, the model measures only central anticholinergic activity (Duvoisin 1976).

Modifications of the Method

Matthews and Chiou (1979) developed a method for quantifying resting tremors in a rat model of limb dyskinesias. The model involved permanent cannulation of the caudate nucleus for the introduction of carbachol. Tremors were quantified with a small transducer and an electronic data collecting system. The system allows the construction of dose–response curves for tremor inhibition by potential antiparkinsonism drugs.

Johnson et al. (1986) developed a procedure for quantifying whole-body tremors in mice. Displacement of a free floating platform by animal movement created a change in resistance across a strain gauge. Administration of oxotremorine, 2.5 mg/kg, i.p., produced numerous high-frequency, high-intensity peaks within 5 min.

Clement and Dyck (1989) constructed and tested a tremor monitor that quantitates soman- and oxotremorine-induced tremors. The device consisted of a force transducer, from which a plastic beaker was suspended containing a mouse. The signal from the force transducer was fed into a tremor monitor and quantitated using the Applecounter from Columbus Instruments.

Coward et al. (1977) recommended *N*-carbamoyl-2-(2,6-dichlorophenyl)acetamidine hydrochloride (LON-954), a tremorigenic agent, as an alternative to oxotremorine for the detection of anti-Parkinson drugs.

Rats treated with 3-acetylpyridine show a selective degeneration of neurons in the inferior olive nucleus and the olivo-cerebellar tract with characteristic motor incoordination and ataxia (Denk et al. 1968; Watanabe et al. 1997; Kinoshita et al. 1998). Similar motor dysfunction is seen in patients with olivopontocerebellar atrophy. To measure the effect of 3-acetylpyridine and the ameliorating effect of TRH agonists in rats, the maximal height of vertical jump after stimulation by a foot shock was measured.

Stanford and Fowler (1997) used a special technique for measuring forelimb tremor in rats induced by low doses of physostigmine. The rats pressed a force-sensing operandum while a computer measured force output and performed Fourier analyses on resulting force-time waveforms.

Several studies indicate that the exposure to the pesticide **rotenone** causes highly selective dopaminergic degeneration and α-synuclein aggregation in rats (Betarbet et al. 2000; Sherer et al. 2003a, b).

Tremulous jaw movements in rats induced by tacrine (Mayorga et al. 1997; Cousins et al. 1999) are considered to reflect symptoms in Parkinson's disease. They are suppressed by dopamine agonists (Salamone et al. 2005).

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MPTP Model of Parkinson's Disease

Purpose and Rationale

N-MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been shown to cause symptoms of Parkinson's disease in exposed individuals. The first observation was made in a relatively young, drug-abusing population. This initiated many clinical and experimental studies (Chiba et al. 1984; Heikkila et al. 1984; Kindt et al. 1988; review by Special 2002). When administered to primates, this compound causes a partial destruction of basal ganglia and a syndrome that resembles Parkinson's disease.

Procedure

Burns et al. (1983) treated eight adult rhesus monkeys weighing 5–8 kg over a period of 5–8 days with cumulative intravenous doses of N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (N-MPTP) up to 10–18 mg/kg. These animals showed a parkinsonism-like disorder (akinesia, rigidity, postural tremor, flexed posture, eyelid closure, drooling) which was reversed by the administration of L-dopa. The pathological and biochemical changes produced by N-MPTP are similar to the well-established changes in patients with parkinsonism.

The N-MPTP intoxication was applied using marmosets by Nomoto et al. (1985, 1988), Temlett et al. (1989), and Kebabian et al. (1992) to evaluate potential anti-Parkinson drugs.

Evaluation

The severity of parkinsonian symptoms is rated by trained observers using a scale of 0 (normal) to 17 (maximum severity) that assesses movement (0, normal; 1, reduced; 2, sleepy), checking movements (0, present; 1, reduced; 2, absent), attention and blinking (0, normal; 1, abnormal), posture (0, normal; 1, abnormal trunk; 2, abnormal trunk and tail; 3, abnormal trunk, tail, and limbs; 4, flexed posture), balance and coordination (0, normal; 1, impaired; 2, unstable; 4, falls), reactions (0, normal; 1, reduced; 2, slow; 3, absent), and vocalizations (0, normal; 1, reduced; 2, absent).

Modifications of the Method

Close and Elliott (1991) studied the behavioral effects of antiparkinsonian drugs in normal and MPTP-treated marmosets following central microinfusions.

Kebabian et al. (1992) tested a selective D_1 receptor agonist with antiparkinsonian activity in MPTP-treated marmosets.

Domino and Sheng (1993) studied the relative potency of some dopamine agonists with varying selectivities for D1 and D2 receptors in MPTP-induced hemiparkinsonian monkeys.

Gnanalingham et al. (1995) used MTPT-treated marmosets and found differential effects with D1 dopamine antagonists as compared with the effects in rats with unilateral 6-OHDA-induced lesions, where 6-OHDA is 6-hydroxydopamine.

Doudet et al. (1993) used intravenous administration of ^{15}O-labeled water and 6-(^{18}F)-L-fluorodopa to assess abnormal striatal activity in monkeys after long-term recovery of unilateral lesions of the dopaminergic nigrostriatal system induced by MPTP. Positron emission tomography (PET) data were examined in relation to behavioral and biological parameters, such as cerebral blood flow.

Belluzzi et al. (1994) induced a hemiparkinsonian syndrome in *Macaca nemestrina* **monkeys** by unilateral infusion of MPTP into the right coronary artery.

Raz et al. (2000) recorded hand tremor and simultaneous activity of several neurons in the external and internal segments of the globus pallidus in **vervet monkeys** before and after treatment with MPTP.

Rollema et al. (1989) compared the effects of intracerebrally administered MPP^+ (1-methyl-4-phenylpyridinium) in three species (**mouse**, **rat**, and **monkey**) by microdialysis determinations of dopamine and metabolites in the striatum.

Asin et al. (1997) tested a selective D_1 receptor agonist in **rats** previously given unilateral 6-hydroxydopamine injections and in **macaques** previously given unilateral, intracarotid infusions of MPTP.

Lange (1989, 1990) described circling behavior in old **rats** after unilateral intranigral injection of MPTP.

Fuxe et al. (1992) studied the protection against MPTP-induced degeneration of the nigrostriatal dopamine neurons in the **black mouse**.

There are marked species differences in susceptibility to the neurotoxic effects of MPTP (Giovanni et al. 1994; Schober 2005).

Grunblatt et al. (2001) described gene expression analysis of mice in the MPTP model of Parkinson's disease using cDNA microarray.

Mandel et al. (2003) reviewed the use of cDNA microarrays to assess Parkinson's disease models and the effects of neuroprotective drugs.

Hamre et al. (1999) performed a quantitative analysis in seven strains of **mice** and found that differential strain susceptibility following MPTP administration acts in an autosomal fashion.

Sedelis et al. (2001) described behavioral phenotyping of the MPTP mouse model on Parkinson's disease.

Muramatu et al. (2003) published an immunocytochemical study on cerebral alterations in an MPTP mouse model of Parkinson's disease.

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Reserpine Antagonism

Purpose and Rationale

Reserpine induces depletion of central catecholamine stores. The sedative effect can be observed in mice shortly after injection, followed by signs of eyelid ptosis, hypokinesia, rigidity, catatonia, and immobility. These phenomena can be antagonized by dopamine agonists.

Procedure

Male NMRI mice of either sex weighing 20–25 g are used. They are injected intraperitoneally with 5 mg/kg reserpine and tested 24 h later. The test compounds are injected 30 min prior to observation. The animals are placed singly onto the floor of a Perspex container (30 × 26 cm, 20 cm high), situated on a Panlab proximity sensor unit. Horizontal movements are recorded for 10 min. Moreover, rearings and grooming episodes are recorded by an experienced observer.

Evaluation

Locomotor activity and grooming scores of drug-treated animals are compared with controls treated with reserpine and vehicle only by analysis of variance.

Modifications of the Method

Rats treated with reserpine develop spontaneous orofacial dyskinesia that has features similar to tardive dyskinesia in humans (Abbott et al. 1991; Nisewander et al. 1994). The incidence of tongue protrusions was recorded to quantify the occurrence of oral dyskinesia.

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Circling Behavior in Nigrostriatal-Lesioned Rats

Purpose and Rationale

Unilateral lesion of the dopaminergic nigrostriatal pathway in the rat by the neurotoxin 6-hydroxydopamine (6-OHDA) induces hypersensitivity of the postsynaptic dopaminergic receptors in the striatum of the lesioned side (Ungerstedt 1971). The rats rotate in a direction toward the lesioned side (ipsilateral) when an indirect acting compound such as amphetamine is administered, but to the opposite direction (contralateral) when a directly acting dopamine agonist, e.g., apomorphine, or the dopamine precursor L-dopa is given. Therefore, this test can be used for the study of central dopamine function and the evaluation of dopamine antagonists and agonists, particularly the activity of novel antiparkinsonian drugs.

Procedure

Male Wistar rats weighing 200–250 g at the time of surgery are used. They are housed individually in a controlled environment with free access to food and water.

The animals are anesthetized with sodium pentobarbital. The head is placed in a stereotaxic device (DKI 900) and positioned according to the atlas of König and Klippel (1963). After a sagittal cut is made in the skin of the skull, a 2-mm-wide hole is drilled with an electrical trepan drill. Care is taken not to lesion the meninges. A 30-gauge stainless steel cannula connected to a Hamilton syringe is aimed at the

anterior zona compacta of the substantia nigra (coordinates anterior 4.1, lateral 1.0, and dorsoventral −2.5 from instrument zero). A total of 8 μg of 6-OHDA in 4 *y*/l of saline is injected at a rate of 4 min. After the intracranial injection, the wound is closed. The animal is allowed several weeks for recovery and for development of the lesion.

Specially constructed opaque plastic spheres attached to solid-state programming equipment serve as test chambers. The number of full turns, either ipsilateral or contralateral to the lesion, is recorded on an automatic printout counter every 15 min for 1- or 2-h-test sessions.

To determine the control values for ipsilateral turning, each subject is administered 2.5 mg/kg of d-amphetamine and immediately placed in the circling chamber for 2 h. Control values for contralateral circling are determined by injecting apomorphine at 1 mg/kg s.c. and recording the rat's circling for 1 h.

Test compounds are given i.p. or s.c. and the animals placed into the circling chambers. Circling is recorded over a 1-h period.

Evaluation

Percent change of drug turns from control turns is recorded. Using various doses ED50 values can be calculated.

Modifications of the Method

Etemadzadeh et al. (1989) described a computerized rotometer apparatus for recording circling behavior. The digital pulses derived from the infrared photocell detector induced by the animal rotations were fed directly to a 20-megabyte microcomputer for online recording and were processed further to the Digital Equipment Corporation's VAX computer with the SAS software system for statistical and graphical analysis.

Hudson et al. (1993) described a 16-channel-automated rotometer system for reliable measurement of turning behavior in 6-hydroxydopamine-lesioned and transplanted rats. The system is preferable to more tedious methods such as videotaping and subsequent manual analysis or various other mechanical systems.

A rotometer differentiating between clockwise and counterclockwise rotations with computerized evaluation is available from Technical and Scientific Equipment (Bad Homburg, Germany; Schwarz et al. 1978).

Carey (1989) tested stimulant drugs as conditioned and unconditioned stimuli in a classical conditioning paradigm using drug-induced rotational behavior in rats with unilateral lesions of dopamine neurons.

The production of asymmetry and circling behavior following unilateral, intrastriatal administration of neuroleptics was discussed by Costall et al. (1983).

Rotational behavior produced by intranigral injections of bovine and human β-casomorphins in rats was described by Herrera-Marschitz et al. (1989).

Perese et al. (1989) created a hemiparkinsonian model in rats in which there is 6-OHDA-induced destruction of the dopaminergic nigrostriatal pathway but sparing of the dopaminergic mesolimbic pathway.

Sauer and Oertel (1994) published a retrograde tracing and immunocytochemical study on the progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine in the rat.

Garrett and Holtzman (1996) compared the effects of apomorphine, d-amphetamine, cocaine, and caffeine on locomotor activity and rotational behavior in rats with unilateral 6-OHDA-induced lesions of the nigrostriatal tract.

McElroy and Ward (1995) reported that the high-affinity and selective dopamine D3 receptor ligand, 7-OH-DPAT, caused 6-OHDA-lesioned rats to rotate in a direction contralateral to the lesioned side similarly to the direct-acting dopamine agonist apomorphine.

Haque et al. (1996) directly infused the neurotrophins NT3 and NT4/NT5 intraparenchymally in close proximity to transplanted nigral tissue placed in the dopamine-depleted striatum of 6-hydroxydopamine-lesioned rats.

A survey on the unilateral 6-hydroxydopamine lesion model in behavioral brain research was prepared by Schwarting and Huston (1996).

DSP-4 [*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine] is highly toxic to noradrenergic neurons (Finnegan et al. 1999). Srinivasan and Schmidt (2003, 2004) showed that parkinsonian symptoms in 6-hydroxydopamine-induced partial degeneration of substantia nigra in rats are potentiated after depletion of nucleus coeruleus noradrenaline by intraperitoneal treatment with DPS-4.

Harro et al. (2003) studied the effect of denervation of the locus coeruleus projections by DSP-4 treatment on [^{3}H]raclopride binding to dopamine D_2 receptors and D2 receptor–G protein interaction in rat striatum.

Löscher et al. (1996) described a mutant rat strain (*CI*) with abnormal circling behavior reminiscent of 6-hydroxydopamine-lesioned rats.

Mele et al. (1997) studied alterations in striatal dopamine overflow by in vivo microdialysis during rotational behavior of rats induced by amphetamine, phencyclidine, and MK 801.

Smith et al. (1996) reported contralateral turning in chronically cannulated rats after stimulation of glutamate receptors by unilateral intrastriatal injections of glutamate receptor agonists, such as kainate or AMPA.

Spooren et al. (2000) tested the effects of a prototypical $mGlu_5$ receptor antagonist on rotarod, locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats using an accelerated rotarod and automated rotameter cylinders (TSE Systems, Bad Homburg, Germany).

Using the same equipment, Breysse et al. (2002) reported that chronic but not acute treatment with a metabotropic glutamate 5 receptor antagonist reverses the akinetic deficits in rats with 6-hydroxydopamine lesions.

Using automated rotameter bowls (TSE Systems, Bad Homburg, Germany), Meissner et al. (2002) found that deep brain stimulation of subthalamic neurons induces contralateral circling in freely moving 6-hydroxydopamine-lesioned rats.

With this equipment, Lebsanft et al. (2005) found that 3,4-methylenedioxymethylamphetamine counteracts akinesia enantioselectively in rat rotational behavior.

O'Neill et al. (2004) described neurotrophic actions of an AMPA receptor potentiator, which may increase brain-derived neurotrophic factor expression, in rodent models of Parkinson's disease.

Inhibition of sinistrotorsion induced in **guinea pigs** by injection of physostigmine into the right carotid artery was proposed as a method of screening central anticholinergic activity by De Jonge and Funcke (1962).

Behavioral quantification of striatal dopaminergic supersensitivity after bilateral 6-hydroxydopamine lesions in the **mouse** was reported by Mandel et al. (1992).

Worms et al. (1986) and Poncelet et al. (1993) studied turning behavior in mice induced by intrastriatal injection of neuropeptides.

Fitzgerald et al. (1992) recommended the **chakragati mouse** (**ckr**), a transgenic insertional mutant which displays lateral circling, locomotor hyperactivity and hyperreactivity, as a model to study aspects of neuropsychiatric disorders associated with dopaminergic abnormalities.

Emonds-Alt et al. (1995) injected 0.3 pg senktide into the striatum of **gerbils** inducing contralateral rotations which were antagonized by intraperitoneal or oral administration of a tachykinin NK3 receptor antagonist.

Vernier and Unna (1963) tested drugs against the tremor induced by stereotactically oriented electric lesions in the region of the subthalamus or the mesencephalic reticular formation in **monkeys**.

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Elevated Body Swing Test

Purpose and Rationale

Borlongan and Sanberg (1995) and Borlongan et al. (1995) proposed the elevated body swing test as a measure of asymmetrical motor behavior of hemiparkinsonian animals in a drug-free state.

Procedure

Male, 8-week-old Sprague Dawley rats are anesthetized with sodium pentobarbital (60 mg/kg i.p.) and mounted in a Kopf stereotaxic frame. They are lesioned by injection of 8 μg 6-hydroxydopamine in 4 μl saline containing 0.02 % ascorbic acid in the left substantia nigra (AP 5.0, ML + 1.5, DV 8.0). The solution is injected over a 4-min period and the needle left in place for an additional 5 min before retraction.

Seven days after the lesion, behavioral testing is performed. The animal is allowed to habituate in a Plexiglas box and attain a neutral position having all four paws on ground. The rat is held about 2.5 cm from the base of its tail and elevated 2.5 cm above the surface on which it has been resting. A swing is recorded whenever the animal moves its head out of the vertical axis to either side. Before attempting another swing, the animal must return to the vertical position for the next swing to be counted. Swings are counted for 60 s over four consecutive 15-s segments. The total number of swings made to each side is divided by the overall total number of swings made to both sides to get percentages of left and right swings. The criterion of biased swing is set at 70 % or higher. At 30 and 45 s, 6-OHDA-lesioned rats exhibit right-biased swings of 70 % or higher compared to normal rats.

Evaluation

A two-way ANOVA is used to analyze swing behavior data across the 15-s segments.

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Skilled Paw Reaching in Rats

Purpose and Rationale

Montoya et al. (1990, 1991), Abrous et al. (1993), Abrous and Dunnett (1994), Nikkhah et al. (1993), and Barnéoud et al. (1996) used the skilled paw reaching test as a model of Parkinson's disease in the rat. The term "staircase test" mentioned in this context has nothing to do with the staircase test described by Thiebot et al. (1973) for evaluating anxiolytic activity in rats (see chapter "► Tests for Anxiolytic Activity").

Unilateral injection of 6-OHDA into the medial forebrain bundle results in an impairment of paw reaching on both sides which can be ameliorated by drug treatment or transplantation of a nigral cell suspension.

The apparatus has been developed after earlier studies by Whishaw et al. (1986) who investigated the contributions of motor cortex, nigrostriatal dopamine, and caudate putamen to skilled forelimb use in the rat.

Procedure

Apparatus

The apparatus consists of a clear Perspex chamber with a hinged lid. A narrower compartment with a central platform running along its length, creating a trough on either side, is connected to the chamber. The narrowness of the side compartment prevents rats from turning around, so they can use only their left paw for reaching into the left trough and their right paw for reaching into the right trough. A removable double staircase is inserted into the end of the box, sliding into the troughs on either side of the central platform. Each of the steps of the staircase contains a small well, and two 45-mg saccharin-flavored pellets are placed in each well.

Learning Procedure

The week before the start of the training period, the rats are deprived of food and their body weight is stabilized at 85 % of the weight of non-deprived rats. At the same time, they are gently manipulated and familiarized with the appetitive saccharin-flavored pellets.

The animals then begin to learn the paw reaching task. For 4 weeks they are placed in the test boxes once per day for 10–15 min. The number of pellets eaten during the test period indicates the rat's success in grasping and retrieving the pellets; the number of steps from which pellets have been removed provides an index of the attempts to reach the food and how far the rat can reach; the number of missed pellets remaining at the end of the test on the floor of the side compartment indicates a lack of sensorimotor coordination in grasping and retrieving the pellets. In addition to these three parameters, it is noted which forepaw the rat used for the first movement to reach the pellet on each test day. A first choice score of +1 corresponds to the paw contralateral to the lesion, a score of −1, to the paw ipsilateral to the lesion. Because rodents exhibit a "pawedness," it must be noted whether there is a preference for one paw.

Lesions

The mesotelencephalic system is lesioned by a stereotaxic unilateral injection of 6-OHDA into the medial forebrain bundle under equithesin anesthesia. 6-OHDA is injected in a volume of 1.5 µl and at a concentration of 4 µg/µl of 0.9 % saline and 0.01 % ascorbic acid twice over 3 min via a 30-gauge stainless steel cannula at the stereotaxic coordinates: $L = 1.6$ mm, $AP = 0$ mm, $V = -7.6$ mm and $L = 1.6$ mm, $AP = -1$ mm, $V = -8$ mm. The coordinates AP and L are estimated relative to the bregma, and V is measured from the level of the dura, with the incisor bar set 5 mm above the interaural line. Following each injection, the cannula is left in place for an additional 4 min to allow the diffusion of the neurotoxin away from the injection site. The sham-operated group receives sham lesions by identical injection of ascorbate-saline solution alone.

Drug Treatment

The animals are injected i.p. with the test drug or saline 30 min before the unilateral 6-OHDA lesion and 24 h thereafter.

Evaluation

Test sessions are performed 4, 5, 7, and 8 weeks after 6-OHDA lesion. The parameters success, attempts, and sensorimotor coordination are subjected to a two-way ANOVA with group as the independent measure and weeks as the dependent measure.

Modifications of the Method

Fricker et al. (1996) investigated the effect of unilateral ibotenic acid lesions in the dorsal striatum, placed at anterior, posterior, medial, or lateral loci, in the staircase test of skilled forelimb use.

Nakao et al. (1996) studied paw reaching ability in rats with unilateral quinolinic acid lesions of the striatum as an animal model for Huntington's disease.

Barnéoud et al. (1996) evaluated the neuroprotective effects of riluzole using impaired skilled forelimb use, circling behavior, and altered dopaminergic metabolism of the

mesotelencephalic system in unilaterally 6-OHDA-lesioned rats.

Fricker et al. (1997) studied the correlation between positron emission tomography, using ligands to the D1 and D2 receptors, and reaching behavior in rats with ibotenic acid lesions and embryonic striatal grafts.

Grabowski et al. (1993), Marston et al. (1995), and Sharkey et al. (1996) tested drug effects on skilled motor deficits produced by middle cerebral artery occlusion in rats using the paw reaching test.

Meyer et al. (1997) described a revolving food pellet test for measuring sensorimotor performance in rats.

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Stepping Test in Rats

Purpose and Rationale

Schallert et al. (1992), Olsson et al. (1995), and Rosenblad et al. (1997) introduced the stepping test as a clinically relevant unilateral model of parkinsonian akinesia. The 6-OHDA lesion induced marked and long-lasting impairments in the initiation of stepping movements with the contralateral paw which can be ameliorated by the systemic application of drugs.

Procedure

6-OHDA Lesion Surgery

Female Sprague Dawley rats receive two stereotaxic injections of 6-OHDA (3.6 μg/μl in 0.2 μg/ml ascorbate saline) into the right ascending mesostriatal dopamine pathway using a 10-μl Hamilton syringe at the following coordinates (in mm, with reference to bregma and dura):

1. 2.5 μl at AP −4.4, L 1.2, V 7.8, tooth bar −2.4
2. 3.0 μl at AP −4.0, L 0.8, V 8.0, tooth bar +3.4 at an injection rate of 1 μl/min

The cannula is left in place for an additional 5 min before slowly retracted.

Experimental Setup for Stepping Test

The tests monitoring initiation time, stepping time, and step length are performed using a wooden ramp with a length of 1 m connected to the rat's home cage. A smooth-surfaced table is used for measuring adjusted steps.

During the first 3 days, the rats are handled by the experimenter to familiarize them with the experimenter's grip. During the subsequent 1–2 days, the rats are trained to run spontaneously up the ramp to the home cage.

The stepping test comprises two parts: first, the time to initiation of a movement of each forelimb, the step length, and the time required for the rat to cover a set distance along the ramp with each forelimb and second, the initiation of adjusting steps by each forelimb when the animal was moved sideways along the bench surface. Each test consists of two tests per day for three consecutive days and the mean of six subtests is calculated.

Initiation Time, Stepping Time, and Step Length

The rat is held by the experimenter with one hand fixing the hind limbs and slightly raising the hind part above the surface. The other hand fixes the forelimb not to be monitored. Time is measured until the rat initiates movement with the forelimb not fixed by the experimenter, using 180 s as break-off point. Stepping time is measured from initiation of movement until the rat reaches the home cage. Step length is calculated by dividing the length of the ramp by the number of steps required for the rat to run up the ramp. The sequence of testing is right paw testing followed by left paw testing, repeated twice.

Adjusting Steps

The rat is held in the same position as described above with one paw touching the table and is then moved slowly sideways (5 s for 0.9 m) by the experimenter, first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand directions of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated twice each day.

The paw contralateral to the lesion is passively dragging when the rat is moved in the forehand direction, while the ipsilateral paw performs frequent stepping movements.

Drug Application

Stepping tests are repeated to determine the baseline weekly after the 6-OHDA lesion. The drug tests are administered for 1 day only. Various drugs can be evaluated in weekly intervals.

Evaluation

Results are expressed as means ± SEM. For statistical evaluation, the data are subjected to one-factor analysis of variance (ANOVA) and Fisher post hoc test.

Modifications of the Method

Schallert et al. (2000), Picconi et al. (2003, 2004), and Centonze et al. (2005) described the limb-use asymmetry test to measure motor performance in rats with 6-OHDA-induced lesions. Forelimb use during exploratory activity was analyzed in a transparent cylinder (20 cm diameter and 30 cm height) for 3–10 min depending on the degree of movement maintained during the trial. The number of supporting wall contacts executed independently with the right of the left forelimb was counted. The percentage of wall contacts executed by the impaired forelimb (contralateral to the lesion) was then subtracted from the percentage of contacts with the nonimpaired forelimb to obtain a limb-use asymmetry score.

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Transgenic Animal Models of Parkinson's Disease

Purpose and Rationale

New insight in the pathobiology of Parkinson's disease has led to the development of transgenic animal models (Gonzalez-de Aguilar et al. 2003; Kirik and Björklund 2003; Levine et al. 2004).

The most prominent models are related to **α-synuclein**. The first transgenic mice that express human α-synuclein were generated by Masliah et al. (2000). These mice displayed a progressive accumulation of α-synuclein and ubiquitin-immunoreactive inclusions in neurons of the neocortex, hippocampus, and substantia nigra. These alterations were associated with a loss of dopaminergic terminals in the basal ganglia and with motor impairments. Since then, several studies on transgenic α-synuclein mice have been performed (van der Putten et al. 2000; Masliah et al. 2001; Giasson et al. 2002; Kirik et al. 2002; Gispert et al. 2003; Hashimoto et al. 2003; Fernagut and Chesselet 2004; Martín-Clemente et al. 2004; Cabin et al. 2005; Frasier et al. 2005; Poon et al. 2005). Masliah and Hashimoto (2002) discussed the possible role of **β-synuclein** for development of new treatments for Parkinson's disease using transgenic animal models.

Ubiquitin carboxyl-terminal hydrolase L1 (**UCH-L1**) is a neuron-specific ubiquitin recycling enzyme, which may be implicated in the pathogenesis of Parkinson's disease (Leroy et al. 1998). Lindsten et al. (2003) developed a model for in vivo analysis of the ubiquitin/proteasome system by generating mouse strains

transgenic for a green fluorescence protein reporter carrying a constitutively active degradation signal.

Loss-of-function mutations in **parkin** are the major cause of early-onset familial Parkinson's disease. To investigate the pathogenic mechanism by which loss of parkin function causes Parkinson's disease, Goldberg et al. (2003) generated a mouse model bearing a germline disruption in parkin. In addition to PARK1 (representing the α-synuclein gene) and PARK2 (representing the UCH-L1 gene), other monogenic forms of Parkinson's disease have been described (Lansbury and Brice 2002; von Bohlen und Halbach et al. 2004).

The ***weaver* mutant mouse** has been proposed as another model of Parkinson's disease (Bankiewicz et al. 1993; Bandmann et al. 1996; Cheng et al. 1997; Ebadi et al. 2005).

Modifications of the Method

Lo Bianco et al. (2002) studied α-synucleinopathy and selective dopaminergic neuron loss in a **rat** lentiviral-based model of Parkinson's disease.

Kirik et al. (2003) described nigrostriatal α-synucleinopathy induced by viral vector-mediated overexpression of human α-synuclein using a primate (adult **marmosets**) model of Parkinson's disease.

Transgenic ***Drosophila*** models expressing human α-synuclein were described by Feany and Bender (2000), Pendleton et al. (2002), and Scherzer et al. (2003).

Brown et al. (2002) published multiplex three-dimensional brain gene expression mapping in a mouse model of Parkinson's disease.

Son et al. (2003) described cloning and expression analysis of the Parkinson's disease gene, **UCH-L1**, and its promoter in **zebrafish**.

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Cell Transplantations into Lesioned Animals

Purpose and Rationale

Human embryonic stem cells may potentially serve as a renewable source of cells for transplantation. In Parkinson's disease, embryonic stem cell-derived dopaminergic neurons may replace the degenerated neurons in the brain. To substantiate this goal, numerous animal experiments were performed. Most authors used rat-rendered hemiparkinsonian by injection of 6-OHDA into the substantia nigra as recipients (Zawada et al. 1998; Mendez et al. 2000; Sawamoto et al. 2001; Björklund et al. 2002; Hao et al. 2002; Kim et al. 2002; Ben-Hur et al. 2004; Burnstein et al. 2004; Jollivet et al. 2004; Levy et al. 2004; Yoshizaki et al. 2004; Rafuse et al. 2005; Richardson et al. 2005).

Takagi et al. (2005) reported that dopaminergic neurons generated from cynomolgus monkey embryonic stem cells functioned in the MPTP primate model of Parkinson's disease. However, complications were reported by Blanchet et al. (2003) using a similar approach.

In spite of almost 20 years of experimental experience, clinical trials with cell transplantation for Parkinson's disease have had disappointing results (Drucker-Colin and Verdugo-Diaz 2004; Linazaroso 2004; Roitberg et al. 2004).

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Transfer of Glial Cell Line-Derived Neurotrophic Factor (GDNF)

Purpose and Rationale

Recombinant viral vectors derived from adenovirus, adeno-associated virus, or *Lentivirus* have been developed into highly effective vehicles for gene transfer to the adult central nervous system. These vectors have been shown to be effective for long-term delivery of glial cell line-derived neurotrophic factor (GDNF) in the nigrostriatal system. GDNF is a strong candidate agent in the neuroprotective treatment of Parkinson's disease. The injection of adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protected nigral neurons in the 6-OHDA-induced degeneration model of Parkinson's disease in rats (Yoshimoto et al. 1995; Bilang-Bleuel et al. 1997; Lapchak et al. 1997; Mandel et al. 1997; Björklund et al. 2000; Chen et al. 2003; Wang et al. 2002; Thi et al. 2004). Jollivet et al. (2004) used intrastriatal glial cell line-derived neurotrophic factor-releasing microspheres. Yasuhara et al. (2005) reported the use of glial cell line-derived neurotrophin factor-producing cells encapsulated into hollow fibers. Kojima et al. (1997) and Cheng et al. (1998) found that glial cell line-derived neurotrophic factor protects against MPTP-induced neurotoxicity in **mice**. Kordower et al. (2000) reported that neurodegeneration was prevented by lentiviral vector delivery of GDNF in primate models

(**rhesus monkeys** treated with MPTP) of Parkinson's disease.

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Animal Models of Neurological Disorders

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Contents

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Huntington's Disease

General Considerations

Purpose and Rationale

Huntington's disease is a neurological disorder characterized by loss of striatal neurons and the motor signs of dyskinesia, dystonia, and chorea, as well as complex neuropsychiatric changes (Hayden 1981). Brouillet et al. (1999) reviewed the different aspects of the replicating Huntington's disease phenotype in experimental animals. There is at present no effective therapy against this disorder. The gene responsible for the disease has been cloned and the molecular defect identified as an expanded polyglutamine tract in the N-terminal region of a protein, named huntingtin (Landles and Bates 2004; Li and Li 2004). Huntingtin interacts with a number of proteins and it has been suggested that alterations in glycolysis, vesicle trafficking, or apoptosis play a role in the pathophysiology of Huntington's disease.

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3-Nitropropionic Acid Animal Model of Huntington's Disease

Purpose and Rationale

An animal model of **Huntington's disease** was recommended by Beal et al. (1993), Brouillet et al. (1993), and Borlongan et al. (1995, 1997a, b). Administration of 3-nitropropionic acid, an inhibitor of the mitochondrial citric acid cycle, produces a very selective striatal degeneration and results in a progressive locomotor deterioration in rodents resembling that of Huntington's disease.

Procedure

Borlongan et al. (1995) injected male Sprague Dawley rats i.p. with 10 mg/kg of 3-nitropropionic acid once every 4 days for 28 days and measured passive avoidance in a step-down apparatus and locomotor activity using the Digiscan Animal Activity Monitor System. Using the same system, Borlongan et al. (1997b) found hyperactivity after two injections, whereas four injections or more lead to hypoactivity.

Lee et al. (2000) evaluated the neuroprotective effect of lamotrigine and MK-801 on rat brain lesions induced by 3-nitropropionic acid by magnetic resonance imaging and in vivo proton magnetic resonance spectroscopy.

Male Sprague Dawley rats received daily i.p. injections of 15 mg/kg of 3-nitropropionic acid for five consecutive days. Test drugs were injected daily before 3-nitropropionic acid injections.

Behavioral changes were recorded daily, and immediately before sacrifice, graded according to the neurological scale described by Guyot et al. (1997). A total of six grades included: Grade 0, normal behavior; grade 1, general slowness in movement due to mild hind-limb impairment; grade 2, prominent gait abnormality with poor coordination; grade 3, nearly complete hind-limb paralysis; grade 4, incapability to move due to four-limb impairment; and grade 5, recumbency.

Magnetic resonance measurements were performed on the sixth day under anesthesia with 60 mg/kg i.p. pentobarbital. An endotracheal tube was set for artificial ventilation with an animal ventilator. The expiratory CO_2 concentration was maintained between 3.5 % and 4.5 % by adjusting the ventilator. Magnetic resonance measurements were performed on a Biospec 4.7 T spectrometer with an active shielding gradient at 6.9 Gy/cm in

500 μs. The rats were placed in a prone position with a custom-designed head-holder. A 20-cm birdcage coil was used for RF excitation, and a 2-cm-diameter surface coil placed directly over the head was used for signal receiving. After magnetic field optimization, a multislice multiecho image was obtained with the following parameters: field of view = 5 cm, four slices (2 mm thick with a 1-mm gap), matrix 256 × 128, TR = 4,000 ms, and initial TE = 20 ms with an echo spacing of 20 ms for six echos. Pixel-by-pixel T2 maps were obtained with commercial analysis software. The average of T2 values of bilateral hippocampus or striatum calculated from multiecho images was applied for statistical analysis.

The point-resolved spectroscopy (PRESS) sequence preceded by three consecutive chemical shift selective saturation (CHESS) pulses for water suppression was used to acquire the localized proton spectra over the striatum. After manually adjusting the transmitter and receiver, magnetic field shimming of the striata and maximizing the suppression of the water signal, spectral data were obtained with the following parameters: TR = 2000 ms, TE = 136 ms, scan no. = 256, spectral width = 4000 Hz. The peak areas of Nacetyl-aspartate, choline, creatine, succinate, and lactate were recognized. The ratios of cerebral metabolites relative to creatine were used for statistical analysis.

Evaluation

The data were expressed as means ±SD. One-way ANOVA followed by a Tukey test was used for evaluating significant difference among sham controls and various treatment groups.

Modifications of the Method

Matthews et al. (1998) found neuroprotective effects of creatine and cyclocreatine against lesions induced by malonate or 3-nitropropionic acid.

Quary et al. (2000) demonstrated major strain differences in response to chronic systemic administration of the mitochondrial toxin 3-nitropropionic acid in rats.

Pubill et al. (2001) reported that orphenadrine (an anticholinergic drug with NMDA receptor antagonist properties) prevented 3-nitropropionic-acidinduced neurotoxicity in vitro and in vivo.

Lastres-Becker et al. (2003) found that compounds acting on the endocannabinoid and/or endovanilloid systems reduce hyperkinesia in rats induced by bilateral intrastriatal injection of 3-nitropropionic acid.

Lee and Chang (2004) used magnetic resonance imaging and spectroscopy to assess 3-nitropropionicacid-induced brain lesions in rats.

Tunez et al. (2004) described a protective effect of melatonin on 3-nitropropionic-acid-induced oxidative stress in synaptosomes in rats.

Hantraye et al. (1992) reported that intrastriatal transplantation of cross-species fetal striatal cells reduced abnormal movements in **baboons** injected with the neurotoxin ibotenic acid.

Palfi et al. (1996) showed that chronic 3-nitropropionic acid treatment replicates the cognitive and motor deficits in Huntington's disease also in **baboons**.

Mittoux et al. (2000) reported restoration of cognitive and motor function by ciliary neurotrophic factor in a primate model (*Macaca fascicularis*) of Huntington's disease.

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Quinolinic Acid Rat Model of Huntington's Disease

Purpose and Rationale

Intrastriatal injection of quinolate, an NMDA receptor agonist, replicates many neurochemical, histological, and behavioral features of Huntington's disease (Beal et al. 1986, 1988, 1991; DiFiglia 1990; Burns et al. 1995; Pérez-Navarro et al. 2000a).

Araujo and Hilt (1997) found that the glial cell line-derived neurotrophic factor attenuates the behavioral and neurochemical deficits induced by quinolinic acid in rats.

Procedure

Female Sprague Dawley rats were anesthetized with pentobarbital (50 mg/kg) before being placed in a stereotaxic apparatus (Kopf). A small hole was made on the left side of the skull 3.0 mm lateral to the midline, through which a hypodermic needle attached to a Hamilton syringe was lowered 5 mm below the dura into the striatum. For each lesioned rat, 200 nmol of quinolinic acid dissolved in 2 μl of phosphate-buffered saline was injected over a 1-min interval through the needle into two sites (0.7 and 1.4 mm anterior to the bregma) in the left striatum, while the contralateral striatum was left intact.

Neurotrophic factors were given 30 min prior to the toxin injections. Recombinant glia-cell-derived neurotrophic factor (Lin et al. 1993; Lapchak et al. 1997), brain-derived neurotrophic factor or neurotrophin-3 was administered intraventricularly. For i.c.v. treatment, a Hamilton syringe or an infusion cannula was lowered through a hole drilled in the skull of anesthetized rats at the following coordinates: 1.4 mm posterior to the bregma, 2 mm lateral to the midline, and 3.5 mm below the dura.

Rotational Behavior

The effects of quinolinic acid lesions and neurotrophic factor treatment on rotational behavior were assessed using a Rota-Count 8 automated rotor system (Columbus Instruments), which monitors both clockwise and counterclockwise turning behavior. One week following surgery, rats were given 5 mg/kg amphetamine i.p. 15 min prior to placement into the apparatus. The total number of turns in successive 10-min intervals was measured in a 1-h period.

Histology

The extent of the lesion produced by quinolinic acid was illustrated using Nissl-stained coronal sections.

D_1 and D_2 dopamine receptor binding was evaluated in homogenized lesioned and unlesioned striata.

Striatonigral [^{3}H]neurotransmitter uptake was measured in synaptosomes from striatal tissue containing putamen-pallidum as well as a portion of the globus pallidus.

Striatal choline acetyltransferase activity was determined in striatal homogenates using a radiochemical assay.

Neuropeptide levels in tissue homogenates were measured using commercial radioimmunoassay kits.

Evaluation

Results are expressed as mean ±SEM of the number of rats, where each sample was the average of duplicate or triplicate measures. Statistical significance was assessed using a one-way ANOVA, followed by post hoc Bonferroni or Dunnett's Multiple Comparisons analysis.

Modifications of the Method

Anderson et al. (1996) reported that the ciliary neurotrophic factor protects striatal output neurons in the quinolinic acid rat model.

Nakao et al. (1996) studied paw reaching ability in rats with unilateral quinolinic acid lesions of the striatum.

Shear et al. (1998) compared intrastriatal injections of quinolinic acid and 3-nitropropionic acid for use in animal models of Huntington's disease.

Hughes et al. (1999) found that administration of recombinant human activin-A has powerful neurotrophic effects on selected striatal phenotypes in the quinolinic acid lesion model.

Pérez-Navarro et al. (1999) reported that intrastriatal grafting of a glia cell line-derived neurotrophic factor-producing cell line protects striatonigral neurons from quinolinic acid excitotoxicity in vivo.

Reggio et al. (1999) found that the intrastriatal injection of an adenosine A_2 receptor antagonist prevents frontal cortex EEG abnormalities in the quinolinic acid rat model.

Nakao et al. (1999) reported that embryonic striatal grafts restore neuronal activity of the globus pallidus in the quinolinic acid rat model.

Pérez-Navarro et al. (2000b) found that neurturin protects striatal neurons but not interneurons in the quinolinic acid rat model.

Bensadoun et al. (2001) found a neuroprotective effect of interleukin-6 and IL6/IL6R chimera in the quinolinic acid rat model.

Gianfriddo et al. (2003) found that adenosine A_{2A} antagonism increases striatal glutamate outflow in the quinolinic acid rat model.

Ryu et al. (2003) described neuroprotective effects of pyruvate in the quinolinic acid rat model.

Scattoni et al. (2004) reported progressive behavioral changes in the spatial open-field in the quinolinic acid rat model.

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Transgenic Animal Models of Huntington's Disease

Purpose and Rationale

Several **transgenic models** of Huntington's disease are described, among them:

The **R6/2 HD mouse model** (Ona et al. 1999; Hickey and Morton 2000; Helmlinger et al. 2002; Keene et al. 2002; Hockly et al. 2003a, b; Zucker et al. 2004; Chou et al. 2005);
The **yeast artificial chromosome (YAC) mouse** (Ainscough et al. 2001; Slow et al. 2003; Al Mahdawi et al. 2004);
The **TgCAG100 mouse** (Laforet et al. 2001; Ariano et al. 2005);
The **HdhQ111mouse** (Gines et al. 2003).

A **transgenic rat model** of Huntington's disease was described by Von Hörsten et al. (2003). Behavioral phenotyping was performed using computerized systems from TSE Systems (Bad Homburg, Germany).

Segalat and Neri (2003) recommended *Caenorhabditis elegans* as a model for human inherited degenerative disorders, such as Duchenne's muscular dystrophy and Huntington's disease.

A **transgenic *Drosophila* model** of Huntington's disease was recommended by Lee et al. (2004) and Agrawal et al. (2005).

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Amyotrophic Lateral Sclerosis

General Considerations

Purpose and Rationale

Amyotrophic lateral sclerosis is an adult-onset neurological disease characterized by the selective loss of motor neurons. The early symptoms, principally limb and bulbar muscle weakness, begin in middle life and progress usually rapidly to death within 2 or 3 years. Clinical subtypes of the disease are defined according to the predominant site of weakness and whether upper or lower motor neurons are primarily involved (Rowland and Shneider 2001; Morrison 2002; Waldmeier 2003). Several experimental models for amyotrophic lateral sclerosis have been developed (Kaal et al. 1999; Anger 1991; Senior 2002).

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Transgenic Animal Models of Amyotrophic Lateral Sclerosis

Purpose and Rationale

Most of the transgenic mouse models are based on familial superoxide dismutase 1 (SOD1) mutations. Of these, the **G93A** mouse is the most investigated (Gurney et al. 1994; Kostic et al. 1997; Trieu and Uckun 1999; Vukosavic et al. 1999, 2000; Azzouz et al. 2000; Li et al. 2000; Spooren and Hengerer 2000; Andreassen et al. 2001; Canton et al. 2001; Jung et al. 2001; Wendt et al. 2002; Azari et al. 2003; Copray et al. 2003; Maragakis et al. 2003; Snow et al. 2003; van Damme et al. 2003; Raiteri et al. 2004; Raman et al. 2004; Waibel et al. 2004). These mice express mutant human SOD1 with a substitution of glycine to alanine in position 93, which induces a severe, progressive motoneuron disease. Several studies were performed with **G37R** (Nguyen et al. 2001; Farah et al. 2003; Robertson et al. 2003), **H46R** (Nagai et al. 2001), and **G85R** (Bruijn et al. 1998; Amedola et al. 2004) mutant mice. Pasinelli et al. (1998) reported cleavage and activation of caspase-1 in these animals, and Lee et al. (2001) found increased vulnerability of cells transfected with these mutant forms of SOD1 to apoptotic stimuli and protection by overexpression of Bcl-2.

Van Damme et al. (2003) tested the AMPA receptor antagonist NBXQ for survival in a transgenic mouse model of amyotrophic lateral sclerosis. Canton et al. (2001) described the pharmacological properties and activity in the SOD1-G53A animal model of amyotrophic lateral sclerosis of a synthetic AMPA antagonist.

Procedure

Transgenic mice [B6SJL-TgN(SOD1-G93A) G1H] heterozygous for the deficient SOD1 gene and wild type littermates were identified by polymerase chain reaction. Animals were treated starting on the 50th day of life, until their death with various subcutaneous doses of the test compound.

The following parameters were examined:

Muscle strength: Animals were tested for muscle strength at intervals using a muscle strength meter. Each mouse was held by the tail and pulled steadily over a metal grill three times in rapid succession. The force was recorded on a force meter. The highest score for muscle strength was taken.

Glutamate uptake: Animals were sacrificed at 150 days and spinal cord rapidly dissected for synaptosomal preparations and high-affinity Na^+ –dependent glutamate uptake was measured. Spinal cords were homogenized in 20 volumes of sucrose (0.32 M) and the homogenate centrifuged at 800 g for 10 min. After centrifugation of the supernatant at 20,000 g for 20 min, the pellet was resuspended and washed in 50 volumes of sucrose buffer. Transport assays were performed in Krebs-HEPES buffer. Duplicate samples, with or without sodium chloride, were incubated at 37 °C for 3 min in the presence of [^{3}H]L-glutamate after appropriate isotopic dilution. The reaction was stopped by addition of ice-cold buffer containing choline and followed by filtration and scintillation counting of the radioactivity retained on filters. High-affinity Na^+-dependent uptake was calculated by subtracting results obtained in choline buffer from that obtained with Na^+ buffer.

Life expectancy: Remaining mice were treated until day of death, which was noted.

Evaluation

Statistical significance of the biochemical and behavioral data was assessed using Kruskal-Wallis non-parametric analysis of variance followed by Dunn's multiple comparison test.

Modifications of the Method

Haase et al. (1998) described therapeutic effects and mechanism of action of adenovirus-mediated transfer of the neurotrophin-3 gene into skeletal muscle of pmn (progressive motor neuropathy) mice.

Schmalbruch et al. (1991) described an autosomal-recessive mouse mutant with progressive motor neuropathy. Homozygotes develop paralysis of the hind limbs during the third week of life. Soon thereafter the forelimbs also become weak, and all mice die 6–7 weeks after birth.

Zhu et al. (2002) reported that minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in transgenic mice.

Howland et al. (2002) described focal loss of the glutamate transporter EAAT2 in a transgenic **rat** model of SOD1 mutant-mediated amyotrophic lateral sclerosis.

Bordet et al. (1999, 2001) reported protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic amyotrophic lateral sclerosis mice overexpressing a mutated form of human superoxide dismutase-1 with a $Gly^{93}Ala$ substitution.

Ralph et al. (2005a, b) reported that a silencing mutant SOD1 using interfering RNA protects against neurodegeneration and extends survival in the G93A model of amyotrophic lateral sclerosis.

Pharmacology and mechanism of action of riluzole, which has demonstrated beneficial effects in amyotrophic lateral sclerosis in clinical studies, have been investigated in several preclinical studies (Doble 1996; Gurney et al. 1998; Jimonet et al. 1999; Snow et al. 2003).

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Spinal Muscular Atrophy

General Considerations

Purpose and Rationale

Spinal muscular atrophy is a common genetic disease of the motor neuron with a high mortality during infancy (Strober and Tennekoon 1999; Iannnaccone et al. 2004; Ogino and Wilson 2004). Among the group of clinically and genetically heterogeneous spinal muscular atrophies, the

autosomal recessive proximal types I-III are the most frequent (Schara and Mortier 2004). They are caused by mutations of the telemetric copy of the survival motor neuron gene (SMN1) on chromosome sq while loss of the centromeric copy (SMN2) does not lead to spinal muscular atrophy (Talbot and Davies 2001; Frugier et al. 2002; Wirth 2002; Nicole et al. 2002).

Transgenic mouse models have been developed for these types of diseases.

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Transgenic Animal Models of Spinal Muscular Atrophy

Purpose and Rationale

Several mouse models of spinal muscular atrophy are described (Bergin et al. 1997; Deniselle et al. 1997; DiDonato et al. 1997, 2001; Schrank et al. 1997; Fricker 2000; Frugier et al. 2000; Hsieh-Li et al. 2000; Jablonka et al. 2000, 2001; Monani et al. 2000a, b, 2003; Cifuentes-Diaz et al. 2001, 2002; Melki 2001; Tucker et al. 2001; Ferri et al. 2003, 2004; Rossoll et al. 2003; Azzouz et al. 2004; Grohmann et al. 2004; Le et al. 2005). These studies are intended to mimic spinal muscular atrophy in humans resulting from loss-of-function mutations in the survival motor neuron gene (Smn). Mice carrying a homozygous deletion of Smn exon 7 display a dramatic and progressive loss of motor axons involving both proximal and terminal regions. The human centromeric survival motor neuron gene (*SMN2*) rescues embryonic lethality in $Smn^{-/-}$ mice. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy.

Chang et al. (2001) reported treatment of spinal muscular atrophy in transgenic mice by sodium butyrate.

Procedure

Cell Culture

Epstein-Barr-virus-transformed lymphoid cell lines from different SMA-type patients with deleted *SMN1* genes were established. Lymphocytes were collected from the whole blood of patients by Ficoll hypaque separation. The buffy coat was collected and washed twice with 5 ml PBS. The pellet was resuspended in 5 ml RPMI medium containing 0.5 ml Epstein-Barr virus, 50 µl phytohemagglutinin (0.5 mg/ml), and 50 µl ciclosporin (0.2 mg/ml). Cells were incubated at 37 °C with 5 % CO_2 until they became viable.

Mice

Five independent human *SMN2* gene transgenic mice were generated and crossed with mice heterozygous for the *Smn* locus knockout. Transgenic mice that were also homozygous for the knockout alleles ($Smn^{-}1^{-}SMN2$) were then generated by crossing with the above mice. These knockout transgenic mice developed progressive motor neuron disease similar to that in human SMA patients. The SMA-like mice were classified

into three groups based on their phenotypes, which were judged by three independent observers. Mice with the most severe pathological form (type 1) did not develop furry hair and died before postnatal day 10; mice with intermediate severity (type 2) showed poor activity and variable symptoms and died at 2–4 weeks; the type 3 mice survived and bred normally, but had short and enlarged tails (Hsieh-Li et al. 2000). SMA-like mice (non-pregnant and pregnant) were supplied with sterile water ad libitum and rodent pellets. The sodium butyrate-treated group received sodium butyrate at a concentration of 0.8 mg/ml or 8 mg/mi in distilled water, beginning immediately after diagnosis or after 15 days gestation in SMA-like pregnant mice. Both groups consumed about 5–10 ml per day per mouse. After 1–12 weeks of treatment, the mice were sacrificed, and their organs or tissues were quickly removed and frozen in liquid nitrogen.

Reverse Transcriptase-PCR Analysis

Reverse transcriptase-PCR analysis was performed according to Hsieh-Li et al. (2000) and Jong et al. (2000).

Subcellular Fractionation

Fresh frozen spinal cord, brain, and skeletal muscle samples (500 mg) from different types of SMA mice were fractionated. Tissues were homogenized with a tight-fitting glass pestle in ice-cold buffer. The nuclei were pelleted by centrifugation at 800 *g* for 3 min. The nuclear pellet was resuspended by trituration in 100 µl of buffer and kept on ice for 15 min followed by centrifugation at 15,000 *g* for 10 min at 4 °C. The supernatant (soluble nuclear extract) was removed, and the insoluble nuclear pellet was further sonicated in sonication buffer.

Western Blot Analysis

Synthetic peptides containing part of human SMN exon 7 (amino acids 279–288) and exon 2 (amino acids 72–84) were used to immunize rabbits and to purify specific antibodies (H2 and H7) from rabbit crude sera with an EAH Sepharose 4B column. Two mouse anti-SR protein antibodies (anti-SRp20 and 16H3) were used to detect the human SR proteins. Proteins were loaded on a 5 % polyacrylamide stacking gel above a 12 % separating gel, and the gel was run with a discontinuous buffer using Laemmli's method. After electrophoresis, proteins were transferred electrophoretically to poly-(vinylidene difluoride) membranes. After the transfer, the membranes were blocked with TBST containing 4 % BSA for 2 h at room temperature. The blots were washed for three 20-mim periods in TBST and then incubated with a 1:32,000 dilution of an anti-rabbit IgG alkaline phosphatase conjugate in TBST for 1 h at room temperature. The reaction was detected by adding 1.5 % 5-bromo-4-chloro-3-indolyl phosphate and 3 % nitro blue tetrazolium in a developing buffer.

Histopathological Analysis

Histopathological analysis was performed according to Hsieh-Li et al. (2000).

Evaluation

Results from multiple experiments were expressed as mean ± standard error. Survival data of treated and untreated mice are presented as a Kaplan-Meier plot using the log rank test. A standard χ_2 test was used to assess differences in the frequency of mild or severe phenotype in the SMA-like mice born from treated and untreated mothers, which analyzed the percentage of type 1 (or 2 + 3) newborn mice as a fraction of the total number of pups.

Modifications of the Method

Lesbordes et al. (2003) reported therapeutic benefits of cardiotrophin-1 gene transfer in a mouse model of spinal molecular atrophy.

Andreassi et al. (2001) found that aclarubicin treatment restored SMH levels to cells derived from type I spinal muscular atrophy patients.

Haddad et al. (2003) reported that riluzole attenuates spinal muscular atrophy disease progression in a mouse model.

He et al. (2005) described an inherited motor neuron disease in domestic cats as a model of spinal muscular atrophy.

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Spinal and Bulbar Muscular Atrophy

General Considerations

Purpose and Rationale

Spinal and bulbar muscular atrophy is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The disease exclusively affects males, while females are usually asymptomatic (Katsuno et al. 2003, 2004).

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Transgenic Animal Models of Spinal and Bulbar Muscular Atrophy

Purpose and Rationale

Spinal and bulbar muscular atrophy is a polyglutamine disease caused by expansion of a CAG repeat in the androgen receptor gene (Adachi et al. 2001; Katsuno et al. 2002; Walcott and Merry 2002). Several transgenic mouse models were developed (Bates and Davies 1997; Abel et al. 2001; MacManamny et al. 2002).

Katsuno et al. (2003) reported that leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy.

Procedure

Animals

Transgenic mice were generated carrying a full-length androgen receptor containing 97CAGs (Katsuno et al. 2002). A full-length human androgen receptor fragment was subcloned containing 24 or 97 CAG repeats (Kobayashi et al. 1998) into a pCAGGS vector (Niwa et al. 1991) digested with *Hind*III. The result was microinjected into fertilized eggs of BDF1 mice. Five founders with AR-97Q were obtained. These mouse lines were maintained by back-crossing with CS7B1/6 mice.

Neurological status was measured with the Rotarod test (Columbus Instruments) and cage activity with the AB system (Neuroscience, Tokyo).

Hormonal Treatment

Leuprorelin acetate was injected subcutaneously at a dose of 25, 50 or 100 μg per mouse every 2 weeks from 5 weeks of age. Control mice were treated with vehicle. Leuprorelin-treated AR-97Q mice were given either leuprorelin only or leuprorelin plus 20 μg testosterone enanthate dissolved in sesame oil s.c. weekly from the age of 13 weeks. Flutamide was injected at a dose of 1.8 mg per mouse once every second day.

Serum testosterone was assayed with a radioimmunoassay.

Protein Expression Analysis

Mice were sacrificed by anesthesia and exsanguination and their tissues snap-frozen with powdered CO_2 in acetone. Tissues were homogenized in 50 mM TRIS, pH 8.0, 150 mM NaCl, 1 % Nonidet-P40,0.5 % deoxychoiate, 0.1 % SDS and 1 mM 2-mercaptoethanol with 1 mM PMSF and 6 μg /mi aprotinine and centrifuged at 2,500 g for 15 min at 4 °C. Each lane of a 5–20 % SDS-PAGE gel was loaded with 160 μg protein from nerve tissue and 80 μg for muscle from the respective supernatant fraction. This was transferred on Hybond-P membranes in a transfer buffer of 25 mM TRIS, 192 mM glycine, 0.1 % SDS and 10 % methanol. After immunoprobing with a rabbit antibody to the androgen receptor, a secondary antibody probing, and detection with the ECL Plus kit (Amersham) was performed. The signal intensity of the bands was quantified using the NIH Image program. The quantitative data of three independent Western blots were expressed as mean ± SD.

Immunohistochemistry and Histology

Deeply anesthetized mice were perfused through the left cardiac ventricle with 20 ml of a 4 % paraformaldehyde fixative in phosphate buffer. The tissues were postfixed overnight in 10 % phosphate-buffered formalin and processed for paraffin embedding. Deparaffinized tissue sections 4 μm in thickness were dehydrated in alcohol, treated with formic acid for 5 min at room temperature and stained with the polyglutamine-specific antibody 1C2 (1:10,000 dilution) (Holmberg et al. 1998). After formalin fixation, tail specimens were washed with 70 % alcohol and decalcified with 7 % formic acid and 70 % ethanol for 7 days followed by paraffin embedding. To assess 1C2-positive cells in muscle, the number of 1C2-positive cells in more than 500 fibers in the entire area was calculated and expressed as the number per 100 muscle fibers. The air dried cryostat sections (6 pm in thickness) of gastrocnemius muscles were stained with hematoxylin-eosin.

Evaluation

Data were analyzed using the unpaired *t*-test.

Modifications of the Method

Adachi et al. (2003) reported that heat shock protein 70 chaperone overexpressions ameliorates phenotypes of the spinal and bulbar muscular transgenic mouse model by reducing nuclear localized mutant androgen receptor protein.

Chevalier-Larsen et al. (2004) found that castration restores the function and neurofilament alterations of aged symptomatic males in a transgenic mouse model of spinal and bulbar muscular atrophy.

Minamiyama et al. (2004) reported that sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy.

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Models of Down Syndrome

Purpose and Rationale

Down syndrome, the most common known genetic cause of mental retardation, is the result of trisomy of chromosome 21. Although the degree of cognitive impairment in Down syndrome can be quite variable, individuals with Down syndrome have significantly lower IQs with impaired speech and language skills as well as specific difficulties in auditory and visual memory, in spatial memory and in the acquisition of conditioned and operant responses. Down syndrome is considered to be caused by trisomy of the human chromosome 21.

Several attempts have been made to find animal models for Down syndrome. Since the mouse chromosome 16 is homologous to the human chromosome 21, mice with trisomy of chromosome 16 were created. Cox et al. (1984) reported production of a viable trisomy 16 diploid mouse chimera as a model of human trisomy 21 (Down syndrome). This model has been extensively used (Epstein et al. 1985; Sweeney et al. 1989; Holtzman et al. 1992; Shetty et al. 1996). However, the value of this model is limited to some extent because trisomy 16 mouse fetuses do not survive as live-born animals.

The **Ts65Dn mouse** carries only a partial trisomy for mouse chromosome 16 in a region that has highly homology to the Down syndrome region of human chromosome 21. These mice survive until adulthood and have therefore been used as animal model for Down syndrome in more recent studies (Davisson et al. 1993; Coussons-Read and Crnic 1996; Klein et al. 1996; Kola and Hertzog 1997; Costa et al. 1999; Cooper et al. 2001; Galdzicki et al. 2001; Granholm et al. 2002; Bimonte-Nelson et al. 2003; Stasko and Costa 2004).

Ema et al. (1999) described an animal model of Down syndrome in mice overexpressing the *mSim2* gene located on chromosome 16. Borella et al. (2003) characterized social behaviors and oxytocinergic neurons in the S-100β overexpressing mouse model of Down syndrome originally described by Friend et al. (1992).

A number of experimental compounds have been developed that result in improved cognitive performance in animal models. Moran et al. (2002) described the effects of piracetam on cognitive performance in a mouse model of Down syndrome.

Procedure

Male 5-week-old Ts65Dn and euploid littermate control mice received daily injection of one of three dose of piracetam (75, 150, and 300 mg/kg) or saline vehicle. Piracetam treatment continued for 4 weeks prior to and throughout the 4-week testing period. Testing consisted of assessments of performance on the visible and hidden platform components of the Morris maze task and assessment of spontaneous activity within computerized activity chambers.

Evaluation

Significant effects were explored with two-way ANOVA, analysis of main effects, and paired *t* comparisons.

Modifications of the Method

Cardenas et al. (2002) established and characterized immortal neuronal cell lines derived from the spinal cord of normal and trisomy 16 fetal mice.

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Models of Wilson's Disease

Purpose and Rationale

Wilson disease is an inherited disorder of copper **metabolism** caused by autosomal recessive mutations of the *ATP7B* gene. The mutant ATP7B proteins lead to an impaired copper efflux, a decreased copper incorporation into ceruloplasmin and to hepatic copper accumulation, resulting in hepatocellular injury and deposition of copper in extra-hepatic tissues. Patients suffer from chronic or acute liver disease, psychiatric symptoms and neurological abnormalities with extrapyramidal symptoms, such as tremor and ataxia. Dpenicillamine has been used for treatment of patients with Wilson disease, but more recently ammonium tetrathiomolybdate has been recommended (Stremmel et al. 1991; Silva et al. 1996; Brewer et al. 2003).

The **Long-Evans Cinnamon rat** has a mutation homologous to the human Wilson disease gene, leading to gross copper accumulation and development of hepatitis. This rat strain has been extensively used as animal model for Wilson disease (Yamaguchi et al. 1994; Suzuki 1995; Adachi et al. 1997; Kodama et al. 1998; Nagano et al. 1998; Nomiyama et al. 1999; Terada and Sugiyama 1999; Klein et al. 1998, 2000, 2004; Komatsu et al. 2000, 2002; Meng et al. 2004).

Klein et al. (2004) reported the effects of tetrathiomolybdate in the treatment of acute hepatitis in Long-Evans Cinnamon rats.

Procedure

Female Long-Evans Cinnamon rats (Charles River, Japan) at an age of between 60 and 100 days were used, whereas female Long-Evans Agouti rats served as controls. Long-Evans Cinnamon (LEC) rats were randomly divided in different groups:

LEC rats without any treatment and without signs of liver disease
Jaundiced LEC rats without any treatment
Jaundiced LEC rats treated once intraperitoneally with 10 mg/kg tetrathiomolybdate

The tetrathiomolybdate-treated rats were sacrificed either 1 or 4 days after treatment.

Tissue Preparation and Subcellular Fractionation

Livers were flushed in situ with ice-cold 0.9 % NaCl in order to remove the blood. A part of the liver was fixed in 4 % neutral formalin, embedded in paraffin, and sections were stained for light microscopy. From homogenates lysosomal and mitochondrial fractions were prepared (Klein et al. 1998, 2000). Electron microscopy and X-ray microanalysis were performed. Metals and metallothionein were quantified.

Evaluation

Student's *t*-test was used for statistical evaluation.

Other Disorders of Copper Metabolism

Besides Wilson's syndrome, other inherited diseases of cellular copper metabolism are known, such as Menkes disease and the toxic milk syndrome in mice (Palida and Ettinger 1991; La Fontaine et al. 2001).

Menkes' syndrome is an X-linked recessive disorder associated with copper deficiency, lethal in early childhood. Menkes' children show impaired synthesis of collagen and elastin. Furthermore, they are severely mentally retarded. Diffuse atrophy, focal degeneration of gray matter and prominent neuronal loss have been detected in the cerebellum. The molecular basis of this syndrome has been identified as a mutated gene (*ATP7A*) encoding for a P-type cation-transporting ATP-ase (Kodama and Murata 1999; Tumer et al. 1999; Rossi et al. 2001).

The mutant **macular** mouse has been used as animal model for Menkes' disease (Tanaka et al. 1990; Murata et al. 1997, 1998; Suzuki-Kurasaki et al. 1997). The **brindled mouse mutant** (*Mobr*) is considered to be the closest

model to Menkes' disease (Grimes et al. 1997; Rossi et al. 2001). The mutant mice are hypopigmented and die at around 15 days after birth, but can be saved by treatment with copper before the 10th postnatal day.

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Models of Cerebellar Ataxia

The pathology mechanisms underlying the signs of hereditary cerebellar ataxia in humans are not well understood (Harding 1984).

Kato et al. (1982) and Yamaguchi et al. (1992) regarded the rolling mouse Nagoya as an animal model of hereditary cerebellar ataxia.

Niimi et al. (1982) made an animal model of cerebellar ataxia by injections of kainic acid into both sides of the cerebellar hemispheres of male rats.

Grüsser-Cornehls et al. (1999) recommended Weaver mutant mice as an animal model of cerebellar ataxia.

Fernandez et al. (1998) induced ataxia in adult rats either with intraperitoneal injection of 50–65 mg/kg 3-acetylpyridine or by bilateral electrocoagulation of the inferior olive. Insulin-like growth factor 1 restored motor coordination in this rat model of cerebellar ataxia.

Ataxia-telangiectasia is a complex multiparametric disease associated with oculocutaneous telangiectasias, cerebellar ataxia, elevated chromosomal aberration frequency, and varied degrees of immunodeficiency. Kaiserlian et al. (1986) described the wasted mouse as a model of this disease.

Clark et al. (2000) reported X-linked transmission of the *shaker* mutation in rats with hereditary Purkinje cell degeneration and ataxia.

Sausbier et al. (2004) described cerebellar ataxia and Purkinje cell dysfunction in mice caused by $C\alpha^{2+}$- activated K^{+} channel deficiency.

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Models of Niemann-Pick Syndrome

Purpose and Rationale

Niemann-Pick disease type C is a progressive neurodegenerative disorder caused by mutations in the *NPC1* gene and characterized by intracellular accumulation of cholesterol and sphingolipids.

Pentchev et al. (1980, 1984) described a genetic storage disorder in BALB/C mice with metabolic block in esterification of exogenous cholesterol. The murine model of Niemann-Pick C disease with mutation in a cholesterol homeostasis gene (**npcl^{-}/$^{-}$mutant mice**) has been used in several studies (Weintraub et al. 1985, 1987; Higashi et al. 1993; Loftus et al. 1997; Erickson et al. 2000; Bascunan-Castillo et al. 2004). Some attempts have been made to improve the clinical course of the Niemann-Pick C disease mouse (Liu et al. 2000). Treatment with cyclodextrins had variable effects in delaying neurological symptoms and in decreasing liver cholesterol storage (Camargo et al. 2001). Zhang et al. (2004) showed that cyclin-dependent kinase inhibitors attenuate protein hyperphosphorylation, cytoskeletal lesion formation, and motor defects in Niemann-Pick type C mice.

Modifications of the Method

Sym et al. (2000) described a model for Niemann-Pick type C disease in the nematode *Caenorhabditis elegans*.

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Models of Gangliosidosis

Purpose and Rationale

The glycosphingolipid lysosomal storage diseases are inherited metabolic diseases in which a gene encoding a lysosomal hydrolase or one of their co-factors is mutated. Two classes of glycosphingolipid storage diseases are known, those in which the storage glycosphingolipid is neutral (**Gaucher** and **Fabry** disease) and the gangliosidoses in which the storage lipid is a ganglioside (glycosphingolipid containing one or more sialic acid residues). The gangliosidoses include the G_{M2} storage disorders, **Tay-Sachs** and **Sandhoff disease** (O'Dowd et al. 1986; Paw et al. 1989; Kolter and Sandhoff 1998; Schröder et al. 1991; Glaros et al. 2005), and the G_{M1} storage disorder termed G_{M1} gangliosidosis or **Morquio** B disease (Matsuda et al. 2003).

Spontaneous gangliosidosis has been described in several animal species, such as **dogs** (Singer and Cork 1989; Kaye et al. 1992; Wang et al. 2000; Yamato et al. 2003, 2004b; Kreutzer et al. 2005), **cats** (Cork et al. 1977; Neuwelt et al. 1985; Muldoon et al. 1994; Kroll et al. 1995; Yamato et al. 2004a; Martin et al. 2005), **sheep** (Skelly et al. 1995; Ryder and Simmons 2001), **deer** (Fox et al. 1999), and **pigs** (Kosanke et al. 1978).

***β*-Galactosidase-deficient mice** were described as an animal model for GM_1-gangliosidoses (Hahn et al. 1997; Matsuda et al. 1997a, b; Oshima 1998; Callahan 1999; Itoh et al. 2001). Mouse models of G_{M2} gangliosidosis are reported by Huang et al. (1997) and Jeyakumar et al. (2003).

No effective treatment of patients is known as yet, but Jeyakumar et al. (1999) found delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with *N*-butyldeoxynojirimycin. Yamaguchi et al. (2003) reported that plasmid-based gene transfer ameliorates visceral storage in a mouse model of Sandhoff disease. Martino et al. (2002) described restoration of the G_{M2} ganglioside metabolism in bone marrow-derived stromal cells from a Tay-Sachs disease mouse model. Furthermore, Martino et al. (2005) reported that a direct gene strategy via brain internal capsule reverses the biochemical defect in Tay-Sachs disease.

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Models of Mucopolysaccharidosis

Purpose and Rationale

Mucopolysaccharidosis type IIIA or **Sanfilippo syndrome** is a lysosomal storage disorder characterized by progressive neurological pathology.

Patients exhibit aggression, disturbed sleep, hyperactivity, and mental decline ultimately resulting in inanition and death. Four mucopolysaccharidosis subtypes result from deficiencies in different lysosomal enzymes that sequentially degrade heparin sulfate: sulfamidase (MPS IIIA), α-*N*-acetylglucosaminidase (MPS IIIB), acetyl-CoA:a-glucosamidine *N*-acetyltransferase (MPS IIIC), and glucosamine-6-sulfatase (MPS IIID).

Mucopolysaccharidosis III has been described in several animal species, such as mice (Li et al. 1999, 2002; Yu et al. 2000; Gografe et al. 2003; Garbuzova-Davis et al. 2005), dogs (Fischer et al. 1998; Aronovich et al. 2000; Ellinwood et al. 2003), emus (Giger et al. 1997; Aronovich et al. 2001), and goats (Thompson et al. 1992).

A naturally occurring mouse model of MPS-IIIA has been discovered (Bhaumik et al. 1999; Bhattacharyya et al. 2001), with pathophysiology and symptoms that resemble the human conditions (Gliddon and Hopwood 2004; Hemsley and Hopwood 2005).

No effective drug treatment for patients with Sanfilippo disease is available; however, experiments in mice showed beneficial effects after transplantation of human umbilical cord blood cells (Garbuzova-Davis et al. 2005), or treatment with lentiviral NAGLU vector (Di Natale et al. 2005) or enzyme replacement (Yu et al. 2000; Gliddon and Hopwood 2004).

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General Anesthetics

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Contents

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Intravenous Anesthesia

General Considerations

Purpose and Rationale

The first agents which could be used as intravenous anesthetics were **barbiturates**. Barbiturates with a duration of action appropriate to the requirements of surgery became available with the introduction of hexobarbital and thiopental (Volwiler and Tabern 1930; Miller et al. 1936). The studies with barbiturates were extended (Butler and Bush 1942; Christensen and Lee 1973). Intravenous anesthetics from other chemical groups were developed, such as **acetamidoeugenol** (Estil, Domenjoz 1959), steroid derivatives (Presuren = **hydroxydione sodium**, Laubach et al. 1955; **alfaxolone**, CT1341, Child et al. 1971), **propanidid** (Goidenthai 1971), **ketamine** (CI-581, Chen et al. 1966; Reich and Silvay 1989), **etomidate** (Janssen et al. 1975), **propofol** (ICI 35868, Glen 1980), and **midazolam** (Pieri 1983; Reilly and Nimmo 1987).

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Screening of Intravenous Anesthetics

Purpose and Rationale

Screening of intravenous anesthetics or hypnotics is performed mostly in mice or rats. Doses for loss of righting reflex and lethal doses are determined. Onset of action and duration of action are the secondary parameters.

Procedure

Male mice weighing 18–22 g are injected intravenously via the tail vein. The anesthetic activity is estimated from the number of animals that lose their righting reflex. The righting reflex is considered lost when the mouse, placed on its back, fails to recover from this position within 1 min. The acute toxicity is based on lethality within a 24-h observation period.

To determine onset and duration of action, groups of 20 mice are used. They are placed in individual observation cages maintained at room temperature (24 ± 1 °C). They are not stimulated during the interval between loss and recovery of the righting reflex. The onset is defined as the complete loss of the righting reflex, i.e., no attempt to move the head or body. Recovery is considered to have occurred when the animal after spontaneous righting would reright itself within 15 s when placed on its back.

Evaluation

The median anesthetic dose (AD_{50}) and the median lethal dose (LD_{50}) are determined from dose–response curves with at least four doses by the method of Litchfield and Wilcoxon (1949).

The data for onset and duration of action are analyzed statistically by Student's *t*-test.

Modifications of the Method

Volwiler and Tabern (1930) determined the minimum effective dose in rats after subcutaneous injection of various barbiturates not being awakened when outer ear passage was tickled with a straw.

Büch et al. (1968) studied the distribution, anesthetic potency, and metabolic elimination of the optical isomers of methylphenobarbital in rats.

Glen (1977) described a method for the laboratory evaluation of the speed of onset of i.v. anesthesia in **mice**. Various clinically used intravenous anesthetics were compared. The technique involves (a) determination of the medium hypnotic dose (HD_{50}) by plotting the probit value of the mice sleeping against dose on a logarithmic scale, (b) plotting mean induction time over a range of doses against the logarithm of the dose, and (c) comparison of induction times at 1.25 HD_{50}. All doses were given over 1 s or 10 s. A 1-s injection was thought to be of most value in the of structure activity effects.

Chen et al. (1966) tested the anesthetic activity and the neuropharmacological spectrum of ketamine (CI-581) in mice, pigeons, and monkeys.

Child et al. (1971) tested the anesthetic activity of alfaxolone (CT1341) in **mice**, **rats**, **rabbits**, **cats**, **dogs**, and **monkeys**.

Janssen et al. (1975) tested onset and duration of anesthesia after etomidate in mice, rats, guinea pigs, and dogs.

New intravenous anesthetics were reviewed by Reilly and Nimmo (1987).

The anesthetic potency of remifentanil in dogs in terms of reduction of enflurane *MAC* was tested by Michelsen et al. (1996).

Dingwall et al. (1993) described the tolerometer as a fast, automated method for the measurement of righting reflex latency.

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EEG Threshold Test in Rats

Purpose and Rationale

The electroencephalographic (EEG) threshold test has been used to determine and compare the potency of several CNS depressant agents (Boiander et al. 1984; Koskela and Wahlstrom 1989; Norberg and Wahlstrom 1988; Norberg et al. 1987). Korkmaz and Wahlstrom (1997) described in detail the protocol of the EEG burst suppression threshold test for the determination of CNS sensitivity to intravenous anesthetics in rats.

Procedure

Adult Sprague–Dawley rats are housed at a reversed light/dark cycle and an ambient temperature of 23 ± 1 °C. Twenty-four hours prior to the EEG threshold test, the rats are placed in a tube restrainer. Twisted stainless steel wire and suitable surgical needles are used to sew the electrodes to the scalp above the frontal cortex. Since generalized changes in EEG recordings are used, this stainless steel material is adequate for recording purposes. Care is taken to prevent irritation of periosteal tissue. Since this procedure causes little discomfort, the use of local anesthetics and general anesthesia can be avoided.

For EEG threshold testing, the rat is placed on a warm cloth and held gently by the assistant. A needle is placed on one lateral tail vein and connected with an infusion pump. Crocodile clips are used to connect the electrodes to the EEG recorder, and a crocodile clip is attached to one of the ears of the rats as a signal ground.

The EEG recording is closely observed by the technician. The changes in the EEG induced by the anesthetic agent are used to measure drug effects on the CNS. The normal EEG in an awake rat has low amplitude and a frequency of approximately 30 cycles/s. During the first part of infusion, an increase in amplitude and a slight decrease in frequency are observed. At this stage of infusion, dependent on the anesthetic agent, jerks or sometimes convulsive episodes may occur. As the infusion continues, the frequency decreases, and burst suppression periods appear. The loss of righting reflex occurs at this stage. When burst suppression lasts 1 second, the threshold criterion which is called "silent second" is reached and the time is recorded. After the threshold determination, the rats are placed in the recovery room.

Evaluation

The threshold dose is calculated by multiplying the time required to reach the threshold criterion with the dose administration rate. Threshold doses are determined for each anesthetic at various dose administration rates indicating the optimal dose administration rate.

Modifications of the Method

Wauquier et al. (1988) studied relationships between quantitative EEG measures and pharmacodynamics of alfentanil in dogs. Before, during, and up to 3 h after infusion, the effects of three doses on six quantitative EEG measures (zero-crossing frequency, root mean square amplitude, spectral edge, relative delta, alpha, and beta power) were assessed.

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Efficacy and Safety of Intravenous Anesthetics

Purpose and Rationale

Besides determination of the ratio between anesthetic and lethal dose, intravenous anesthetics have to be tested for their influence on the cardiovascular and pulmonary system. Borkowski et al. (1990) described a method to compare intravenous anesthetics in rabbits.

Procedure

Adult New Zealand White rabbits with a mean weight of 4.5 kg are used. To provide access for direct blood pressure measurement and arterial blood samples, an 18-gauge catheter is implanted into the left carotid artery under halothane anesthesia. Following a minimum 24-h recovery period, the rabbit is placed in a sling and a pneumograph fitted around the rabbit's caudal thorax at the level of 10th to 12th ribs to monitor respiratory rate and pattern. From the arterial catheter blood is withdrawn for blood gas analysis. Then the catheter is connected to a blood pressure transducer. A 10-min acclimatization period is allowed before control measurements are recorded. Each rabbit serves as its own control in that cardiopulmonary parameters and responses to noxious stimuli are determined before anesthesia is induced. The right marginal ear vein is catheterized with a 22-gauge catheter, which is secured with adhesive tape, flushed with physiological sterile saline, and used for the administration of the anesthetic agents.

One-third of the dose of the anesthetic to be tested is injected manually over a 1-min period. When the rabbit is relaxed it is removed from the sling and placed in left lateral recumbence on a heating blanket. The degree of muscle tension and reaction to noxious stimuli are determined while the rabbit is in the sling and at 15 min intervals following anesthesia. The assessments performed include those of jaw tone, leg muscle tone, palpebral reflex, corneal reflex, ear pinch reflex, and pedal withdrawal reflex. Jaw tone is evaluated subjectively by pulling the lower jaw open by an index finger. Leg muscle tone is evaluated by flexion and extension of the right rear leg according to subjective scores. The corneal reflex is tested by placing a moistened cotton swab on the cornea. The palpebral reflex is tested by touching the medial canthus with a dry cotton swab. Assessment of the ear pinch reflex is performed by applying a compression force with an alligator clip. The pedal withdrawal reflex is determined by applying the same clip on the right rear fifth digit at the distal phalanx.

Cardiopulmonary parameters and rectal body temperature are determined while the rabbit is in the sling and also at 15 min intervals following induction of anesthesia with the rabbit in lateral recumbency. Heart rate, mean arterial blood pressure, respiratory rate, and respiratory pattern are calculated from tracings from the physiological recorder. Arterial blood pH, partial pressure of oxygen (PaO_2), and partial pressure of carbon dioxide ($PaCO_2$) are determined from arterial blood samples.

Evaluation

The heart rate, mean arterial blood pressure, respiratory rate, pH, PaO_2, and $PaCO_2$ are analyzed using a two-factor analysis on repeated measures. The control values are treated as covariate to allow standardization of the inherent variation between rabbits. The single *t*-test for paired differences is used to compare control values to data obtained during the later testing intervals. The standard error of the mean (SEM) is calculated for each variable at each time interval. Data for muscle tone and responses to noxious stimuli are calculated as frequency percentages. The Fisher's exact test is used to compare between treatments. For all of the statistical analyses, a p-value of less than 0.05 is considered significant.

Modifications of the Method

Details of anesthesia in the rabbit were also described by Murdock (1969).

Peeters et al. (1988) performed a comparative study of four methods for general anesthesia in rabbits.

Glenn (1980) examined the anesthetic activity of propofol (ICI 35868) in mice, rats, rabbits, cats, pigs, and monkeys including cardiovascular and respiratory parameters and EEG studies.

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Inhalation Anesthesia

General Considerations

Purpose and Rationale

The efficacy and safety of new inhalation anesthetics has to be evaluated in pharmacological experiments. Robbins (1946) defined the anesthetic AD_{50} as the concentration of anesthetic at which 50 % of mice failed to right themselves for 15 s when placed in a rotating bottle with a known concentration of anesthetic. The concentration of the anesthetic that caused apnea in 50 % of the mice in 10 min was defined as the LD_{50} and the ratio LD_{50}/AD_{50} as index of safety.

Wolfson et al. (1972) recommended brain anesthetic concentration for construction of anesthetic indices.

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Screening of Volatile Anesthetics

Purpose and Rationale

A simple technique for preliminary testing of anesthetic agents is the introduction of a measured amount of volatile liquid into a covered glass container of known volume. If the density and molecular weight of the liquid are known, the volume % concentration of the anesthetic mixture can be calculated (assuming 1 mol of vapor = 22.4 l). Mice or rats are introduced into the chamber, and the quality of anesthesia is noted. Conditions are then adjusted until the anesthetic concentration has been established.

Procedure

Male NMRI mice weighing 20–25 g or male Wistar rats weighing 250–300 g are used. A wide-mouth, screw-cap glass jar of 3 l volume is flushed with oxygen for 1 min and a measured amount of the volatile substance placed on the bottom through a suitable syringe. The amount is calculated to give 1.25 vol.% concentration of vapor in the jar (or a logarithmic multiple of 1.25 %, i.e., 0.63, 2.5, 5.0, 10.0). The jar is closed and evaporation of the substance facilitated by gentle rotation of the jar. One rat or five mice are quickly placed from a beaker into the jar, and the jar is immediately closed. Every 15 s the jar is gently rotated and the time noted for each animal to become anesthetized (loss of righting reflexes). The procedure is repeated until all animals are anesthetized. Induction should occur not sooner than 30 s and not later than 5 min. The animals are allowed to remain in the anesthesia jar for 10 min, with testing of righting reflexes until they are quickly removed into room air. The time of recovery to righting or walking is recorded for each animal. Postanesthetic analgesia is tested by

gently pressing the base of the tail every min until recovery has occurred. Recovery time is defined as the time in min until the mouse spontaneously moves in upright position. If induction time is shorter than 30 s or longer than 10 min, the concentration of anesthetic is decreased or increased until the proper concentration is found.

Evaluation

The results are reported as mean induction time and mean recovery time. Twenty-four-hour survival rate is recorded for latent toxicity.

Modifications of the Method

Burns et al. (1961) used a simplified mouse test apparatus with a small container and an open-circuit technique.

Raventós (1956) used cats, dogs, and monkeys to evaluate the cardiovascular effects of fluothane.

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Determination of Minimal Alveolar Anesthetic Concentration (MAC)

Purpose and Rationale

The term "**minimum alveolar anesthetic concentration**" (**MAC**) was coined by Merkel and Eger (1963) as an index to compare various anesthetic agents.

The use of *MAC* which represents the partial anesthetic pressure in the brain has gained wide acceptance (Eger et al. 1965; Quasha et al. 1980).

For **man**, Saidman and Eger (1964) defined *MAC* as the point at which 50 % of the patients moved in response to a surgical incision.

A method for determining minimum alveolar concentration of anesthetic in the **rat** was published by Waizer et al. (1973). Kashimoto et al. (1997) determined the minimum alveolar concentration of sevoflurane in rats. Eger et al. (1999) studied maximum alveolar anesthetic concentration of fluorinated alkanols in rats and discussed the relevance to theories of narcosis. Eger et al. (2003) studied additive minimum alveolar concentration (MAC) effects of halothane and isofluroane in rats.

Issues in the design and interpretation of minimum alveolar anesthetic concentration (MAC) studies were discussed by Sonner (2002).

Procedure

Minimum alveolar anesthetic concentrations (MAC) are determined in Sprague Dawley rats weighing 300–450 g. Each rat is placed in an individual gas-tight plastic cylinder closed at both ends by rubber stoppers. The stoppers are pierced with holes for various purposes. A rectal temperature probe (temperature maintained between 36 °C and 38.5 °C) and the rat's tail are drawn separately through holes in the rubber stopper closing the distal end of the cylinder. Delivered gases at an average inflow rate of 1 L/min to each rat enter through ports at the head (proximal) end of the cylinder and exit at the tail (distal end), a flow to minimize rebreathing (inspired $CO_2 < 10$ mmHg). Exiting gases are scavenged.

The anesthetics are introduced from conventional vaporizers. For the determination of MAC, an initial concentration is used that permits movement of the rats in response to noxious stimulation. A tail clamp is applied for one minor until the animal moves, and the anesthetic partial pressure is measured by gas chromatography. If the animal moves, the partial pressure is increased by 0.2 % or 0.3 % atmospheres. After equilibration for

30 min, the tail clamp is applied again and the anesthetic partial pressure measured by gas chromatography. This procedure is repeated until the partial pressures bracketing movement-nonmovement are determined for each rat.

Evaluation

MAC is defined as the average of the partial pressures that just prevented movement in response to clamping of the tail. Differences between anesthetics are accepted at $P < 0.05$.

Modifications of the Method

Fang et al. (1997) found that maturation decreases ethanol minimum alveolar anesthetic concentration (*MAC*) more than desflurane *MAC* in **rats**.

Gong et al. (1998) assessed the effect of rat strain on susceptibility to anesthesia and convulsions produced by inhaled compounds.in five different rat strains. Strain minimally influenced anesthetic and convulsant requirements of inhaled compounds in rats.

Doquier et al. (2003) studied the minimum alveolar anesthetic concentration of volatile anesthetics in rats as tools to assess antinociception in animals.

Determination of the minimal alveolar concentration (*MAC*) of halothane in the New Zealand white **rabbit** was published by Davis et al. (1975).

Determination of an anesthetic index (Apnea/MAC) in experiments in **dogs** has been proposed by Regan and Eger (1967).

Murphy and Hug (1982)and Hall et al. (1987) used the reduction of enflurane *MAC* values in **dogs** as parameter for the anesthetic potency of fentanyl or sufentanyl, respectively.

Seifen et al. (1987) used *MAC* values for comparison of cardiac effects of enflurane, isoflurane, and halothane in the **dog heart-lung preparation**.

Ide et al. (1998) used airway occlusion in **cats** as a noxious respiratory stimulus that induces a visceral sensation of choking for determination of minimum alveolar anesthetic concentrations during halothane, isoflurane, and sevoflurane anesthesia. These values were compared with *MAC* values for somatic noxious stimuli such as toe pinch or tetanic stimulus. The authors recommended this method as a new concept for *MAC* determination.

Eger et al. (1988) determined minimum alveolar concentration of fluorinated anesthetics in **pigs**.

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Efficacy and Safety of Inhalation Anesthetics

Purpose and Rationale

To assess the safety margin of an inhalation anesthetic not only the ED_{50} values but also the maximally effective dose and the dose with a minimal danger of fatal outcome should be determined. In particular, *cardiovascular parameters* are observed (Kissin et al. 1983).

Procedure

Male Sprague Dawley rats weighing 300–350 g are placed into a clear chamber with the tail protruding from a special opening. An anesthetic-oxygen nonhumidified mixture is directed into the chamber at a rate of 4 l/min. The inhalation anesthetics, e.g., halothane or isoflurane, are vaporized in Draeger vaporizers, and the level in the chamber is monitored with a gas analyzer which is calibrated with a mass spectrometer. Rectal temperature is monitored and maintained at 37 °C with a heating pad. Each rat is exposed to only one predetermined concentration of anesthetic for 30 min, at which time the presence or absence of the end point of anesthesia is determined. For the lethal end point, rats are tracheotomized and ventilated at 60 strokes/min through an endotracheal catheter. Tidal volume is adjusted to maintain $PaCO_2$ at 40 ± 5 mmHg.

As end points of anesthesia are used

- Loss of righting reflex. The test is regarded as positive if the animal fails to right itself with all four feet on the floor within 15 s after being placed in a side position.
- Prevention of purposeful movement response to a noxious stimulus. The animals are stimulated for 60 s by placement of a 1-kg weight on the middle of the tail. Only the purposeful movement of the head or legs is considered to be a response.
- Prevention of the heart rate increase to a noxious stimulus (ECG signals). An increase in heart rate of greater than 1 % is regarded as a positive response.
- The end point for the lethal effect is 7 mmHg in the femoral artery with artificial respiration.

With each of the anesthetics, four series of experiments are performed: determining the righting reflex, purposeful movement response, heart rate response, and lethal effect. The concentrations of the test compounds and the standard are spaced equally between the abovementioned doses.

After determination of the heart rate effect and the lethal effect, the rats are sacrificed for

determinations of brain tissue concentrations. The whole brain is removed and tissue anesthetic concentration determined by gas chromatography.

Evaluation

For calculation of the dose-effect curves, the probit method of statistical analysis is used.

For the assessment of anesthetic safety, not only the therapeutic ratio (LD_{50}/ED_{50}) but also the standard safety margin

$$SSM = (LD_5 - ED_{95})/ED_{95} \times 100$$

is used. This represents the percentage by which the ED_{95} has to be increased before LD_5 is reached.

Critical Assessment of the Method

The standard safety margin has definitive advantages over therapeutic ratio. In contrast to the LD_{50}/ED_{50} index, the standard safety margin is influenced not only by the distance between central points of the anesthetic and lethal dose-effect curves but also by the slope of these curves.

Modifications of the Method

A similar concept based on response to tail clamping, respiratory arrest, and cardiovascular failure in the **rat** was published as anesthetic index by Wolfson et al. (1973).

Another attempt to determine anesthetic requirements in rats was published by White et al. (1974).

Kissin et al. (1984) studied the morphine-halothane interaction in rats.

Fukuda et al. (1996) investigated the effects of sevoflurane and isoflurane on bupivacaine-induced arrhythmias and seizures in rats.

Kanaya et al. (1998) compared myocardial depression by sevoflurane, isofluorane, or halothane in **cultured neonatal rat ventricular myocytes**. Changes in beating rate and amplitude during exposure to the anesthetics were measured.

Chaves et al. (2003) used noninvasive electrocardiography in **mice** to study the effects of intravenous and inhalation anesthetics and of age.

Krantz et al. (1941, 1953) described an anesthetic index between surgical anesthesia (cornea and wink reflexes abolished) and respiratory failure in **dogs**.

Van Poznak and Artusio (1960a, b) determined the anesthetic properties of fluorinated compounds in dogs using a face mask for the induction of anesthesia and a cuffed endotracheal tube later on. ECG (lead II) and EEG were monitored.

Steffey and Howland (1978) determined the potency of enflurane in dogs in comparison with halothane and isoflurane.

Johnson et al. (1998) compared isoflurane with sevoflurane for anesthesia induction and recovery in adult dogs.

Salmempera et al. (1992) studied in dogs the potency of remifentanil, a short-acting opioid analgesic, which is used as anesthetic adjunct by variable-rate infusion. Enfluorane minimal alveolar concentration was measured by the tail-clamp method in dogs before and after sequential infusion of various doses of remifentanil. The plasma concentration causing a 50 % reduction of enfluorane minimal alveolar concentration was determined.

Kataoka et al. (1994) studied the negative inotropic effects of sevoflurane, isoflurane, enflurane, and halothane in canine blood-perfused papillary muscles.

Hirano et al. (1995) compared the coronary hemodynamics during isoflurane and sevoflurane anesthesia in dogs.

Mutoh et al. (1997) compared the cardiopulmonary effects of sevoflurane with those of halothane, enflurane, and isoflurane, in dogs.

Hashimoto et al. (1994) examined the effects of sevoflurane and halothane on the effective refractory period and ventricular activation in a canine myocardial infarction model.

The effects of desflurane, sevoflurane, and halothane on postinfarction spontaneous dysrhythmias in dogs were examined by Novalija et al. (1998).

Cardiopulmonary effects in **cats** were studied for desflurane by McMurphy and Hodgson (1996) and for sevoflurane by Hisaka et al. (1997).

Saeki et al. (1996) determined the effects of sevoflurane, enflurane, and isoflurane on baroreceptor-sympathetic reflex in **rabbits**.

Hanagata et al. (1995) found that isoflurane and sevoflurane produce a dose-dependent reduction in the shivering threshold in rabbits.

Antognini and Eisele (1993) determined anesthetic potency and cardiopulmonary effects of enfluorane, halothane, and isofluorane in **goats**.

The effects of multiple administrations of sevoflurane to cynomolgus **monkeys** were evaluated by Soma et al. (1995).

The effect of inhalation anesthetics on the **respiratory system** was investigated in several studies:

Mazzeo et al. (1996) compared the relaxing effects of desflurane and halothane at various MACs on isolated proximal and distal airways of dogs precontracted with acetylcholine.

Hashimoto et al. (1996) compared the bronchodilating effect of sevoflurane, enflurane, and halothane in dogs using a superfine fiberoptic bronchoscope. The dogs were anesthetized with pentobarbital, paralyzed with pancuronium, and the lungs were mechanically ventilated. The endotracheal tube had an additional lumen to insert the superfine fiberoptic bronchoscope (outer diameter 2.2 mm) which was located between a second and third bronchial bifurcation to continuously monitor the bronchial cross-sectional area of third- or fourth-generation bronchi. Bronchoconstriction was produced by histamine injection and infusion. The bronchial cross-sectional area was printed out by a video printer at the end of expiration and was calculated on a computer using an image program after various *MAC*s of the different inhalation anesthetics.

Mitsuhata et al. (1994) induced systemic anaphylaxis in dogs sensitized to Ascaris suum by intravenous injection of the antigen and measured pulmonary resistance and dynamic pulmonary compliance. Sevoflurane was as effective as isoflurane in attenuating bronchoconstriction associated with anaphylaxis in dogs.

Cervin and Lindberg (1998) examined the short-term effects of halothane, isoflurane, and desflurane on mucociliary activity in the rabbit maxillary sinus in vivo.

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NMRI Methods in Psychoneuropharmacology

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Contents

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General Considerations

Purpose and Rationale

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin–lattice relaxation time (T_1) and the spin–spin relaxation time (T2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T2 images are sensitive to water, and because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T1 values, whereas relatively loose structures, such as gray matter or lesions, have higher T1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected (0.1–0.3 mmol/kg) and, in areas of increased blood–brain barrier

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permeability, leaks into the brain parenchyma, causing local enhancement of the T_1-weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

Besides general applications to body composition in animals (Mitchell et al. 2001; Changani et al. 2003; Mirsattari et al. 2005) NMR studies contributed to evaluation of drugs in psychoneuropharmacology.

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NMRI Psychopharmacological Studies in Rats

NMRI Study of Experimental Allergic Encephalomyelitis in Rats

Purpose and Rationale

Duckers et al. (1997) studied the effect of a neurotropic treatment on cortical lesion development in experimental allergic encephalomyelitis in rats by longitudinal in vivo magnetic resonance imaging methods.

Procedure

Animals

Female Lewis rats weighing approximately 190 g were used for in vivo proton MRI. The rats were housed individually in Macrolon cages and had free access to rat chow and water during the 5-month study. The rats were divided at random into three groups. In two groups of rats chronic experimental allergic encephalomyelitis was induced by s.c. injection of myelin/adjuvant. The third group was injected with vehicle.

Immunization of Animals

Two hours before inoculation, spinal cords from Duncan Hartley guinea pigs were dissected and homogenized in an equal volume of phosphate-buffered saline (PBS) (pH 7.3; 1 g spinal cord in 1 ml PBS). Equal parts of guinea pig spinal cord homogenate in PBS and complete Freund's adjuvant (CFA) were mixed as described by Lassmann (1983) and Wisniewski and Keith (1977).

Anesthetized rats were sensitized by injecting 0.3 ml of the CNS emulsion in the dorsum pedis of the hind limbs. Age-matched controls were challenged similarly except that the emulsion solely contained PBS in CFA. The day of inoculation was designated day 0 post inoculation (dpi 0).

Peptide Treatment

For the neurotrophic treatment, an ACTH4–9 analogue devoid of corticotrophic activity [Org2766, HMet(O2)-Glu-His-Phe-D-Lys-Phe-OH] was used. The heptapeptide has well-documented neurotrophic properties, as demonstrated previously in different animal models for peripheral and central nerve damage (Bär et al. 1990; Strand et al. 1991). The rats with chronic experimental allergic encephalomyelitis were given s.c. injections, in the neck, of either the ACTH4–9 analogue (75 μg/kg in 0.5 ml saline) or 0.5 ml saline every 48 h. Age-matched control animals received saline injections every 48 h. Treatment was started on the day of inoculation and continued until the experiment ended 21 weeks after inoculation.

Neurological Status of the Rats

The clinical status of the rats was evaluated every 48 h by weighing the rats and scoring their neurological status on a 0–9 ordinal scale. The scores represent the following neurological symptoms: grade 0, no visible neurological symptoms; grade 1, loss of tail tip reflex; grade 2, flaccid tail; grade 3, moderate paraparesis, manifested as minor locomotion disturbances; grade 4, severe paraparesis accompanied by lordosis, severe disturbed locomotion, and severe weight loss; grade 5, one paralyzed hind limb; grade 6, complete paralysis; grade 7, paralysis from diaphragm upward; grade 8, tetraplegia, only head movements possible; grade 9, death due to chronic experimental allergic encephalomyelitis.

T2-Weighted and Short Tau Inversion Recovery Magnetic Resonance Imaging

Animals were examined by using MRI at 5, 10, and 20 weeks post inoculation. The rats were anesthetized with Hypnorm (containing 10 mg/ml fluanisone and 0.315 mg/ml fentanyl citrate, dose 0.6 ml/kg s.c.) and Stesolid (diazepam). After intubation, the rats were mechanically ventilated with 70 % oxygen and 30 % N2O with 0.5 % v/v halothane.

The ^{1}H-MRI experiments were performed with a SISCO 200/400 system (SISCO, Palo Alto, Calif., USA) operating at 4.7 T and equipped with actively shielded gradients (max. gradient 18 Mt/m). In all experiments, an Alderman-Grant-type radio frequency coil with an inner diameter of 5 cm was used. For accurate and reproducible positioning, animals were mounted in a stereotaxic holder positioned on the coil support. For each animal, a sagittal T1W scout image [echo time (TE) 20 ms, repetition time (TR) 600 ms] outlined the start (border of frontal lobe and olfactory bulb) and end point (border of cerebellum-spinal cord) for subsequent coronal multislice experiments used to quantify the extent of tissue damage.

All MRI experiments were based on a spin-echo sequence with 5-ms sinc-modulated radiofrequency pulses. A total of 11 T2W coronal multislice images per animal was obtained (slice thickness 1.1 mm, interslice gap 0.7 mm), using a TR of 2 s and a TE of 60 ms; two transients were averaged. The field of view was 5×5 cm^2; 128 points were acquired in the phase encoding direction and 512 points in the read-out direction. After processing, these were presented in a square matrix of 512×512 pixels.

Subsequently, multislice coronal short tau inversion recovery (STIR) images were recorded as described above (TR/TE 2000/60, number of transients 2), except that the spin echo sequence was preceded by a 180° inversion pulse followed by an inversion time of 250 ms. In pilot experiments, this inversion time was found to yield optimal contrast between compromised and healthy brain tissue.

In order to measure the coronal relaxation time T2 on a pixel-by-pixel basis, six rats (two age-matched control rats and four rats with chronic experimental allergic encephalomyelitis) were used in a series of T2W imaging experiments with a TE of 20, 40, 60, and 80 ms and a TR of 6 s. In this experiment, which was performed at week 21 post inoculation, all other parameters were identical to those of the abovementioned T2W imaging experiment. The series of T2W images at a given slice position were used to reconstruct a two-dimensional map of T2 by fitting the data on a pixel-by-pixel basis to monoexponential decay functions. T2 values of cortex tissue, thalamus, and ventricles were read from the T2 map and averaged among animals. T2 values of lesioned tissue were calculated for lesions which extended throughout the thickness of the MRI slice.

All MRI data were analyzed using MRI-specific software (Image Browser, SISCO, Sunnyvale, Calif., USA) by two different investigators who were blind to the treatment given to the animals. A filter was positioned over the region of interest (cortex and subcortical regions) in MRI slices. The filters were preset at fixed values, which appeared to yield an optimal differentiation between areas of relatively low and high

signal intensity in pilot experiments. Pixels with relatively high signal intensities (e.g., higher than the filter setting) were added to obtain the total surface area of the brain regions with high signal intensities in that given slice. These surface areas with high signal intensity were summed for all slices for each animal and converted to total lesion volume (in μl). In the analysis, interslice gaps were not included in the calculation of the total lesion volume.

Evaluation

Application of the drugs, evaluation of the clinical status, and the MRI were all performed by different investigators who were blind to the treatment given to the animals. Treatment codes were broken at the end of the experiment after data analysis had been completed. The distribution of clinical scores in the different treatment groups was analyzed using a nonparametric Wilcoxon rank-sum test. Treatment effects on lesion volume measured by MRI were tested by an analysis of variance for repeated measurements (MANOVA) followed by additional Student's t-tests (two-paired). $P < 0.05$ was considered to be significantly different.

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NMRI Study of 3-Nitropropionic Acid-Induced Neurodegeneration in Rats

Purpose and Rationale

Effective clinical therapies for neurodegenerative diseases are currently lacking because the precise mechanisms underlying cell death are still obscure. It is hypothesized that a defect in mitochondrial energy metabolism may be the basis of secondary excitotoxic neuronal death in neurodegenerative diseases. 3-Nitropropionic acid is an irreversible succinate dehydrogenase inhibitor which blocks both the Krebs' cycle and mitochondrial electron transport. 3-Nitropropionic-acid-intoxicated rats serve as the animal model for one neurodegenerative disease, Huntington's disease. Chyi and Chang (1999) applied noninvasive diffusion- and T_2-weighted magnetic resonance imaging to study the temporal evolution and spatial distribution of brain lesions which were produced by intravenous injection of 3-nitropropionic acid in rats.

Procedure

Animal Preparation

Male Sprague Dawley rats were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg). Anesthesia was maintained by serial supplement of pentobarbital (5 mg/50 min, i.v.) 2 h after the initial injection. One femoral vein was cannulated for chemical administration, and an endotracheal tube was set for artificial ventilation with an animal ventilator. A muscle relaxant, gallamine, was injected intravenously to avoid spontaneous ventilation and movement during the image acquisition period. The initial dose of gallamine was 12 mg and the maintaining dose 6 mg/h. A bolus injection of 3-nitropropionic acid was intravenously administered.

Image Acquisition

Experiments were performed on a 4.7 T spectrometer (Spectrospin, Biospec 4.7 T, Fäilanden, Switzerland) with an active shielding gradient at 5.6 G/cm in 500 μs. A 70-mm birdcage RF coil was used, and rats were placed in a prone position with a custom-designed head-holder. Four transverse images (2-mm thick) along anterior-posterior direction were obtained using a 5-cm field of view and 128 × 256 matrix. The offsets were set corresponding to the sections −4.3, −1.8, +0.7, and +3.2 mm to the bregma. A fast spin echo sequence was applied for T_2-weighted images (T_2WIs) with relaxation TIME = 4000 ms, effective echo TIME = 80 ms, and echo train LENGTH = 8. For diffusion-weighted images (DWIs), the pulsed field gradient method was employed with a relaxation time of 2000 ms, echo time of 59 ms, *S* of 20 ms, A of 27 ms, and b-value of 1300 s/mm^2. The diffusion-sensitive gradients were applied in the read (x) direction before and after the 180° pulse. The controlled T_2WIs and DWIs were acquired prior to 3-nitropropionic administration. Then 2.5 min after the injection of 3-nitropropionic, sequential T_2WIs and DWIs were continuously performed for 8 h.

In a separate preparation, DSC MRI was performed immediately after diffusion-weighted MRI at 5 min before and 4 h after 3-nitropropionic acid administration. A 2-mm-thick transverse single slice was chosen and centered at 0.5 mm anterior to the bregma where the striatal lesion sites were easily observed on the DWIs. A series of 30 gradient-echo images with a 64 × 256 matrix size, relaxation TIME = 30 ms, echo TIME = 10 ms, and $a = 15°$ were acquired. The acquiring time for each gradient-echo image was 1.92 s. The bolus of susceptibility contrast agent, gadopentic acid (0.3 mmol/kg, Schering, Berlin, Germany), was injected intravenously 10 s after the start of image acquisition.

Evaluation

Data processing was performed using a commercially available image analysis software (MRVision, MRVision, Menlo Park, Calif., USA). According to Paxinos and Watson (1986), selected regions of interest (ROIs) in the striatum, hippocampus, cortex, and corpus callosum were defined. A signal-to-noise ratio (SNR) of a ROI was defined as the ratio of the mean signal intensity (SI) in this ROI to the mean SI in an adjacent background area. The relative contrast of a ROI was calculated according to the following formula: $|SI_A SI_C| / SI_C$, where SI_A is the mean SI in this ROI and SIC is the mean SI in the cortical ROI on the same image (Knowles and Markisz 1988). The SIC was selected as reference because its SNR remained constant throughout the experimental period. The rCBV maps were generated by the integral under the AR_2* transit curves on a pixel-by-pixel basis. The SNR, relative contrast, and regional rCBV determined from the rCBV map were all unitless ratios.

Results were presented as means ± SEM. Statistical analyses were performed using paired Student's t-test to compare controlled and 3-nitropropionic-acid-treated data. $P < 0.01$ was considered statistically significant.

Modifications of the Method

Using magnetic resonance imaging and in vivo proton magnetic resonance spectroscopy, Lee et al. (2000) evaluated the neuroprotective effect of lamotrigine and MK-801 on rat brain lesions induced by 3-nitropropionic acid.

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NMRI Studies of Brain Activation in Rats

Purpose and Rationale

Brain functional magnetic resonance imaging (fMRI) is capable of revealing in vivo biochemical, structural, and functional information that reflects the physiological integrity of the brain (Jenkins et al. 1996). In general, brain fMRI includes four different aspects of MR methods including tissue water movement (diffusion-weighted MRI) (Le Bihan et al. 1986), capillary flow (perfusion-weighted MRI) (Williams et al. 1992; Kim 1995), neuronal activation (blood oxygenation level-dependent (BOLD) MRI) (Ogawa et al. 1992), and cerebral metabolites (magnetic resonance spectroscopy) (Brown et al. 1982). In all of these methods, the BOLD effect, based on the difference in magnetic susceptibility between paramagnetic blood deoxyhemoglobin and surrounding tissues, is frequently employed to in vivo map functional changes during brain activation (McCarthy et al. 1993; Turner et al. 1993).

Chang and Shyu (2001) described a functional magnetic resonance imaging (fMRI) study of brain activations during non-noxious and noxious electrical stimulation of the sciatic nerve of rats.

Procedure

Male Sprague Dawley rats were initially treated with atropine (6 mg/kg, i.p.), then anesthetized with methohexital sodium, 65 mg/kg, i.p. A PE-240 tube was inserted via tracheotomy for artificial ventilation. A PE-50 catheter was placed in the femoral vein for the administration of drug. α-Chloralose (50 mg/kg) was given intravenously. After the sciatic nerve was exposed and isolated, a cuff electrode made from silicon tube and stainless steel wires was placed around the nerve. The surrounding muscle and skin were carefully sutured, and paraffin oil was filled around the electrode to prevent short-circuiting. Artificial ventilation was applied and CO2 concentration monitored and controlled within the range of 3.5–4.5 %. Rectal temperature was measured and maintained at about 37 °C by a feedback-controlled warm-air system. After the head-holder was in place, muscle relaxant (gallamine triethiodide, 50 mg/kg, i.v.) was given for muscle relaxation.

Electrophysiological Experiment

Laminectomy was performed between T12 and L5. Dorsal roots at L4 and L5 level were carefully isolated and cut. Each of the peripheral ends of the roots was ligated with a cotton thread previously soaked with normal saline. A silver–silver chloride electrode hooked on the end of the root was used to record the evoked field potentials extracellularly ipsilateral to the stimulating sites. The extracellular field potentials were amplified by a high-input impedance amplifier. All analog signals were displayed on either an analog or digital storage oscilloscope. Twenty sweeps of evoked compound potentials were averaged for each stimulus intensity. The threshold current for inducing minimal muscle twitch was tested before applying the muscle relaxant. The electrical current was delivered by a constant current pulse generator. The electrical pulse was a square wave, 0.5 ms duration, 2 Hz. This muscle twitch threshold current was used to compare with the threshold for inducing the minimal compound action potentials. The intensity was increased as a multiple of the threshold current value.

Imaging Acquisition in fMRI Experiments

MR experiments were performed on a Biospec BMT 47/40 4.7 T system equipped with an actively shielded gradient system (up to 5.9 G/cm in 500 μs). A 70-mm birdcage RF coil was used, and rats were placed in a prone position with a custom-designed head-holder. T1-weighted spin-echo images were scanned in transverse and sagittal planes to position the animal properly so that images were centered 1 mm posterior to the bregma. For identifying anatomical location, T2-weighted spin-echo images with four slices in the transverse plane were acquired using a repetition time (TR) of 4 s, an echo time (TE) of 80 ms, a field of view (FOV) of 5 cm, and a slice thickness (SLTH) of 2 mm. The acquisition matrix

was 256 × 128 with a matrix of 256 × 256 after zero-filling. Thus, the images covered the rat brain from 3 mm anterior to the bregma to 5 mm posterior to the bregma. At the same locations of T_2-weighted spin-echo images, a series of 40 gradient-echo images per slice for four slices in the transverse plane were collected for baseline and stimulation sets with a TR of 180 ms, a TE of 20 ms, a flip angle of 22.5°, a FOV of 5 cm, and a SLTH of 2 mm. The acquisition matrix was 256 × 64 with a matrix of 256 × 256 after zero-filling. The total scanning time for each image was 23 s. In the baseline set, the stimulation was turned off during the first eight image acquisitions. The electrical stimulation was switched on in two stimulation sets during acquisition of images 9–16 and 25–32. The stimulator was switched off for the rest of the period. The stimulation consisted of rectangular pulses of 0.5 ms duration and a 2 Hz frequency current. The same muscle twitch measurement as described in the electrophysiological study session was conducted to determine the intensity of the threshold current. In each image acquisition session, only one stimulation intensity was used. Stimuli with varied threshold-multiplied intensities were applied randomly in three different sessions in each rat. At least 30 min waiting time was allowed between each acquisition session.

Physiological Data Acquisition System in fMRI Experiments

Inside the magnet, animals were placed in an acrylic head-holder for accurate positioning of the location of brain structures. The integrated physiological data acquisition system we used was processed and controlled by a program written in Microsoft BASIC language on an IBM personal computer (Shyu et al. 1996). The data acquisition and control signals were channeled through serial ports and a PCL-818 (Advantech, A/D converter and digital input/output) interface card. All digitized physiological data, timing events, and trigger pulses from each image acquisition were displayed in real time to view the animal's condition. The same data were placed in a data array and stored on a hard disk for offine analyses, plotting, and printing.

Evaluation

Image data processing and analyses of significance of signal changes were performed using MRVision (MRVision, USA) running on a Silicon Graphics INDY workstation. The series of multisliced images were realigned in the vertical and horizontal direction related to the first image in the series. The analyses of the aligned data were based on the cross-correlation processing strategy (Bandettini et al. 1993). The correlation coefficient between each pixel of the series of images and a model of a square wave (whose OFF-ON-OFF-ON-OFF period was the same as the stimulation pattern) was calculated. An associated *t*-statistic image was generated by the fitting function. The *t*-statistic image was used to determine whether the correlation coefficient for each pixel was significantly different from zero. The same analysis procedure was carried out in each of the four slices. A critical t-value for each pixel for the desired level of significance (e.g., $P < 0.01$) and degrees of freedom (total images-2) was calculated and an output image, an activated map consisting of clusters of pixels whose values exceeded the level of significance, generated. This image, white color in appearance, was then overlaid with a high-resolution T2-weighted image, resulting in an image showing the significantly activated region in white overlapping the anatomical image. The region of interest (ROI) from the t-statistic image was selected according to its corresponding location in the T2-weighted image. The stereotaxic atlas of the rat (Paxinos and Watson 1998) was used as the reference for determining the corresponding anatomical structures in T2-weighted images. The time course of signal intensities of 40 images of each of the four slices was obtained in the selected ROI. The extent of the functional activation was obtained by calculating the percentage change of averaged signals between the OFF period and the first and the second ON periods. Each chosen ROI corresponded to an anatomical structure that appeared in different slices, thus the signal intensity changes of each ROI in different slices could be averaged. The area of each ROI as depicted by pixel numbers appearing in different slices was summed. The mean percent signal increases and

total area of each ROI activated by different intensities were compared using one-way ANOVA with $P < 0.05$ or <0.001 considered to be significant. Post hoc examination of the difference between groups was performed by the Student-Newman-Keuls test.

Modifications of the Method

Kerskens et al. (1996) compared ultrafast perfusion-weighted MRI of functional brain activation in rats during forepaw stimulation with T2*-weighted MRI.

Several other studies using MRI were performed in rats:

Wrynn et al. (2000) reported an in vivo magnetic resonance imaging study of the olfactory bulbectomized rat model of depression. The magnetic resonance imaging (MRI) investigation demonstrated alterations in signal intensities in cortical, hippocampal, caudate, and amygdaloid regions in olfactory bulbectomized animals but not in sham-operated controls. Ventricular enlargement was also evident in olfactory bulbectomized animals.

Itoh et al. (2004) described magnetic resonance and biochemical studies during pentylenetetrazole kindling development and the relationship between nitric oxide, neuronal nitric oxide synthase, and seizures. The daily administration of pentylenetetrazole was associated with an increase in the amount of neuronal nitric oxide synthase (nNOS). NO generation was measured directly by in vivo and ex vivo electron paramagnetic resonance on rats undergoing progressive convulsions. Morphological changes in the brain structure of rats were measured by magnetic resonance imaging during epileptic convulsions induced by repetitive administration of pentylenetetrazole.

Watanabe et al. (2006) described mapping of the habenulo-interpeduncular pathway in living **mice** using manganese-enhanced 3D MRI. Six hours after intracerebroventricular microinjection of $MnCl_2$, T1-weighted 3D MRI (2.35 T) at 117 μm isotropic resolution revealed a continuous pattern of anterograde labeling from the habenula via the fasciculus retroflexus to the interpeduncular nucleus.

Hasegawa et al. (2003) described diffusion-weighted imaging in kainic-acid-induced complex partial status epilepticus in **dogs**. A cannula was stereotactically inserted into the left amygdala. One week after surgery, all dogs were imaged using MRI. Preinjection imaging consisted of T2-weighted (T2W) imaging, fluid attenuated inversion recovery (FLAIR), and DWI. Two weeks after surgery, five dogs received intra-amygdaloid KA microinjections. MRI was carried out at 3, 6, 12, 24, and 48 h after onset of complex partial status epilepticus.

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NMRI Psychoneuropharmacological Studies in Primates

Functional NMRI Studies in the Brain of Rhesus Monkeys

Purpose and Rationale

Zhang et al. (2000) and Andersen et al. (2002) published methodological details for functional MRI studies in awake rhesus monkeys.

Procedure

Adult female rhesus monkeys (*Macaca mulatta*) were maintained on a 12-h light: 12-h dark cycle in individual cages with water available ad libitum. They had to be adapted to an MRI environment.

The goal was to elicit cooperative involvement of the rhesus monkeys through positive reinforcement. The animals were first acclimated to working with the trainers in their home cage room. Visual contact, vocal familiarity, and positive reinforcements (juice rewards and preferred foods) were used to promote bonding and establish a working relationship between subject and trainer. A pole-and-collar system was used to handle and train monkeys. The monkeys were trained to voluntarily exit their home cages with use of positive reinforcements and the pole-and-collar system. After subjects were sufficiently trained, they were introduced to the primate-training chair. Food was removed from the subject's home cage approximately 20 h prior to a training session. Training sessions lasted 45–75 min and were conducted at least twice a week per subject until no major movements were observed.

Apparatus

The animals were adapted to the MRI-compatible chair constructed from nonferromagnetic materials and designed to comfortably position an adult rhesus monkey in a prone, sphinx-like

position within the magnet bore. The head-holder was designed to restrict head motion without having to surgically attach a head-holder to the skull. The head frame was placed over the monkey's head and secured to the training chair using four nylon screws. The monkey's head rested comfortably on a padded chin support that acted as a stabilizer and cushion. Its nose and mouth were positioned outside the head-holder for comfort and ease of breathing. Under local anesthesia (1 % lidocaine, 2.0 ml on each side), two MRI-compatible pins were inserted through the overlying skin and connective tissue to contact the bony cranium. Earbars/earplugs were constructed that followed the natural angle of the rhesus ear canal and secured firmly to the chair-mounted head-holder. Earbars/earplugs were used both for MRI and training sessions to reduce head movement and protect the animals from the high ambient MRI noise levels. Two separate boxes in front of the MRI-compatible chair accommodated the front limbs of the monkey.

fMRI Procedures

The scans were conducted on a Siemens VISION (VB33A) 1.5 T MRI scanner using the body coil to transmit radio frequency and an 8-cm surface coil placed above the monkey's head for RF signal reception. The anatomic structures of interest were visualized using a 3D FLASH sequence with 1 mm isotropic resolution (TR/TE = 21/6 ms, flip ANGLE = 30°, image matrix SIZE = 128 × 128 × 90, field of VIEW = 128 mm, BANDWIDTH = 195 Hz per pixel). No correction was made for the fall-off in signal intensity with distance from the surface coil in the anatomic images, but sufficient sensitivity was available to accurately visualize anatomic structures throughout the entire slice.

Functional MR images were acquired continuously using a FLASH 2D multiple gradient recalled echo (MGRE) navigator sequence. Slices were acquired interleaved for three noncontiguous positions in the coronal plane, the first slice covering the area of the putamen and the caudate, the second one covering globus pallidus, and the last one including the substantia nigra (Zhang et al. 2000). An 11-echo MGRE sequence was used to map on a pixel-by-pixel basis the local transverse relaxation rate R_2 (Menon; Andersen; Chen and Chen). R_2* is the local transverse relaxation rate for the measured MR signal intensity, S (TE) = S_0 exp($-R_2$*TE), where TE is the echo delay time. It is the parameter that affects contrast in a T_2*-weighted image sensitized to local susceptibility effects such as those caused by changes in the blood oxygenation level. Darker regions in T_2*-weighted images have larger baseline R_2* values reflecting a more rapid MR signal decay rate (R_2* = 1/T_2*). The increase in blood oxygenation level associated with brain "activation" causes a decrease in the value of the R_2* parameter. There are several advantages of using R_2*. As an intrinsic MR parameter, its value is not affected by the nonuniform sensitivity pattern of the surface coil used for signal reception, provided the images at all echo times TE have sufficient signal level, well above the intrinsic noise floor. Also, R_2^* is insensitive to changes in the absolute image intensity, S_0, that may occur over the duration of a long scan. A disadvantage is that the rate of acquisition is slower, although a single-shot multiecho EPI method was recently developed for quantitative R_2* mapping in functional MR imaging (Posse et al. 1999).

The last TE images were acquired without phase encoding gradients to serve as a navigator echo for detection of head motion occurring during the acquisition of data for reconstruction of single R_2^* images. The FLASH 2D acquisition parameters were TR = 250 ms, TE = 7–75 ms, image acquisition matrix SIZE = 112 × 128, rectangular field of VIEW = 112 × 128 mm, slice THICKNESS = 3 mm, flip ANGLE = 40°, BANDWIDTH = 156 Hz per pixel. Local shimming of the static magnetic field $B0$ in the basal ganglia was used to optimize sensitivity to the BOLD effect. For each animal, 20 min of baseline fMRI data were collected, after which 0.1 mg/kg apomorphine was injected subcutaneously. Data collection then resumed for another 20 min. In cases where replicate scans were obtained in the same animal, there was at least a 1-week interval between scans.

Evaluation

The calculation of R_2* values was carried out on a pixel-by-pixel basis by fitting the gradient echo signal decay to a first-order model $\ln(S) = \ln(S_0)$-R_2*TE. The corresponding percentage signal changes, $\Delta S/S$, reported in our activation studies are determined from ΔR_2* as the values that would have been observed at a representative single echo time of TE = 45 ms (Blamire et al. 1992; Menon et al. 1993). The latter time is chosen to match the relaxation time $T2^*$ in brain tissue for maximum contrast [assuming the signal intensity S is maintained constant across studies ($\Delta S/S \approx -\Delta R_2$*TE)]. While R_2* itself does not report on the BOLD effect directly (only the temporal contrast due to changes ΔR_2* does), repeatability in the measurement of baseline R_2^* values in specific brain regions of a given animal helps ensure consistency during replicate scans over a period of months. However, the baseline R_2^* in some tissues, such as the globus pallidus, does change slowly over a period of years due to metal deposition (e.g., iron) with aging. While there are differences between brain areas in their baseline R_2^* value, we have not seen a significant correlation between baseline and drug-evoked changes (ΔR_2*). Thus, quantitative measures of changes in R_2* can be detected regardless of baseline values. Additionally, any departure from a log-linear signal decay behavior can be mapped to help yield information about the intra- or extravascular origin of the signal (Chen et al. 1996). All of the analyses have been conducted using MAT:LAB software on Unix/Linux workstations (Mathworks, Natick, Mass., USA).

The fMRI data needed for computation of the R_2^* maps were acquired at a rate of one set consisting of three image slices every 30 s. Rigid-body motion between successive time points was not corrected since most effects were in the through-slice direction. Prior to administration of any drug, a total of 40 time frames were collected over 20 min for the baseline state. Following the injection of drug, an additional 40 images were collected to track the dynamic response. First, the time-course data for each pixel were adjusted by centering so as to have a zero mean during the baseline period comprising the first 40 time frames. The sample covariance matrix of dimension 80×80 was then computed from the measured R_2* response for all image voxels in the brain, and the principal component time-course profiles were determined as the eigenvectors. The associated pixelwise scores across the slice images represented the spatial modes/patterns.

Information in the navigator echo data from all three slices was used to detect line-to-line changes and subsequently discard image time frames where motion had occurred during the acquisition. Additionally, formal statistical methods of influence analysis for PCA were used to flag and subsequently remove outlying observations that exerted undue influence and contributed disproportionately in the partitioning of variance (Andersen et al. 2002). These observations typically corresponded to spikes at points in the time series data and present as artifacts in the corresponding R_2* maps. In a typical fMRI study of an awake rhesus monkey, on the order of 5–10 time frame observations out of a total of 80 would be detected by the navigator echo and 10–20 flagged as statistical outliers in the influence analysis for PCA. From the trimmed data set, the sample covariance matrix and principal component time course profiles were then recomputed yielding a far more robust representation.

The first principal component, which also represents the largest source of variance in the data, was then used to model the temporal response in each voxel across the brain image slices. This approach of filtering and fitting enables both an analysis of the dynamic nature of the temporal response to administration of a drug in a single animal and the development of an unbiased measure of activation that can be used in the analysis of data combined across multiple animals. From the filtered data, the change ΔR_2* representing the fMRI activation response to a drug was determined as the difference between the mean R_2* value across 20 images post drug administration during the period of peak response and the mean value within the 40 images acquired as baseline prior to administration.

Modifications of the Method

Stefanacci et al. (1998) and Dubowitz et al. (2001) reported MRI studies in conscious monkeys.

Howell et al. (2001) described an apparatus and behavioral training protocol to conduct positron emission tomography (PET) neuroimaging in conscious rhesus monkeys reporting the development and standardization of PET neuroimaging protocols in conscious rhesus monkeys and their application to the characterization of the acute effects of cocaine on cerebral blood flow. Specific attention was devoted to the development of an effective and comfortable head restraint device to be used in the imaging of conscious monkeys. The restraint device was designed to attach to a standard primate chair to facilitate frequent immobilization. Subjects received extensive behavioral training prior to neuroimaging in order to ensure their comfort and minimize potential stress associated with the imaging protocols. Functional changes in cerebral blood flow were characterized in three subjects with the positron-emitting tracer O water following acute i.v. administration of cocaine. Regions of interest were defined on MRI scans with a high degree of accuracy.

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Functional NMRI Studies in the Brain of Common Marmosets

Purpose and Rationale

Massacesi et al. (1995) and Genain and Hauser (1997) described actively and passively induced experimental autoimmune encephalomyelitis in common marmosets as a model of multiple sclerosis. Hart et al. (1998, 2004) used this model for detailed MRI studies with histopathological characterization of MRI-detectable white matter lesions.

Procedure

Eleven marmosets (*Callithrix jacchus*) were used for this study. The body weight of the monkeys at the start of the experiment ranged between 295 and 320 g. During the experiments, the monkeys were individually housed in spacious cages

with padded shelter on the bottom. The daily diet consisted of commercial food pellets for nonhuman primates, supplemented with rice and fresh fruit. Drinking water was provided ad libitum

Induction of Experimental Autoimmune Encephalomyelitis (EAE)

Myelin was isolated from human brain white matter, which was kindly provided by Dr. Rivka Ravid of the Dutch Brain Bank (Amsterdam, the Netherlands). The myelin concentration in the stock solution was 30 mg/ml on a dry-weight basis and 1.3 mg/ml protein on a protein basis as measured according to Bradford (1976).

In a first group of five monkeys the myelin stock solution was emulsified in an equal volume of enriched complete adjuvant. Enriched complete adjuvant was prepared by mixing incomplete adjuvant (DIFCO Laboratories, Detroit, Mich., USA) with 6 mg/ml desiccated mycobacteria (*Mycobacterium tuberculosis*, H37A, DIFCO) followed by brief sonication. Under ketamine anesthesia, each monkey was injected intradermally on the back with 600 µl of emulsion divided over four spots, two in the inguinal and two in the axillary region. In addition, 1 ml phosphate-buffered saline (pH 7.4) containing 1010 heat-inactivated *Bordatella pertussis* particles was injected immediately after immunization and 48 h later.

In a second group of six monkeys, the original immunization protocol was modified in two respects. First, the *Mycobacterium* concentration in the antigen-adjuvant emulsion was reduced from 1.5 to 0.5 mg/ml to diminish the severity of ulceration around the injection sites. Second, *B. pertussis* administration as second adjuvant was omitted to obtain a milder EAE in which patterns of EAE reactivity can be associated with the presence of certain mhc alleles and cytokine profiles.

Clinical Diagnosis

The clinical course of EAE was recorded daily by a trained observer using semiquantitative scoring: 0 = no clinical signs; 0.5 = apathy, loss of appetite, altered walking pattern without ataxia; 1 = lethargy and/or anorexia; 2 = ataxia; 2.5 = para- or monoparesis and/or sensory loss and/or brainstem syndrome; 3 = para- or hemiplegia; 4 = quadriplegia; 5 = spontaneous death attributable to EAE. The highest per-day scores were averaged over 1 week. Moreover, each monkey was weighed at least three times per week to obtain a more objective score of the clinical well-being.

MRI

MRI was performed at the Bijvoet Center of Utrecht University, the Netherlands. For each monkey, T2- and T1-weighted magnetic resonance (MR) images were recorded, the latter also with contrast enhancement after intravenous injection of gadolinium-diethylenetriamine-penta-acetic acid (DTPA) (triple dose). The time points for performing MRI after immunization were chosen during periods of clinically active EAE.

In Vivo MRI

In preparation for the experiment, the monkeys were anesthetized with ketamine/Vetranquil (9/1 v/v). During scanning, each monkey was placed on a 37 °C water-filled heating pad to prevent hypothermia. The head of the monkey was fixed in a custom-built stereotaxic apparatus made of metal-free plastics to ensure reproducible positioning in the magnetic field and to minimize movement artifacts. The stereotaxic apparatus was placed inside a saddle-type radiofrequency coil.

MRI was performed on a SISCO 200-MHz spectrometer (Varian, Palo Alto, Calif., USA) equipped with an actively shielded gradient (maximum gradient 3.2 G/cm, 33 cm inner diameter).

Postmortem MRI

T2-weighted MR images were recorded from formalin-fixed brains to enable the determination of the exact localization of the lesions that were detected in vivo. In both scannings the same orientation points were chosen for slice localization. Because movement artifacts were absent and long acquisition times can be used, images of very high contrast can be obtained.

Slice Orientation and Scanning Procedure

First, a sagittal scout scan was made. The posterior and anterior positions of the corpus callosum were chosen as orientation markers for precise localization of the slices for the in vivo and postmortem MRI. A T2 [echo time (TE)/repetition time (TR), 60/2500 ms-]-weighted multislice scan (20 slices of 1 mm thickness) was obtained followed by a T1-weighted scan (TE/TR, 25/1000 ms) with the same spatial prescription. Gadolinium-DTPA (Magnevist; Schering, Berlin, Germany) was injected intravenously (0.3 mmol/kg) and allowed to circulate for 10 min to ensure adequate distribution. Next, the T1-weighted MRI was repeated to attain a postcontrast data set. Each slice was recorded with a matrix of 256 × 128 data points and a field of view of 5 × 5 cm. The data set was analyzed on an Apple Macintosh Performa 630 using the public domain NIH Image program.

The T1-weighted MR images had an unexpected gray-white matter contrast. In images recorded with the NMR machines that are now used in clinical settings, which are 1.5 T or less, a contrast conversion of the white and gray matter is normally seen on T1-weighted versus T2-weighted images. In T1-weighted images the NMR signal of the white matter is hyperintense in comparison with that of the gray matter, whereas in T2-weighted images the white matter signal is hypointense compared with that of the gray matter.

In both the T1- and T2-weighted images recorded on our 4.7-T machine, white matter is hypointense as compared with gray matter, and lesions are visible as hyperintensities. This is not a unique feature of the marmoset brain, given that it was also observed in cat brains. The most likely explanation for this discrepancy is that with the high magnetic field used, T1 values of gray and white matter converge, whereas T2 values are unaffected by the strength of the field. In our T1-weighted images, the intensities of white and gray matter are most likely determined by the longer T2 value of gray matter and the different proton densities of both tissues. This phenomenon did not affect the detection of gadolinium enhancement, because even in long TR/short TE images ("proton-density"-weighted) contrast enhancement can be observed.

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Part VI

Drug Effects on Learning and Memory

Behavioral Methods Used in the Study of Learning and Memory

F. Scott Hall and Yasir Saber

Contents

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Introduction

The methods associated with the study of learning and memory functions are vast. Our understanding of the neural mechanisms underlying learning is new and evolving, at least in comparison to many of the techniques and basic principles of learning, which have been known for some time and have been summarized in some detail (Gallistel 1990; Mackintosh 1974). This chapter will discuss the major techniques currently being used in the main species used to study learning and memory in animal models, concentrating on techniques in mice and rats. Where appropriate, discussion of methods in other species will be considered with respect to particular methods, as well as some general discussion of other species used in learning and memory research. The goal of this chapter will primarily be to characterize the behavioral methods that are used to study learning and memory functions in behavioral pharmacology, behavioral neuroscience, and behavioral genetics. These techniques are used in basic science studies as well as in pharmaceutical drug development. Secondarily, methods will be described that induce various types of impairments in learning and memory function, as these are commonly used to understand those conditions and to develop drugs that may improve learning and memory function. These methods include both treatments that produce impairments of learning and memory function, as well as models of genetic conditions that alter these functions.

The study of learning and memory function naturally involves a variety of aspects of cognition, as well as aspects of motivation since those things that are remembered best are those that have the greatest hedonic valence. This chapter will not consider all of the various methods used to study cognitive performance (attention, executive function, impulsivity, etc.) except to the extent that those methods are also used to study learning and memory function explicitly. Different aspects of learning function and memory function will be assessed, which may be rather broadly classified: classical conditioning, instrumental

learning, spatial learning, discrimination learning, and implicit (e.g., motor skill learning). In many experimental protocols used to assess learning and memory function, the role of different forms of learning often overlaps. With regard to mnemonic function, one of the primary approaches to the assessment of different aspects of memory function is to administer drugs during different parts of the learning process, e.g., prior to acquisition, consolidation, or retrieval. More recently, as interest in memory reconsolidation and extinction has increased, drug administration has been used to modify these processes as well. These memory processes associated with many types of learning naturally have quite different underlying neural mechanisms. This chapter will consider the majority of learning processes commonly used in animal studies, but it will not address all of the biological mechanisms underlying these processes.

Learning and memory processes vary substantially with the nature of the experimental subjects, including species, strain, sex and age, among many other factors. Learning has a definite ontogeny associated with the development of the brain, particularly the cerebral cortex (Myslivecek 1997; Spear et al. 1979), and a definite decline with aging (Gallagher and Pelleymounter 1988), which has been associated with specific neural changes associated with aging (Foster 1999). There are both genetic (Pedersen and Gerritsen 2015) and environmental (van Praag et al. 2000) contributions to the development of learning abilities. The well-established neurodevelopmental effects of environmental enrichment are mediated by epigenetic modifications during development (Sweatt 2009), as are likely many effects that occur early in life. For example, it is has long been known that there are specific developmental periods at which learning and memory function are optimal, but, moreover, periods during which experience is necessary to develop particular abilities, including aspects of learning and higher cognitive functions (King et al. 2014; McKone et al. 2012), although certainly the degree of importance of experience depends on the particular aspect of learning considered. Abilities for many aspects of learning and memory mature gradually over the course of development (Campbell and Spear 1972).

Much work in learning and memory is directed toward conditions that result in impaired learning and memory function. In addition to "normal" declines during aging (Gallagher and Pelleymounter 1988), there is certainly much emphasis in this field on pathological conditions, including Alzheimer's disease and other dementias (Webster et al. 2014). Research also emphasizes many other circumstances that cause brain insult, either during development or in adulthood (Henry et al. 2007; Rice 1987). The different pathological conditions that produce impairments of cognition, learning, and memory function are too many to discuss here in detail, but as these models are a main emphasis of drug development efforts in this field, several of the more important conditions will be discussed.

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Classical Conditioning

Classical conditioning (Pavlov and Anrep 1927) involves association between a neutral stimulus (which becomes a conditioned stimulus (CS)) with a stimulus that has intrinsic aversive or rewarding properties (an unconditioned stimulus (UCS)). Generally speaking, such stimuli can be described as having intrinsic biological significance to the organism, and hence, responses to such stimuli are innate, whereas conditioned stimuli do not have such innate significance, but acquire such significance through their association with unconditioned stimuli. The CS-UCS pairing can occur once, with a very strong UCS, resulting in one-trial learning, or can occur more gradually with a weaker UCS, resulting in more gradual learning (see Mackintosh (1974) for a broad discussion of such methods and the history and theory of classical conditioning). Depending upon the circumstances of the particular procedure, animals will come to associate different stimuli with an aversive or rewarding stimulus. Classical conditioning approaches for appetitive or rewarding stimuli are generally less commonly used. Some of the earliest versions of such procedures examined classically conditioned responses to aversive stimuli, such as freezing. Other examples of widely used approaches include eyeblink conditioning in rabbits and fear-potentiated startle responses in rodents. Depending upon the particular temporal relationships between the UCS and a discrete stimulus, such as a light or tone, conditioning may occur to either the discrete stimulus or the environment.

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Fear Conditioning

Purpose and Rationale

The most common fear conditioning procedure in rodents involves the association between an aversive UCS (most commonly an electric shock) and a CS. The innate response to the UCS is freezing behavior, a cessation of all movements except involuntary movements (e.g., breathing). After pairing the CS with the UCS, the CS comes to evoke freezing behavior, and the measure of this behavior assesses the learning and memory for the association (for review, see Kim and Jung (2006)).

Procedure

Mice or rats of both sexes, different ages, and different strains are used, and all of these factors influence fear conditioning (Dalla and Shors 2009; March et al. 2014; Shechner et al. 2014). The procedure begins with an initial period of

habituation to the apparatus for 3–10 min. The conditioning apparatus varies substantially in size but is commonly about 18 × 18 × 30 cm in mice and 30 × 30 × 30 cm in rats. Conditioning proceeds in a manner similar to other types of classical conditioning procedures for aversive stimuli with a series of 3–10 CS-UCS pairings separated by an intertrial interval of 60–120 s. Subjects remain in the apparatus for a period of time after the last conditioning trial, comparable to the initial habituation period. The conditioned stimulus is most commonly a tone (2,000–4,000 Hz, 10–30 s, 70–80 dB). The shock parameters vary a little from rats to mice (rats, 0.5– 1.0 mA, 0.5–1 s; mice, 0.15–0.5 mA, 0.5–1 s). The stimulus parameters can vary substantially outside of these common parameters however; more intense stimuli accelerate conditioning and can produce single-trial learning (Drew et al. 2010). The UCS occurs at the offset, or just before the offset, of the CS. Depending upon the experimental circumstances, conditioning occurs to the CS, the experimental environment, or both. Conditioning in response to the CS and the conditioning context are then assessed on subsequent days. CS conditioning is assessed in an experimental chamber that differs in dimensions and appearance from the conditioning chamber. The CS is replayed in this environment and freezing behavior assessed. Contextual conditioning is assessed in the original conditioning apparatus in the absence of the CS. Recent examples of the use of this procedure include Bernardi and Spanagel (2014), Chen et al. (2014), Czerniawski et al. (2012), Diamantopoulou et al. (2012), Dubroqua et al. (2015), Schaap et al. (2013), Skorzewska et al. (2015), and Soya et al. (2013).

Evaluation

The primary measure used to assess learning in this procedure is the determination of freezing behavior, either by measuring the absolute duration of freezing or by assessing freezing at intervals (typically 10 s). The behavior is then expressed as the percent of the total time spent freezing. The freezing behavior is defined as immobile behavior with the only movements being from whisker movements and breathing. This is commonly determined by an observer that is blind to treatment conditions, although more often now this procedure has been automated using infrared beams to measure movement or analysis of video recordings. Post-conditioning behavior is usually compared to preconditioning behavior for contextual conditioning. For CS conditioning, it is common to compare a period immediately before the CS to the time during the presentation of the CS.

Modifications of the Test

It has become common to examine the extinction of conditioned freezing after it is established, by repeated exposure to the conditioned stimulus (Bernardi and Spanagel 2014), and subsequently the reinstatement and reconsolidation of fear memories can be studied (Vouimba and Maroun 2011; Yamada et al. 2009). Fear conditioning itself is not a unitary process and is subserved by different brain systems. Although the conditioning parameters are most commonly established so that the UCS begins with, or just before, the termination of the CS, there can also be a delay between the end of the CS and the UCS. In this procedure, “delay” or “trace” conditioning, the length of the interval affects whether conditioning occurs to the CS or to the environmental context (Czerniawski et al. 2012). Finally, a recent study demonstrated that fear conditioning, using very similar behavioral procedures, could be produced by optogenetic stimulation of fear circuits (Kwon et al. 2014).

Critical Assessment of the Method

As sounds are being used as conditioned stimuli, the equipment is generally contained within soundproof chambers to prevent extraneous noise from affecting the experiments. White noise is sometimes used as well. A main concern, however, when comparing between different

strains of rodents in particular, is baseline differences in freezing behavior and sensitivity to shock, which should be assessed prior to conditioning experiments or as part of the initial conditioning study.

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Fear-Potentiated Startle

Purpose and Rationale

The acoustic startle response is a reflexive behavior that relies upon a simple polysynaptic neural circuit (Davis 1990; Koch 1999). The startle response can be modified by a variety of sensorimotor gating phenomena, including conditioned aversive stimuli, a procedure termed fear-potentiated startle.

In this procedure, the presentation of a classically conditioned aversive stimulus increases the magnitude of the startle response when it immediately precedes the startle stimulus.

Procedure

Mice or rats of both sexes, different ages, and different strains are used, and these factors influence fear-potentiated startle (Dalla and Shors 2009; Pardon et al. 2002; Yap and Richardson 2007). Conditioning proceeds in a manner similar to that described for other types of classical conditioning of aversive stimuli, typically the pairing of a tone or light CS with a shock UCS. The US and UCS parameters are similar to other types of aversive conditioning. Subsequently the CS is played back during assessment of acoustic startle in response to a 50-ms 95–120-dB white-noise, startle stimulus. Conditioning proceeds as for other methods, except that the subject is placed in a small holding apparatus with a wire floor to which an electric shock can be applied. The holding chamber is placed inside a sound-attenuating chamber, on top of an accelerometer that measures the force of the movement associated with the startle response. The holding apparatus varies substantially in size and can be tubular or a rectangular chamber (e.g., a $6 \times 6 \times 5$ cm box for mice or a $16 \times 8 \times 9$ cm chamber for rats). Subsequently the subject is placed back in the apparatus and series of startle stimuli are presented. A series of initial trials (about 10) produces habituation of the startle response to a stable baseline. Subsequent trials are mixed presentations of the startle stimulus alone or in combination with the CS. Some recent examples of the use of this procedure include Burman et al. (2010), Daldrup et al. (2015), Jones et al. (2005), Lehmann et al. (2010), Powers et al. (2010), Schwienbacher et al. (2006), Smith et al. (2011), and Trivedi and Coover (2006).

Evaluation

The measure used to assess learning in this procedure is the percent potentiation of the baseline startle response by the CS.

Modifications of the Test

As for other conditioning procedures, the acquisition, expression, extinction, and reinstatement of this response can be studied. In particular, it has become useful to study the extinction of this response (Pare et al. 2004), as it may relate to attempts to extinguish this type of learning in humans that is associated with anxiety disorders and posttraumatic stress disorder (Lissek and van Meurs 2014).

Critical Assessment of the Method

Similar considerations exist for this model as for other aversive conditioning procedures. Baseline differences in startle and pain reactivity should be assessed, although these are unlikely to provide a major confounding influence since the primary measure is percent difference from each subject's own baseline.

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Eyeblink Conditioning

Purpose and Rationale

The classically conditioned eyeblink response (or nictitating membrane response) is a widely used model system for studying associative learning ((Gormezano et al. 1962) for review, see Longley and Yeo (2014)), mediated by the cerebellum (McCormick and Thompson 1984a, b). It has been studied in most mammalian species, including humans, but is most associated with studies in the rabbit. It involves classical conditioning of the reflexive eyeblink response using a puff of air (the UCS) and a conditioned stimulus (commonly a light stimulus). This approach has also been used in models of age-related memory disorders based on cholinergic hypotheses, including aluminum toxicity, scopolamine-induced memory impairments, and in aged animals (Solomon and Pendlebury 1988; Woodruff-Pak and Li 1994; Woodruff-Pak et al. 1994; Yokel et al. 1988).

Procedure

The eyeblink response is studied in many species but particularly in rabbits (*Oryctolagus cuniculus*). One example of a preparation for measuring this reflex involves suturing a small loop of surgical nylon to the nictitating membrane. On the following day, the subject is placed in a Plexiglas restrainer. The eyes are held open by metal clips. The rabbit is fitted with a head mount supporting a minitorque potentiometer that converts the physical movement of the nictitating membrane into an electrical signal. The potentiometer is attached to the nylon loop on the nictitating membrane. The potentiometer converts reflexive movements of the nictitating membrane into electrical signals that are recorded with a resolution of 0.06 mm of membrane extension every 5 ms. When an air-puff is used as the UCS, an outlet nozzle is attached to the head-mount over the nictitating membrane. The animal is then positioned in a ventilated, sound-attenuating chamber facing a stimulus panel containing the apparatus for

producing the appropriate stimuli (a speaker, lights, etc.). Examples of CS used in such experiments are a 1,000-ms, 1-kHz, 84-dB tone. Examples of UCS include a 350-ms air-puff (3 psi) and a 100-ms, 3-mA, 60-Hz shock delivered to wound clips attached to the parietal cortex.

After a period of adaptation to the apparatus, repeated conditioning trials are conducted over a period of 7 days or so. During this time, no stimuli are presented over the 60-min sessions. The next phase involves repeated conditioning trials, 30–60 trials per day, presented over several weeks of daily training. The conditioning trials each day include a mix of conditioning trials in which both the US and the UCS are presented and CS alone trials presented in a pseudo-randomized fashion. As for other forms of classical conditioning, the UCS can occur with the offset of the CS or with a trace interval of between 125 and 500 ms. An intertrial interval of about 60 s separates individual trials. A response is defined by a particular threshold of movement, often at least a 0.5-mm extension of the nictitating membrane. Responses occurring during the tone or light-conditioned stimulus, but before the unconditioned stimulus, are recorded as conditioned response; those occurring after the unconditioned stimulus onset are recorded as unconditioned response. Recent examples of the use of this method include Carretero-Guillen et al. (2013), Kehoe et al. (2010), Siegel (2014), and Zbarska and Bracha (2012).

Evaluation

The data are analyzed in terms of the percent of responses to the CS or in terms of a ratio between the number of responses during the first half of the CS to the sum of that period plus an equivalent time immediately preceding the start of the CS. The magnitude and the latency of the response can also be measured.

Critical Assessment of the Method

The rabbit nictitating membrane response has been widely used (Schindler et al. 1990). As a highly reflexive response, it is open to few potential confounds. Since the same reflex can be measured across a wide range of species, including humans, it is highly translationally relevant. However, as a cerebellar response, treatments producing changes in the nictitating membrane response may not necessarily be informative about other types of learning or conditions producing memory impairments in humans.

Modifications of the Test

Variants of the test include the use of different types of conditioned and unconditioned stimuli. Trace conditioning, delay conditioning, and long-delay conditioning of the nictitating membrane response have been done (Kehoe et al. 2010; Solomon and Groccia-Ellison 1996). As for most other tests of learning, assessment of different types of memory processes has been done, based on drug administration at different times during the procedure, to examine acquisition (Woodruff-Pak et al. 2002), consolidation (Cooke et al. 2004), retention (el-Zahaby et al. 1994), extinction (Kehoe and White 2002), and reacquisition (Napier et al. 1992).

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Conditioned Taste Aversion

Purpose and Rationale

Conditioned responses resemble the unconditioned responses with which they are associated. Certain types of responses are intended to mediate very specific types of responses, such as the eyeblink response. Another example of this type of conditioned response is conditioned taste aversion (CTA). When animals are ill within a short time after experiencing a novel tastant, they subsequently avoid that tastant. This was initially demonstrated with gamma radiation (Garcia et al. 1955) and subsequently with lithium chloride (Nachman 1963). The procedure is now commonly used to measure the aversive conditioned effects of drugs of abuse (Riley and Tuck 1985; Verendeev and Riley 2012). Most drugs of abuse have been shown to also have aversive effects and to produce CTA, although these effects generally occur at slightly higher doses than the positively valenced hedonic effects of those drugs. Recent examples of the use of this method include Cobuzzi et al. (2014), Jones et al. (2009), Roma et al. (2008a, b), Serafine et al. (2012), and Wetzell et al. (2014).

Procedure

Rats and mice of both sexes, different ages, and strains are used in this procedure. The procedure begins with placing the subjects on water deprivation and giving them a daily period of fluid access for 1 h. Subsequently, subjects are given access to a novel tastant, most often saccharin solution, instead of water. Ten to fifteen minutes after fluid access, the subjects are exposed to an aversive stimulus (e.g., lithium chloride or a drug). Repeated pairings are made between the stimulus and the tastant, which gradually reduces consumption, despite the fluid deprivation. A test session is given after the conditioning sessions, or sometimes at intervals between conditioning sessions, in which fluid containing the tastant and water are both available. During this test session, the avoidance of the tastant is generally enhanced by the availability of water.

Evaluation

During conditioning trials, as the CTA is developing, the primary measure is the volume consumed (per unit body weight). In the two-bottle assessments, the percent preference for the novel tastant is the primary measure. Dose-response curves are used to identify the threshold for the development of CTA.

Modifications of the Test

The number of conditioning sessions is modified depending upon the strength of the aversive stimulus to get slower or faster acquisition of CTA. In an interesting modification of this procedure, subjects are immediately placed in a conditioned place preference apparatus after the drug administration (Roma et al. 2006). This allows simultaneous assessment of CTA and conditioned place preference from the same stimulus, reflecting separate learning about the aversive and rewarding components of the drug effects.

Critical Assessment of the Method

When comparing across groups, it is essential to determine preferences for the tastants independent of any conditioned effects, as these will influence the dependent measures in the CTA test.

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Instrumental Conditioning

Purpose and Rationale

Whereas classical conditioning identifies associations between stimuli that predict outcomes, instrumental conditioning identifies associations between actions and outcomes (Thorndike 1911). For a broad discussion of such methods and the history and theory of instrumental conditioning, see Mackintosh (1974). In particular, it must be noted that there is a certain theoretical debate about the nature of what is learned in instrumental conditioning (Rescorla and Solomon 1967), and it may involve multiple types of associative processes. In any case, the key practical consequence of such learning is that particular actions are reinforced that produce particular outcomes. Instrumental learning approaches are used to assess a great variety of cognitive functions and often involve the acquisition of a particular response (an operant) in a dedicated type of apparatus (an operant box (Skinner 1938)) in order to receive a reinforcer. Subjects are generally in a deprived state, either for food, water or for both, when these are used as reinforcers. Non-deprived animals can also be used, particularly when the reinforcer is highly desired, such as positively reinforcing drugs (e.g., cocaine, morphine, etc.) or the opportunity to copulate. Many instrumental learning techniques are used more to examine cognitive performance than to examine learning per se, but of course, learning is an inherent part of the procedure, and it will often be determined whether subjects "acquire" a particular operant task or whether they take longer to "reach criterion." Many cognitive tasks involve a progression through more and more complex cognitive tasks or to greater and greater task demands, where the subjects have to do more work for each reinforcer earned. Some instrumental tasks are rather simple, including avoidance-type tasks, while others are quite complex and take a great deal of time to learn. For such tasks, it is common to begin subjects on a simpler task, such as a continuous reinforcement schedule for operant responding and then to move them to more demanding ratio or interval schedules in which more responses or longer intervals are required to receive the reinforcer.

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Avoidance Learning

Fear conditioning has already been discussed. In contrast to such learning about relationships between stimuli in order to predict outcomes, animals also learn that actions produce particular outcomes that are desirable or undesirable, e.g., action-outcome associations (Colwill and Rescorla 1986). In the case of avoidance learning, the experimental circumstance is arranged such that actions of the experimental subjects can prevent the occurrence of an aversive event, allowing

avoidance. This can occur either by inhibiting a natural response, such as exploration, which is thus termed "inhibitory avoidance," or by learning an active response, which is thus termed "active avoidance." Although many aspects of these behaviors are based on species-specific defense reactions, such as freezing behavior, avoidance behavior is still highly flexible (Crawford and Masterson 1982). The learning involved in these paradigms is similar to that which occurs during drug conditioning involving an aversive drug (such as β-carbolines (Di Scala and Sandner 1989)), in which a drug is paired with an initially neutral environment, leading to later avoidance of that environment when given a choice between that environment and another environment (e.g., conditioned place avoidance (CPA)). Although different in temporal characteristics in particular, CPA is also similar in some respects to the classical conditioning procedure conditioned taste aversion, discussed previously. Although not considered here, the association of a conditioned stimulus with a positively valenced hedonic stimulus can induce approach, such as occurs in the conditioned place preference paradigm (CPP). CPP and CPA are usually used to assess the reinforcing effects of drugs of abuse, so are not discussed here, but it must be remembered that at their core, all of these procedures involve associative learning and the use of that information to guide later behavior. For an in-depth examination of conditioned place preference, see Tzschentke (2007).

Inhibitory avoidance involves learning to inhibit movement, into another area of the apparatus or down from a raised platform, for instance. By contrast, active avoidance learning requires that the animal learn to move to another location in order to avoid the aversive stimulus (shock) based on the appearance of a CS signaling the imminent onset of a shock UCS (Herrnstein 1969). A typical learning pattern in the active avoidance task begins with an escape response initially, in which the animal experiences the aversive stimulus (shock), but escapes the shock to the safe location. On subsequent trials, the subject learns to avoid the shock, moving in response to the CS prior to the shock presentation. Active avoidance can be done in a discrete trial procedure (one-way avoidance) or in a more continuous procedure in which successful escape or avoidance initiates another trial after a certain intertrial interval (two-way avoidance).

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Step-Down Inhibitory Avoidance

Purpose and Rationale

One of the simplest types of avoidance tasks is the step-down inhibitory avoidance task (SDIA), which is one of the earliest learning tasks used in rats (Hudson 1939) and mice (Jarvik and Essman 1960). Rodents typically spend most of their time in any environment near the walls and avoid open spaces. Initial exploration and preference is almost always for areas that are enclosed or nearest walls. When a rodent is placed on a slightly elevated platform in a square or rectangular compartment, the floor of which comprises an electrified grid floor, it steps down almost immediately to the floor to explore the chamber. The platform is usually rectangular or square, but circular platforms placed in the center of the

apparatus have also been used. The platform can be located in the center or to one side of the chamber. In most procedures, the subject receives a shock upon stepping off the platform and the trial is terminated. Behavior on subsequent trials is assessed to determine memory of this experience, which would increase the latency to step down from the platform.

Procedure

Mice or rats of both sexes, different ages, and different strains are used. The dimensions of the text box, the platform, and the stimulation parameters vary slightly. A square or rectangular test box is used that most commonly has dimensions of 50 × 50 × 25 cm in both rats and mice. The grid floor (0.3–0.5 cm bars, 0.5–1 cm apart, size depending somewhat on the size of the test subjects) is connected to a scrambled shock generator that delivers foot shocks through the grid floor. Earlier studies used a number of shock parameters, but these are most commonly 0.5 mA, 50 Hz for 2 s in both rats and mice. The procedure consists of several phases, although not all studies may include all phases. The first phase is **familiarization/habituation** in which the animal is placed in the apparatus and allowed to explore for a period of time with no shock. Many studies skip this habituation step and begin directly with the second phase. The addition of the habituation phase may be necessary for subjects (due to strain characteristics for instance) that are slow to explore a novel environment. Additional prior handling may also help to reduce initial step-down latencies and reduce variance across subjects. Lighting conditions may also affect baseline latencies. The second phase is the **conditioning** phase. The animal is placed on the platform, and immediately after the animal has descended from the platform, an unavoidable footshock is applied (50 Hz; 0.5 mA; 2 s) and the animal is returned to the home cage. The third phase is a **retention** test, in which the animal is placed in the apparatus as before and the step-down latency once again determined. Usually no shock is received during this test. This test may occur less than 24 h after conditioning (usually 60 min) to assess STM or 24 h or more after conditioning to assess LTM. During these tests, a time cutoff is used because some animals do not step down from the platform at all. The latency is usually 300 s, but this varies from 60 to 300 s across studies. Recent examples of the use of this procedure include Aguggia et al. (2013), Fowler et al. (2011), Furini et al. (2014), Garcia-Gutierrez et al. (2013), Goncalves et al. (2012), Harvey et al. (2012), Moojen et al. (2012), Ortega-Alvaro et al. (2011), Santos et al. (2014), Souza et al. (2013a, b), Vignisse et al. (2011), and Zarrindast et al. (2013).

Evaluation

The primary measures used to assess learning in SDIA are based on a comparison between the step-down latencies during the initial trial compared to subsequent trials. Shorter intervals (minutes) assess STM, while longer intervals (24 h or more) assess LTM. The most commonly used outcome measure is latency, although early studies often used "percent avoidance" according to some criterion, such as staying on the platform for a certain period of time. Although the task is usually terminated after the initial descent from the platform, the shock can be left on so that the frequency and duration of descents can be measured over the course of repeated conditioning trials.

Modifications of the Test

The main modifications of the test adjust the parameters for learning according to the test subjects, for instance, lower shock parameters and a smaller platform in younger animals (Myslivecek and Hassmannova 1991). Those authors examined the ontogeny of SDIA in young rats. Aspects of the test conditions were changed with age, including the shock parameters (1.1–1.9 mA) and the size of the platform (2 × 6 × 2 cm to 5 × 12 × 5 cm) from 2 to 13 weeks. If the shock parameters are reduced, learning can be assessed over a number of conditioning trials rather than just one trial (Cheng et al. 2011). The test has been used to examine differences in learning and memory function resulting from genetic differences and after various environmental and pharmacological

treatments (see references for examples). In pharmacological studies, the timing of the treatments, before or shortly after the conditioning trial, or prior to the retention trial, would affect initial learning, memory consolidation, or retrieval/performance.

Critical Assessment of the Method

The variability of the method can be quite high as it relies upon a small number of measurements of a rather simple behavior, stepping down from a platform. As long as the conditions are sufficient to prevent extraneous stimulation and the animals are handled carefully, it is not necessary to test large groups of animals per experimental condition (eight rats or ten mice per condition). There are some critical parts in the experimental procedure: (i) Placing the animal on the platform needs to be gently done since the tendency of the animal is to escape contact with the human. Habituation to handling and the apparatus helps mitigate this factor. (ii) The electric shock must be applied at the first contact of the animal with the floor. The duration and intensity of the shock should be constant, which is not a problem with commonly available commercial equipment. (iii) The room should be soundproof, and white noise is preferable (60–70 dB).

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Step-Through Inhibitory Avoidance

Purpose and Rationale

This method for establishing inhibitory avoidance involves conditioned avoidance of one compartment of a two-chambered apparatus. Like step-down inhibitory avoidance, it produces conditioning with a single shock pairing. The technique was derived from that of Hudson (1950) whereby shock delivered on entering a feeding cage led to avoidance of the chamber. Step-through inhibitory avoidance methods follow the general procedure described by Essman and Sudak (1964). This method is based on rodent's tendency to avoid a brightly light area and favor movement into a darker area. When a rodent is placed in a small brightly illuminated chamber, it will quickly move through a small opening into a darker chamber. As the subject steps through the opening, a guillotine door automatically closes, blocking access to the first chamber, and the subject receives an electric shock and the trial is terminated. As in step-down inhibitory avoidance, assessments of performance at subsequent time points assess short-term and long-term memory.

Procedure

Rodents of both sexes, various ages, and strains are used. The apparatus generally consists of two compartments of equal size, although unequal sizes are sometimes used, one of which is brightly illuminated and the other of which is dimly illuminated. The apparatus is often a bit larger for rats than for mice, although in each case, the most common arrangement is two $15.5 \times 12.5 \times 11.5$ cm compartments. The apparatus has a grid floor that is connected to a scrambled shock generator as for other types of aversive conditioning, in particular as for step-down inhibitory avoidance. Similar shock parameters are used as for other procedures that have already been described. As for step-down inhibitory avoidance, there are three experimental phases: a habituation phase, a conditioning phase, and a retention phase. Recent examples of the use of this procedure include Akar et al. (2014), Banfi et al. (1982), Broekkamp et al. (1986), Essman and Sudak (1964), Fekete and De Wied (1982), Fornari et al. (2012), Frye et al. (2005), Hock et al. (1989), Morris and Gold (2012), Sanchez-Resendis et al. (2012), and Zhang et al.(2011).

Evaluation

The methods of evaluation for step-through inhibitory avoidance are the same as step-down inhibitory avoidance (see the previous section), primarily the comparison of the latency on the training trial compared to subsequent trials.

Modifications of the Test

Modifications of this procedure include using other aversive stimuli for conditioning, such as the inescapable 10 °C cold water of Hughes (1976). Some early versions of step-through inhibitory avoidance used a much larger starting

chamber (50 × 50 × 35 cm), with a small opening in one side (6 × 6 cm) leading into a smaller, darker compartment (15 × 15 cm). Upon entering the smaller compartment, the subject would receive a shock and the procedure would proceed as described above. The idea behind the unequal sizes is to encourage entrance into the darkened compartment. As for some examples of step-down inhibitory avoidance, lower shock levels can be used that do not produce complete avoidance, so that the learning is more graded and the number of transitions into the dark compartment, and the proportion of time in each compartment, can be measured. Examples of this approach include Banfi et al. (1982), Bures and Buresova (1963), and Kurtz and Pearl (1960).

Critical Assessment of the Method

Issues with the step-through inhibitory avoidance procedure are the same as for the step-down inhibitory avoidance procedure (see the previous section).

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One-Way Avoidance

Purpose and Rationale

One-way avoidance involves an apparatus in which one side is a "start" side and the other is a "safe" side. The subject begins on the "start" side to which the shock will be applied, but can escape to the other side, the "safe" side after the initiation of the shock, or prior to the initiation of the shock in response to a CS (Hock and McGaugh 1985; Lubar and Perachio 1965; Munn 1950).

Procedure

Mice or rats of either sex, and different strains, are used. The design of the apparatus can be as a runway with a wire-grid floor and guillotine doors that cut off part of the runway to define a start box and a "safe" area or a target box with a runway in between the two areas that the subject needs to traverse to escape or avoid shock. One door opens to initiate each trial and when the target box is reached, the trial is terminated and a guillotine door closes. Alternatively, it is more common now to use an apparatus similar to that for step-through inhibitory avoidance, with a wire-grid floor for shock presentations. Conditioning is usually done using an auditory CS similar to those used in conditioned freezing experiments. As in those other methods, after an initial period of habituation to the entire open apparatus, typically 5 min, the subject is placed in the "start" side of the apparatus with the guillotine door closed. To signal the initiation of the test, the light turns on in the compartment containing the test subject, defining the starting area. After a period of time, 10 s or so, the CS is applied and the door is simultaneously opened. Shock occurs at the termination of the CS after 5 s or so. The subject either escapes to the other side, which is not shocked, after the initiation of the UCS, or avoids the shock by moving prior to receiving the UCS. The trial is terminated by closing the guillotine door. After a certain intertrial interval (usually variable between about 50 and 70 s), the subject is returned to the start side to initiate another trial. Training continues until the animal attains a criterion of avoidance responses, commonly 90 %. As with other methods of this type, STM and LTM can be assessed by assessing the subject at different time points. Some recent examples of this procedure include Boivin and Beninger (2008), Duffy et al. (2008), Gebhardt et al. (2013), and Maul et al. (2008).

Evaluation

The primary measure is trials to criterion or performance (e.g., % of trials in which there is an avoidance response). The latency to respond is often also measured, as are errors (escape responses in which the animal receives the shock).

Modifications of the Test

The main variation of this task includes the use of a two-compartment box as opposed to a runway apparatus, which has already been discussed. As for other aversive conditioning tasks, different types of stimuli can also be used for the CS, such as a light. The number of trials and the criterion for learning can be varied, usually dependent upon the strength of the UCS; a weaker UCS leads to more protracted learning. There have also been attempts to automate this task, "jump-up avoidance" (Martinez et al. 1992; McKean and Pearl 1968; Tenen 1966). In this variant, the "safe zone" of the runway or box is an elevated Plexiglas platform. A retractable barrier controls access to the platform. After a successful escape or avoidance response, this barrier moves outward forcing the animal back onto the grid, which is no longer electrified, initiating another trial.

Critical Assessment of the Method

Same as for other procedures of this type.

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Two-Way Avoidance

Purpose and Rationale

Two-way (or shuttlebox) avoidance is a continuous task where from trial to trial the "safe" side shifts to the opposite side. Thus, it is a more difficult task than the one-way avoidance task. This task has become far more common than the one-way avoidance task because it is easier to automate.

Procedure

Rats and mice of both sexes and different strains are used in this test. It is conducted in a similar two-compartment apparatus as that described for other types of avoidance with an electrified grid floor for applying shock (the UCS). The apparatus has the facility to present various visual and acoustic stimuli, similar to those previously described for other types of aversive conditioning. After being allowed a period of time (about 5 min) to habituate to the apparatus with the guillotine door open and the houselights off in both compartments, the door shuts, confining the subject to one side. After 20 s, the houselight is turned on in the compartment containing the animal and the door is opened. A tone CS is presented, and 5 s later, shock is applied to the grid floor in the illuminated compartment. As with the one-way version of this task, the subject can escape the shock after it comes on, or it can avoid the shock by crossing to the other side after the presentation of the CS, but prior to the presentation of the UCS. The shock is terminated when the subject escapes, after the initiation of the shock, or does not come on at all when the subject makes an avoidance response during the CS. In either case, when the animal crosses to the other side, the door closes. After a variable intertrial interval of 30–90 s, another trial is initiated. The light is now switched on in the compartment that the subject now occupies (the previously dark compartment) and the door opens. This compartment is now the starting compartment where the shock will be applied, and the subject needs to move to the other compartment to avoid a shock in the starting compartment. As before, the shock is applied at the termination of the CS. The subject now has to cross into the compartment in which it previously was shocked, which is part of the reason that this is a more difficult task. This process continues for a number of trials, commonly 30 or 40 trials per day, until the animal reaches a preestablished criterion, commonly 90 % avoidance responses. As for other types of aversive conditioning, the rate of learning is determined in part by the shock parameters. Recent examples of the use of this task include Choi et al. (2010), Datta et al. (2009), Ilango et al. (2011), Lichtenberg et al. (2014), and Wietzikoski et al. (2012).

Evaluation

The data from this task are evaluated in the same manner as for one-way avoidance.

Critical Assessment of the Method

The task is more difficult to learn than the one-way avoidance task because of the shifting safe area, which may make the task much more stressful during early acquisition. This may impact particularly upon the learning of more emotional or anxious subjects. This is, in fact, an issue with all aversive conditioning – in practical terms it can be difficult to dissociate the impact upon the emotional perception or reaction to the aversive stimulus from other factors that may influence learning.

Modifications of the Method

As with other methods of this type, retention can be tested at various intervals after the test criterion is met to examine memory, and drugs may be administered at different points to examine difference aspects of memory function. Extinction, reinstatement, and reconsolidation of the avoidance response can also be determined.

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Operant Learning

Purpose and Rationale

Operant techniques are widely used in the study of learning, memory, and cognition. They involve instrumental conditioning using an operant, which is often a somewhat arbitrary behavior. The types of learning and cognitive functions that can be studied using operant techniques are myriad, and these techniques are especially widely used in the study of drug reinforcement. This description will include just the basic outline of such techniques.

Procedure

Operant procedures have long been described in great detail (Mackintosh 1974) and very standard apparatus exist for both rats and mice. Different types of operants are used; lever presses in rats and mice, and nose pokes in mice are most common. Operant boxes have dimensions that are typically about 30 × 25 × 20 cm for rats and slightly smaller about 16 × 14 × 13 cm for mice. The rest of the apparatus varies substantially in composition, with one or more levers (or other operant devices) and various means of producing conditioning stimuli (lights, speakers to emit tones, and/or tubes to emit odors) that signal the appropriate response, the availability of the reinforcer, or other aspects of the task. There is also some means of delivering the reinforcer; a food pellet can be delivered into a tray accessed by a panel, fluid can be pumped into a tray, a drug delivered via an indwelling intravenous catheter, an access door opening to give access to a

sexually receptive conspecific, etc. Some operant tasks are more continuous in nature, such as the animal continually making responses, on a particular schedule of reinforcement, for food or another reinforcer. Other operant tasks consist of a series of discrete trials, often initiated by a particular response by the subject and/or signaled by a distinctive event, such as turning on a light in the top of the chamber. A more specific example of such a procedure is considered in the subsequent section on visual discrimination.

Evaluation

In a continuous type of operant task, the dependent measures are usually the number of responses or the number of reinforcers earned. In some operant procedures, there are two operants, one that produces a desired reinforcer and the other that produces no effect, or an effect that is not desired; in such a case, the ratio of the two responses can be compared. This is common in drug self-administration where the number of responses on a lever producing a drug injection is compared to the number of presses for a lever producing a saline injection. In a discrete trial procedure, the primary measure is usually the number of trials to meet a particular criterion, such as 80 % correct responses. During the acquisition of a response, the number of correct and incorrect responses is also typically measured, as well as the type of incorrect responses (e.g., emitting the wrong response, a premature response, omitting a response, etc.).

Modifications of the Test

Operant procedures are used in many different ways, some of which are discussed in subsequent sections. The most important recent innovation is the use of touch screens to replace much of the previous mechanical and electrical parts of the apparatus (Aggleton et al. 1997; Romberg et al. 2013). Stimuli can be shown on the screen, which registers when the subject touches the screen in the appropriate (or inappropriate) location, resulting in the delivery (or nondelivery) of a reinforcer.

Critical Assessment of the Method

One of the main issues with operant learning approaches is to dissociate clearly the effects of treatments on the ability to respond and the desire to respond. Similarly, impairments in motor or attentional function can also affect performance and need to be dissociated from effects on learning or memory. In practice there are often measures within the task that allow assessments of such confounds, including the latency to collect the reinforcer, the latency to respond to a stimulus, omission of responses, and so forth. Separate assessments of motor, sensory, and motivational effects of treatments are also recommended, in particular for drugs with a less well-known pharmacology or for genetically modified mice (see Crawley (2007) for a discussion of these issues with regard to genetically modified mice, although the same issues apply to pharmacological studies).

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Discrimination Learning

In the types of classical and instrumental learning discussed above, although associations with conditioned stimuli come to affect behavior, that

behavior involves singular potential outcomes. In the case of instrumental learning, only one response was possible (or a nonresponse). Another important type of learning occurs when choices must be made, based on the presence of multiple potential stimuli that can be used to guide behavioral choices. In a discrimination experiment, one stimulus (the S+) is reinforced, while another stimulus (S-) is not. Any type of stimulus can be used in such experiments that the animal can perceive, including locations, for instance, the different arms of a T-maze. If only one of the stimuli is available on each trial, the procedure is termed successive discrimination. If both stimuli are available on the same trial, but subject must make a specific response according to their disposition, such as entering the black arm of the T-maze, regardless of which side it is on in a particular trial, it is termed a simultaneous discrimination. The following examples illustrate just a few of the types of procedures that involve discrimination learning. An exhaustive survey of the types of discrimination studies can be found in Gilbert and Sutherland (1969) (see also Mackintosh (1974)).

As has already been noted, many studies of instrumental learning are conducted in order to examine different types of cognitive process, a full summary of which is beyond the scope of the present work. Discrimination learning in particular, whether it is primarily spatial in nature or based upon operant learning, is a component of a number of procedures used to study different aspects of cognition, including different aspects of response selection, executive function, and attention. For a discussion of this sort of strategy, see Bussey et al. (2012), a consideration of the use of a battery of methods to study cognitive deficits in schizophrenia.

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Spatial Discrimination

Purpose and Rationale

In one simple type of discrimination learning, the subject learns to distinguish between stimuli that differ in spatial location, arms of a maze, or lights on the left or right side of an operant box. Many other configurations are naturally possible, but the key element in all procedures of this type is that the feature that distinguishes the CS+ from the CS- is spatial location. In an operant box, a left-right basis for the discrimination is produced by arrangement of the apparatus with all of the salient features (lights, levers, etc.) on one wall. In a maze, a left-right basis for the discrimination is produced because the choice points are literally a "T" or "Y" in the maze. The example below is for a maze version of a spatial discrimination.

Procedure

Rats and mice of both sexes and different strains are used. Subjects are food deprived and food is used for reinforcement. The apparatus used is usually a simple T- or Y-maze. The apparatus for rats is larger (arms 50 × 10 cm) than the apparatus for mice (arms 30 × 10 cm). The last 10 cm (or more sometimes) of each arm is separated from the rest of the apparatus by a guillotine door. The bottom of the T in the T-maze or one of the arms in the Y-maze is a start box. Each trial is initiated by opening the guillotine door. A food cup is placed at the end of each of the top arms of the T-maze or the two opposite arms of the Y-maze. One of the arms contains food for each trial and acts as a CS+, and the other does not and acts as a CS-. Thus, as the animal leaves the start

box and reaches the choice point, it may turn right or turn left, one of those directions is always correct, resulting in receipt of a reinforcer. Common reinforcers are food pellets, about 100 mg, or a liquid reinforcer such as 50:50 sweetened condensed milk, about 0.7 mL. In either case, when the subject passes into the last 10 cm of one of the arms, a choice is made and registered as correct or incorrect. The subject is then confined there for about 30 s (during which the subject consumes the reinforcer if the correct choice was made). The subject is then returned to the start box and another trial initiated. Multiple trials are run each day (20–40), or spread out over multiple days, and subsequent retention, extinction, etc., for the response can be determined. As for other procedures of this type, prior habituation to handling, the apparatus and the food rewards are all important. For a more detailed description of this method, see Deacon (2006), and for a discussion of the consideration of reward sizes versus the number of trials per session, Gaskill et al. (2011). Recent examples of the use of this procedure include Derenne et al. (2014), Hallock et al. (2013), and Sanderson et al. (2012).

Evaluation

For maze versions of spatial discriminations, the number of correct and incorrect responses is measured, as well as the number of sessions (days) necessary to meet a particular criterion. The choice latency can also be assessed, which is the latency to enter the end of one of the arms.

Critical Assessment of the Method

The ecology of rodents makes these animals especially proficient in spatial discrimination learning, which is usually mastered in a few trials. Most of the initial errors are not due to the inability of the animal to remember the correct solution, but rather to its tendency to explore alternative pathways. Differences in anxiety levels between groups can influence the rate of learning. Habituation is a critical factor, as are lighting conditions. The environment should not be aversive; concurrent evaluation of anxiety differences between subjects using other methods may be warranted. This would be indicated by reduced exploration of the maze and longer choice and reinforcer consumption latencies. Anxiety is one example of various confounding issues of this type, including the different types of species-typical behaviors, which can confound animal test of learning and memory (Thorpe et al. 2004).

Modifications of the Test

The conditioned spatial discrimination tasks in mazes vary substantially, including the nature of the stimuli, the nature of the reinforcers, the design of the maze, and the general experimental environment that contributes to spatial learning aspects of the task. Barnes (1979) and others introduced the radial maze as a modification of spatial discrimination. This method is now well established and widely used in many different ways. The test can also be automated in a variety of ways (for example, see Gaskill et al. 2011). Altering the nature of the stimuli results in variants of the task, including those in which an initial trial demonstrates which arm is reinforced, delayed matching to position (Hammond et al. 2012), as well as delayed alternation (Fowler et al. 2013). Such tasks can be used to examine working memory in this task. Other versions have been developed in which the maze is partially filled with water and the subject needs to choose the correct arm to escape the maze, called the water (or "paddling") version of the T- or Y-maze (Fidalgo et al. 2012; Murray et al. 2013).

There are of course many other types of mazes used to assess different aspects of spatial learning and memory (Sharma et al. 2010). Many of these can be used to study spatial discrimination learning, as well as other types of spatial learning, depending upon the nature of the mazes and the experimental protocols. Indeed, one approach involves a series of discriminations rather than using spatial maps. How the maze is solved by the subject can be addressed in a variety of ways, such as rotating the maze to change global room cues.

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Visual Discrimination

Purpose and Rationale

There is certainly a visual component to spatial discrimination tasks, as well as most other tasks assessing spatial learning and memory, although the learning of many spatial tasks certainly involves other types of learning. Other qualities of stimuli, including visual, auditory, olfactory, and tactile differences, can be used in discrimination experiments. A summary of all of these approaches is beyond the scope of the present work. It is most common in visual discrimination experiments to use an operant approach. Visual stimuli are particularly useful in this regard as they can be presented in a very specific manner and made to differ in a number of qualities in a quite precise manner. The first experiments of this type would teach animals (rodents, but also a wide range of other species) to respond to lights in different locations in a different manner, or the same light when flashing at different frequency, and so forth. As an example of this type of approach, the procedure for a simple type of visual discrimination is described below.

Procedure

Rats and mice of both sexes and different strains may be used. Other species are also commonly used in these types of experiments as discussed in subsequent sections. The apparatus consists of a standard operant chamber for rats or mice, depending upon the experimental subjects. All experimental control and recording of

responses is via computer control of the apparatus. Two retractable levers are placed on each side of a food tray covered by a panel used to measure entries to obtain the reinforcer. Reinforcers can be dispensed into the tray (either food pellets or fluid aliquots via a dipper or pump). Food pellets can be standard food pellets, although more desirable foods are often used. Solutions are often sucrose or saccharin solutions, or sometimes flavored solutions such as strawberry-flavored Ensure™. In one arrangement, lights are placed above the food panel and the two levers, and a houselight is placed in the ceiling of the apparatus. The food tray can also be illuminated from behind. As for most tasks of this type, even a simple discrimination, the subjects are trained on a series of progressively more difficult tasks, the number of stages depending on the difficulty of the ultimate task. Initially subjects are trained to press either lever to receive a reinforcer on a continuous reinforcement schedule. Once stable responding is observed (based on some criterion, such as >50 responses within 15 min), training begins on the visual discrimination. The discriminanda are slow and fast flashing lights (0.83 Hz vs. 5 Hz for 2.4 s, all three lights). Each trial begins with a 4 s illumination of the houselight. Following the presentation of the CS, the levers enter the chamber for 6 s and remain until a response is made or for 6 s and then are retracted. Correct responses result in illumination of the food tray and receipt of a reinforcer. Incorrect responses or omissions result in a 6 s delay, signaled by turning off the houselight, before the 5 s intertrial interval in which the houselight is off (i.e., incorrect responses increase the interstimulus interval). Subjects are tested for 128 trials per day, with equal numbers of each condition. Subjects take 30–40 sessions to acquire the discrimination depending on the criterion. At this point, various types of manipulations can be made to assess performance and motivation, including changing the stimulus duration and properties. Examples of the use of this type of discrimination procedure include Marston et al. (1993), Muir et al. (1992), Reading et al. (1991), and Winters et al. (2004).

Evaluation

The number of correct and incorrect responses is determined, including omissions, as well as the latency to respond and to collect the reinforcer. One of the main measures of learning is the number of sessions required to meet a criterion of performance, commonly 80 % or 90 % correct responses.

Critical Assessment of the Method

Visual discrimination procedures of this type are much more time-consuming than other methods, but such methods can be used to address more complex forms of learning that may have greater translational application for some conditions (Bussey et al. 2012). Approaches that are more complex than the one described here may be used to separate different aspects of various higher cognitive functions that contribute to learning, including various aspects of attention and response control (Bari and Robbins 2013; Lustig et al. 2013).

Modifications of the Test

Much more complex sorts of visual discrimination are of course studied than the one described above. The development of touch screen methods for rats, mice, and other species greatly facilitates the ability to use a range of complex stimuli in such studies (Bussey et al. 1997; Horner et al. 2013; Romberg et al. 2013) and can be used to assess a wide range of cognitive functions. The five-choice serial reaction time task, and subsequent modifications, has a similarly wide applicability to the assessment of learning, memory, and cognition (Bari et al. 2008; Carli et al. 1983; Harrison et al. 1997). Such approaches can also be used to study recognition memory (Steckler et al. 1998), although certainly there are other methods that involve primarily physical exploration of objects (Bevins and Besheer 2006).

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Spatial Learning

Spatial learning is certainly a component of many of the learning and memory tasks that have already been considered here. In any particular task, however, even in what might be considered to be an overtly "spatial" task such as a maze, the actual stimuli that are used by the subject to solve the task (i.e., escape an aversive situation or acquire a

reinforcer) may or not be spatial in nature. In a classical maze such as the Stone's maze (Stone 1929), if the task involves a relatively simple series of turns, the pattern is learned rather than the animal needing to use global cues to navigate. Similarly, in several versions of the T-maze, left-right direction, the texture of the floor, or the color of the walls can guide responses, so that only a limited degree of spatial learning may occur. The tests considered in the next sections are considered to require spatial learning, although certainly depending upon the specific testing conditions, other aspects of learning may be involved or even dominate. It would be presumed that these tests would all engage hippocampal-dependent mechanisms involved in spatial memory and cognitive maps to one degree or another (Silva et al. 1998). These are, of course, just a few of the many tests that examine spatial learning, but are among the most common and most widely used (Paul et al. 2009; Sharma et al. 2010).

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Spatial Habituation Learning

Purpose and Rationale

One of the core features of the response of an animal to any novel stimulus is that repeated exposure, in the absence of reinforcement, leads to reduced exploratory interactions, i.e., habituation (Hinde 1970; Thompson and Spencer 1966). Habituation is a non-associative memory process that has a different basis across brain systems. It has been documented at the synaptic level in invertebrates (Pinsker et al. 1970a, b). Habituation is found for both discrete types of sensory inputs, as was the case for the classical studies in aplysia, and for places. The rate of habituation reflects growing familiarity with the environment and involves alterations in gene expression that are associated with reduced cell firing in the hippocampus (Guzowski et al. 2006). In many contexts, habituation occurs within a single exposure to a stimulus (within-session habituation, an STM process) and with repeated exposures to the same stimulus (between-session habituation, an LTM process). Spatial habituation learning refers to both processes with respect to a particular environment. This type of habituation implies a degree of recognition or familiarity with the specific physical characteristics and spatial relationships of the environment.

The behavioral assessment of habituation can be done in virtually any apparatus, but is commonly performed using an open-field apparatus (e.g., Hall et al. 2000). Rodents, in particular, explore environments through locomotion and physical interaction. The typical pattern is for the level of exploration to be high initially and to decrease thereafter. The degree of exploration is impacted by a variety of competing effects, including the subject's motivational state (e.g., hunger, thirst, etc.) and the degree to which the environment evokes anxiety (a more open and brightly lit environment inhibits exploration). The physical complexity of the environment, including the presence of objects, also affects exploratory tendencies (Berlyne 1960).

Procedure

An open-field apparatus is usually square in shape (rats, 100 cm^2; mice, 40 cm^2) made of painted wood or Plexiglas, with 30 or 40 cm high walls. The color of the apparatus is often chosen based upon the color of the experimental subjects so that

they are easy to see (particularly for video recordings or monitoring). The lighting conditions can vary from very brightly lit to very dimly lit (often using a red light), but illumination should be arranged so that it is evenly distributed over the apparatus, which is open at the top. This is in part determined by the tendency of the subjects (particularly with regard to strain) toward anxiety; a more dimly lit environment may be necessary to get optimal exploration levels in certain strains. The subjects are exposed to the apparatus repeatedly on different days for a period of around 15–30 min. The apparatus is cleaned between each trial with dilute (0.1 %) acetic acid. It is very useful to transport the subjects to the testing room 30 min before testing, which will reduce anxiety and increase exploration. The most traditional means of measuring behavior is by an observer blind to treatment of the subjects. It is far easier to measure certain specific behaviors in this manner, but more difficult to measure others. It is far more common now to use a computerized analysis of the path the animal takes using automated software from any number of vendors. These programs can also now measure specific behaviors based on video recordings (such as freezing or rearing), which required an observer previously. As is the case for most behavioral tests in rodents, the open-field test is more often conducted during the light phase, which is not optimal in nocturnal animals. In subjects with low levels of activity, it is better to conduct the test in red light or during the dark phase. Repeated exposures (3–5) occur over a period of days spaced 24 h apart, although shorter intervals can be used to assess STM. Recent examples of the use of this test include Brenes et al. (2009), de Oliveira Alvares et al. (2005), Deacon et al. (2009), Lee et al. (2004), Milot and Plamondon (2008), and Popovic et al. (2014).

Evaluation

In earlier versions of the test, the open field was divided into 25 squares by placing colored lines on the apparatus. The number of "line crosses" was then measured as an index of locomotion. Computerized systems allow the measurement of the actual distance traveled by the subjects over time. This activity can also be divided between the center and periphery of the apparatus as a measure of anxiety and the quality of the movements analyzed mathematically to determine the average length and repetitiveness of the movements (Geyer et al. 1986). In terms of habituation, the primary measures are for locomotion and for rearing. The rates of decrease in these measures over time, within-sessions and between-sessions, are the primary measures of habituation.

Modifications of the Test

Emotionality, including freezing and defecation, and anxiety (percentage of time spent in the center of the apparatus) are commonly measured in the open field. Habituation can also be measured in response to objects placed in the open field or in another enclosed apparatus. As noted by Berlyne (1960), one of the critical determinates of exploration is complexity, so that altering complexity will increase exploratory tendencies.

Critical Assessment of the Test

The open-field paradigm is a well validated, simple, and time economical test, which has been widely used to examine the neurobiological foundations subserving spatial memory, general activity, and emotionality in rodents with different approaches including lesions, drugs, electrophysiology, neuroanatomy, and neurogenetics. The major confounds involve anxiety, which may require adjusting the procedure as discussed above.

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Radial Arm Maze

Purpose and Rationale

The radial arm maze (RAM) was developed by Olton and Samuelson (1976) to assess spatial learning and memory. The maze involves eight arms radiating away from a center platform. The ends of some of the arms are baited with food. Over repeated trials, the subject must navigate the maze to retrieve the food. Optimal performance (e.g., learning) is based on retrieving the food with the fewest number of errors (e.g., entering un-baited arms or reentering arms). The rat uses spatial information provided by distal room cues to navigate through the maze. Depending on the particular experimental procedures used in a particular RAM experiment, spatial working memory or working/reference memory can be assessed (Olton and Samuelson 1976; Vorhees and Williams 2014). Performance of the task can be either dependent on the prefrontal cortex or on the hippocampus (Floresco et al. 1997), depending upon the specific conditions of the task. In reference memory versions of the RAM, information is retained for extended periods of time (LTM), while in working memory versions of the RAM, the subject needs only to remember which of the arms have already been entered (working memory).

Procedure

The test is performed in mice and rats of different ages, sexes, and strains. The size of the apparatus varies substantially, but is larger for rats than mice. The wooden or Plexiglas apparatus consists of central platform, from which the maze arms (rats: 10 × 50 cm, with 13 cm high walls; mice:

5 × 35 cm, with 9 cm high walls) radiate in eight evenly spaced directions. The entire maze is elevated above the ground. Depending on the task, all of the arms may be used or only a few. The maze is well illuminated and distinctive distal cues are placed around the room. Food pellets (or other appetitive reinforcers) are placed at the ends of one or more of the arms.

In the simplest version of the task, used to assess working memory, all of the arms are baited. The subjects are food deprived prior to the test (typically 85–90 % of their free-feeding weight). Initially the subjects are allowed to freely explore the apparatus with food placed at the ends of the arms. Subjects are subsequently trained on a daily basis, and the session is terminated after eight choices, with the number of reinforcers collected and the number of errors being the measure of performance. Subjects are trained until meeting a criterion of 80 % performance. Alternatively, the subject can be allowed to collect all eight pieces of food and the total number of errors measured, and the criterion may be a certain threshold for the number of errors. The RAM can also be used to assess spatial working and reference memories, by baiting only some of the arms. This requires the use of global room cues to orient to particular arms and prevents the development of a simple strategy to retrieve all of the food. In this case, errors include both entering un-baited arms and reentering arms that were previously entered (Petkova et al. 2014; Vorhees and Williams 2014).

Evaluation

The number of errors, entries to non-baited arms, or re-entries into arms that were previously entered is the measure of learning and performance. If a limited number of arm entries are possible, then the percent of correct responses is measured.

Modifications of the Test

Several different versions of this test have been used. The difficulty of the test may be altered by increasing or decreasing the number of arms used. The test may also be conducted by removing the subject between arm entries (or confining it for a period of time once an arm is entered) to introduce delays that require the maintenance in STM of the arms that have been entered. Delay procedures within repeated trials can also be used to examine the learning of win-stay (return to the same arms) or win-shift (go to different arms) strategies (McDonald and White 2013). A non-appetitive (aversive) version of the RAM has been created by filling the maze with water so that the subject must swim through the maze to find an escape platform at the end of each arm (Buresova et al. 1985; Penley et al. 2013; Vorhees and Williams 2014). In this procedure, after 20 s, the escape platform would be lowered, but the subject could find another platform at the end of an arm that had not been previously entered.

Critical Assessment of the Test

Depending upon the specific arrangements of the procedure, subjects can achieve learning criteria in ways other than by using working or reference memory when overtrained, including the development of a chain or serial strategy. Delay procedures can help to prevent the development of a strategy. The appetitive procedure, like many others, is also dependent upon the motivational state of the animal. Finally, olfactory cues can confound the results (Dubreuil et al. 2003; Vorhees and Williams 2014).

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The Morris Water Maze

Purpose and Rationale

Richard Morris developed an open-field water maze that is now the most used method for assessment of hippocampal-dependent spatial memory and learning (Morris 1981, 1984). Spatial navigation must be used in the task to find a hidden platform and thus escape from the water. No proximal cues are present, so the subjects, rats or mice, must use distal cues to find a hidden platform. A spatial navigation strategy produces the optimal performance in the test (e.g., the shortest latency to find the platform and the shortest distance). Learning in this test is dependent on the integrity of the hippocampus and entorhinal cortex (Vorhees and Williams 2014).

Procedure

Rodents (mice and rats) can be used in this method. Both species have a natural capability for swimming. The apparatus consists of a circular pool, the dimensions depending on the species tested, for mice about 120 cm in diameter and for rats about 240 cm in diameter. If mice are tested in a larger pool, they may fail to locate the target platform (Schaefer et al. 2011). The platform diameter which is usually 5–10 cm should be consistent with pool diameter. The size of the pool in recent articles has tended to become smaller in comparison with what was used in early studies. The pool has no proximal cues that can be used by the animal to find the escape platform, so that distal cues must be used to navigate. The pool stays in the same relation to these cues throughout testing. The pool is filled with room temperature water to about 20 cm lower than the upper edge of the pool. To obscure the location of the platform, white milk powder was originally dissolved in the water. This has been widely replaced with nontoxic acrylic paint. The pool is divided into four quadrants (west, south, east, and north), with one of them containing the platform that submerged by about 1 cm below the water surface (Darcet et al. 2014). Multiple trials are conducted each day (4–8 depending upon the rate of acquisition in the particular strain being studied), over a series of days, by dropping the subject in a different location in the pool for each trial (equidistant from the platform), with a maximum latency of 60 s. Generally speaking, on the first trial, the subject will rarely find the platform, partly because the initial tendency is to search near the walls of the pool. If the subject does not find the platform, it is placed on the platform for 30 s, usually by gently guiding it toward the platform. Over trials, the subject abandons its initial thigmotaxis and learns the platform location, eventually proceeding in a straight line to the platform. This is the acquisition phase of the experiment. The day after the last acquisition day, a probe test is conducted by removing the escape platform. The time that animal spends in the quadrant that had platform in comparison with the time spent in the other quadrants reflects retention of the spatial

memory. For further details, see Vorhees and Williams (2006). Recent examples of the use of this method include Able et al. (2006), Fan et al. (2010), Jiang et al. (2014), McAuliffe et al. (2006), and Skelton et al. (2006).

Evaluation

The latency to reach the escape platform is measured during the training days. Originally this was done by hand timing, but is now automated using any video tracking software. This software will also calculate the distance, average distance from the platform and swim speed. During the probe trial, the primary measure is the percent time in the appropriate quadrant, as well as the number of crossings over the previous platform location.

Modifications of the Test

Especially when mice are used in this method, it is preferred to add an initial training session that consists of a visible platform before starting acquisition of the hidden platform trials. This accelerates their learning trajectory (Vorhees and Williams 2014). The test may also be done with proximal cues (Morris 1984).

Critical Assessment of the Test

Some inbred strains of mice should not be used in this test because they either float or show persistent thigmotaxis (swim near the edge of the pool) (Vorhees and Williams 2014). These types of effects may also be found in transgenic mice. The distance traveled to the platform is a better measure than latency, as it is independent of swim speed. Swim speed should be assessed as a measure of motor impairment.

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Non-rodent Species Used in Learning and Memory Studies

The preceding sections discussed a variety of methods used to study learning and memory functions in rodents (rats and mice). This emphasis is based upon the preponderance of studies in this field that have used these species as experimental subjects. This is not, in any way, meant to imply that important contributions to the study of learning and memory generally, and in particular to certain aspects of learning and memory function, have not been made by studies in other species.

Nonmammalian species have certainly contributed substantially to our understanding of learning and memory at a mechanistic level, beginning with aplysia (Pinsker et al. 1970a, b). There are a number of advantages to invertebrate species, not the least of which is cost. Of course, the large differences in brain structure for creatures far lower on the phylogenetic scale than mammals must be taken into account, but the advent of genomics clearly narrowed the gap. With the sequencing of the *C. elegans* genome (Consortium 1998), it became clear that the major transition in terms of genetic diversity came in the step from unicellular to multicellular life and that gene homologues for most human genes would be found in many species outside of the mammalia. These species, including most especially *C. elegans* (Sasakura and Mori 2013), *Drosophila* (Wright 2014), and zebrafish (Levin and Cerutti 2009) are now important models used in studying the behavioral genetics of learning and memory and in modeling cognitive disorders and aging.

A wide range of non-rodent species have been used in studies of learning and memory, including dogs (Macpherson and Roberts 2010), guinea pigs (Philippens et al. 1992), pigs (Sondergaard et al. 2012), ferrets (Rabe et al. 1985), squirrel monkeys (Weller et al. 2006), capuchin monkeys (Fragaszy and Cummins-Sebree 2005), and many other species. Of course the most well-studied experimental animal subjects outside of rodents are the primates. Studies in rhesus macaques, in particular, were essential for understanding of the basic contributions of the amygdala, hippocampus, the basal forebrain, frontal cortex, and temporal cortex to memory function (Bachevalier et al. 1999; Barbas 2000; Levy and Goldman-Rakic 2000; Voytko 1996). Studies in this species also contributed to studies of memory decline in aging (Hara et al. 2012; Walker et al. 1988). As studies in the larger primates have been more difficult and expensive to conduct, other primate species have also been used to study memory and cognition. One of the most successful of such efforts has been with the common marmoset (Roberts 1996; Roberts et al. 1988), which has produced many insights into the function of the prefrontal cortex and neurological impairments of memory function associated with Parkinson's disease and Alzheimer's disease. One of the major advantages of studies in primates, aside from a generally faster rate of learning for complex cognitive tasks, is better (more human-like) manual dexterity. Thus, many studies of object discrimination (delayed matching to sample, delayed non-matching to sample, etc.) can more closely model similar procedures in humans.

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Models of Learning and Memory Impairments

Many of the animal models developed to study learning and memory deficits have emphasized Alzheimer's disease (Webster et al. 2014) and aging (Solomon and Pendlebury 1988). Most of the early work in these areas emphasized the role of the loss of acetylcholine in learning and memory deficits in these conditions (Antuono et al. 1979; Pepeu et al. 1986). This was based upon pathological findings in Alzheimer's patients, although losses of other neuronal populations were certainly known to occur in that condition. However, the well-known amnesic effects of the acetylcholine antagonist scopolamine were certainly influential in this thinking, and this idea was supported by a wealth of evidence demonstrating mnemonic impairments in animals treated with scopolamine (Carli et al. 1999; Entlerova et al. 2013; Heo et al. 2014; Klinkenberg and Blokland 2011; Philippens et al. 1992; Popovic et al. 2014; Scavio et al. 1992; Singer and Yee 2012; Wilson 2001; Woodruff-Pak and Hinchliffe 1997). Indeed, the scopolamine model continues to be widely used in studies of mnemonic impairments. Findings with scopolamine were further supported by initial lesion studies in the basal forebrain, which contains the cell bodies of the main cholinergic projections to the hippocampus and cerebral cortex

(Miyamoto et al. 1985; Pepeu et al. 1986). However, more selective lesioning techniques that specifically targeted acetylcholine neurons did not replicate these broad effects on memory function (Everitt and Robbins 1997; Robbins et al. 1997), although certainly other effects were observed.

Changes in views of the role of acetylcholine in mnemonic function, and the role of the loss of acetylcholine in memory impairments associated with Alzheimer's disease, were occurring at the same time that genetic findings were suggesting that aberrant versions of genes encoding β-amyloid were responsible for the accumulation of amyloid plaques in the brains of Alzheimer's patients (Butterfield 2002). The development of genetically modified mouse models for aberrant human genes was important in evaluating the role of these genetic changes in the disease and in impairments in learning and memory function (Barnes et al. 2004; Butterfield 2002; Deacon et al. 2009; Souza et al. 2013). However, recent data has indicated that the accumulation of β-amyloid may be less central to the process than previously thought, while other data suggested that aberrant Tau proteins may be more critical determinants of cognitive impairments (Castillo-Carranza et al. 2015). These studies clearly indicate the importance of genetic models in current attempts to understand conditions producing impairments in learning and memory function. These models are by no means limited to the study of Alzheimer's disease. Genetic models have been developed for everything from Fragile X syndrome (Berry-Kravis 2014) to Parkinson's disease and indeed for most major (and minor) conditions that produce impairments in learning and memory functions.

Animal models of learning and memory function are by no means limited to genetic causes of dysfunction either. Numerous models have been developed to model the consequences of different types of toxicity and other adverse events that might contribute to impairments in memory and cognition during development or afterward. Some of these conditions model other neurological conditions that produce cognitive impairments (Archer et al. 2011), including Parkinson's disease. For instance, chronic low-dose exposure to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in impairments in a number of tests of learning and memory function (Fox and Brotchie 2010; Roeltgen and Schneider 1994; Schneider et al. 1994). A wide range of other neuronal insults have been modeled, including neonatal hypoxia (McAuliffe et al. 2006; Young et al. 1986), exposure to aluminum (Sethi et al. 2008; Yokel et al. 1988), ischemia in adult animals (Gordan et al. 2012; Hunter et al. 1998), and traumatic brain injury (Fujimoto et al. 2004; Henry et al. 2007; Moojen et al. 2012), among many others.

The models that have been developed in recent years address a very wide range of underlying causes of learning and memory impairments and moreover reflect a growing appreciation of the complex causality underlying all of these conditions, both genetic and environmental. The history of the evolving hypotheses concerning the causes of Alzheimer's disease, in particular, emphasizes the need for both translational and reverse-translational approaches; that is, researchers engaged in research using animal models need to propose hypotheses to be tested in humans, and researchers conducting human research need to propose hypotheses to be tested in animal models. One of the greatest handicaps in creating animal models has always been a lack of understanding of the underlying causes of disease in humans. The reciprocal process outlined here can contribute to focusing both human and animal research in the most appropriate directions, particularly when attention is paid to the correspondence between the types of learning and memory processes evaluated in the tests of learning and memory in human subjects and in animal models.

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Non-behavioral Methods Used in the Study of Learning and Memory

F. Scott Hall

Contents

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In Vitro Methods

In Vitro Inhibition of Acetylcholine-Esterase Activity in Rat Striatum

Purpose and Rationale

The purpose of this assay is to screen drugs for inhibition of acetylcholine-esterase activity. Inhibitors of this enzyme may be useful for the treatment of Alzheimer's disease.

Acetylcholinesterase (AChE), which is sometimes called true or specific cholinesterase, is found in nerve cells, skeletal muscle, smooth muscle, various glands, and red blood cells (Nachmansohn and Rothenberg 1945; Koelle et al. 1950; Ellman et al. 1961). AChE may be distinguished from other cholinesterases by substrate and inhibitor specificities and by regional distribution. Its distribution in brain roughly correlates with cholinergic innervation, and subfractionation shows the highest level in nerve terminals.

It is generally accepted that the physiological role of AChE is the rapid hydrolysis and inactivation of acetylcholine. Inhibitors of AChE show marked cholinomimetic effects in cholinergically innervated effector organs (Taylor 1996) and have been used therapeutically in the treatment of glaucoma, myasthenia gravis, and paralytic ileus. However, recent studies (Christie et al. 1981; Summers et al. 1981; Davies and Mohs 1982; Atak et al. 1983) have suggested that AChE

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inhibitors may also be beneficial in the treatment of Alzheimer's dementia.

Augustinsson (1971) reviewed a number of methods for assaying cholinesterase activity and concluded that the method described by Ellman et al. (1961) was one of the best. The method described is a modification of Ellman's procedure.

Procedure

Reagents

1. 0.05 M phosphate buffer, pH7.2
   (a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O.
   (b) 13.40 g $Na_2HPO_4 \cdot 7H_2O$/100 ml distilled H_2O.
   (c) Add (a) to (b) until pH reaches 7.2.
   (d) Dilute 1:10.
2. Substrate in buffer
   (a) 198 mg acetylthiocholine chloride (10 mM)
   (b) q.s. explain to 100 ml with 0.05 M NaH_2PO_4, pH7.2 (reagent 1)
3. DTNB in buffer
   (a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   (b) q.s. to 100 ml with 0.05 M NaH_2PO_4, pH7.2(reagent1)
4. A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent 3). Drugs are serially diluted (1:10) such that the final concentration (in cuvette) is 10^{-4} M and screened for activity. If active, IC_{50} values are determined from the inhibitory activity of subsequent concentrations.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, and corpora striata dissected free, weighed, and homogenized in 19 volumes (approximately 7 mg protein/ml) of 0.05 M NaH_2PO_4, pH7.2 using a Potter–Elvehjem homogenizer (Kontes, Vineland, NJ). A 25 µl aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and reincubated for 10 min at 37 °C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for IC_{50} determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

Blank:	0.8 ml PO_4 buffer/DTNB 0.8 ml buffer/substrate
Control:	0.8 ml PO_4 buffer/DTNB/enzyme 0.8 ml PO_4 buffer/substrate
Drug:	0.8 ml PO_4 buffer/DTNB/drug/enzyme 0.8 ml PO_4 buffer/substrate

Blank values are determined for each run to control for nonenzymatic hydrolysis of substrate, and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program (Beckman DU-50 series spectrophotometer, kinetics Soft-Pac™ module operation instructions: 1–7 also calculates the rate of absorbance change for each cuvette.

Evaluation

For IC_{50} determinations, substrate concentration is 10 mM diluted 1:2 in an assay yielding a final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\%\,\text{Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC_{50} values are calculated from log–probit analysis.

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In Vitro Inhibition of Butyrylcholine-Esterase Activity in Human Serum

Purpose and Rationale

This assay can be used in conjunction with the acetylcholine-esterase assay to determine the enzyme selectivity of various cholinesterase inhibitors.

Butyrylcholine esterase (BChE), which is sometimes called pseudocholinesterase, preferentially hydrolyzes butyrylcholine. This enzyme is found in the highest amounts in serum, but its physiological role is not known (Chemnitius et al. 1983; Walker and Mackness 1983). Ethopropazine and tetra-isopropyl pyrophosphoramide (ISO-OMPA) are selective inhibitors of butyrylcholinesterase. In an ex vivo experiment with the selective BChE inhibitor, ISO-OMPA, it was shown that inhibition of butyrylcholinesterase was not correlated with any significant acute cholinomimetic effects.

Procedure

Reagents

1. 0.05 M phosphate buffer, pH7.2
   (a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O.
   (b) 13.40 g $Na_2HPO_4 \cdot H_2O$/100 ml distilled H_2O.

(c) Add (a) to (b) until pH reaches 7.2.
(d) Dilute 1:10.

2. Substrate in buffer
   (a) 225.8 mg s-butyrylthiocholine chloride
   (b) q.s. to 100 ml with 0.05 M phosphate buffer, pH7.2(reagent 1)
3. DTNB in buffer
   (a) 19.8 mg 5, 5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   (b) q.s. to 100 ml with 0.05 M phosphate buffer, pH7.2(reagent1)
4. A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent3). Drugs are serially diluted (1:10) such that determined from the inhibitory activity of subsequent concentrations.

Enzyme Preparation

A vial of lyophilized human serum (Precilip, Biodynamics, Houston, Texas) is reconstituted in 3 ml of distilled water. A 25 ml aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and preincubated for 10 min at 37 °C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for IC_{50} determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

Blank:	0.8 ml PO_4 buffer/DTNB 0.8 ml buffer/substrate
Control:	0.8 ml PO_4 buffer/DTNB/enzyme 0.8 ml PO_4 buffer/substrate
Drug:	0.8 ml PO_4 buffer/DTNB/drug/enzyme 0.8 ml PO_4 buffer/substrate

Blank values are determined for each run to control for nonenzymatic hydrolysis of substrate, and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

Evaluation

For IC_{50} determinations, substrate concentration is 10 mM diluted 1:2 in assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\%\,\text{Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC_{50} values are calculated from log–probit analysis.

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Ex Vivo Cholinesterase Inhibition

Purpose and Rationale

This assay is used to determine the dose–response relationship and duration of action of cholinesterase inhibitors in vivo.

Cholinesterase inhibitors, including physostigmine (Christie et al. 1981; Davis and Mohs 1982; Thal et al. 1983) and tacrine (Summers et al. 1986), have been shown to improve cognitive functions in Alzheimer's disease. Physostigmine is a potent, but nonselective inhibitor of cholinesterase (Taylor 1996), and has a short duration of action. Tacrine also inhibits both acetylcholine esterase (true) and butyrylcholine esterase (pseudo), but is more potent as an inhibitor of the pseudoenzyme (Heilbronn 1961).

The mechanism of inhibition of these two drugs is quite different. Physostigmine is a competitive inhibitor and blocks the active site of the enzyme by carbamylation of a serine hydroxyl

group at the esteratic site of the enzyme (Taylor 1996; O'Brien 1969). This covalently bound carbamyl group then dissociates from the enzyme much more slowly than the acetyl group left by the natural substrate, but the inhibition is not irreversible like that of the organophosphates. The inhibition characteristics of physostigmine, i.e., submicromolar affinity for the enzyme and covalent binding of the inhibiting group, are ideal for ex vivo studies. Tacrine, however, is a mixed competitive inhibitor of cholinesterase (Heilbronn 1961), with lower apparent affinity than physostigmine for the enzyme (based on IC_{50} values at saturated substrate concentrations). Tacrine binds to the anionic site of cholinesterase through weak hydrophobic interactions (Steinberg et al. 1975).

Ideally, a dose–response for cholinesterase inhibition is determined first. Then a dose which gives a reasonable effect (>50 % inhibition if possible) is chosen to do the time-course experiment. The effects on brain acetylcholinesterase activity are examined in striatal tissue, using 5 mM acetylthiocholine as a substrate (Ellman et al. 1961). Effects on butyrylcholine-esterase activity may be determined in plasma samples, with 5 mM butylthiocholine used as a substrate.

Procedure

Reagents

1. 0.05 M phosphate buffer, pH7.2
   (a) 6.85 g $NaH_2PO_4 \cdot H_2O$/100 ml distilled H_2O.
   (b) 13.40 g $Na_2HPO_4 \cdot 7H_2O$/100 ml distilled H_2O.
   (c) Add (a) to (b) until pH reaches 7.2.
   (d) Dilute 1:10.
2. DTNB in buffer
   (a) 19.8 mg 5, 5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
   (b) q.s. to 100 ml with 0.05 M phosphate buffer, pH7.2 (reagent 1)
3. Substrate in buffer
   (a) 198 mg acetylthiocholine chloride (10 mM)
   (b) q.s. to 100 ml with 0.05 M phosphate buffer, pH7.2 (reagent 1)

Drug Treatment

Groups of four male Wistar rats are dosed i.p. or p.o. with vehicle or the test drug. For the initial dose–response study, the rats are given varying doses of drug based on toxicity reported in primary overt effects testing and sacrificed at either 30 min or 1 h after dosing. The animals are observed and the occurrence of cholinergic signs is noted (piloerection, tremors, convulsions, salivation, diarrhea, and chromodacryorrhea). For the time-course study, a dose of the test drug is given which gave significant inhibition of cholinesterase activity.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, and corpora striata dissected free, weighed, and homogenized in 4 volumes of 0.05 M phosphate buffer, pH7.2, using a Potter–Elvehjem homogenizer (Kontes, Vineland, NJ). A 12.5 ml aliquot of the homogenate is added to 1 ml 0.05 M phosphate buffer, pH7.2/DTNB (reagent 2).

Assay

1. Enzyme activity is measured with the Beckman DU-50 spectrophotometer.

Reagents are added to the blank and sample cuvettes as follows:

Blank:	0.8 ml PO_4 buffer/DTNB (reagent 2) 0.8 ml PO_4 buffer/substrate (reagent 3)
Control:	0.8 ml PO_4 buffer/DTNB/enzyme from control animal 0.8 ml PO_4 buffer/substrate
Drug:	0.8 ml PO_4 buffer/DTNB/enzyme from treated animal 0.8 ml PO_4 buffer/substrate

Blank values are determined for each run to control for nonenzymatic hydrolysis of substrate, and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

2. Substrate concentration is 10 mM diluted 1:2 in the assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration.

Evaluation

The percent inhibition at each dose or time is calculated by comparison with the enzyme activity of the vehicle control group.

$$\%\,\text{Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

Ex vivo time-course experiments for physostigmine are as follows:

Physostigmine, 0.3 mg/kg, i.p.		
Time (h)	% Inhibition striatum	Cholinergic signs
0.25	48.6	P, T, D
0.5	28.5	P, T, D
1.0	27.0	P, T
2.0	7.6	P, T
4.0	1.4	P, T

P = piloerection, T = tremors, D = diarrhea

Modifications of the Method

Antagonism of physostigmine-induced lethality in mice has been used by Gouret (1973) as a general indicator of central or peripheral anticholinergic activity. A low dose of physostigmine can be used for detecting procholinergic activity.

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Molecular Forms of Acetylcholinesterase from Rat Frontal Cortex and Striatum

Purpose and Rationale

Different molecular forms of acetylcholinesterase can be isolated from animal tissues after solubilization in buffers containing various detergent and salt

concentrations. The number of forms isolated, their relative amounts, and molecular characteristics depend on the tissue source and the conditions used for solubilization of the membrane-bound enzyme (McIntosh and Plummer 1973; Reiger and Vigny 1976; Trevor et al. (1976). Bon et al. (1979) have classified these forms as globular (G_1, G_2, and G_4) and asymmetric (A_4, A_8 and A_{12}), where the subscripts indicate the number of catalytic subunits. The G_1 and G_4 forms, having sedimentation coefficients of approximately 4S and 10S, respectively, are the major forms contained in bovine caudate nucleus (Grassi et al. 1982). Under conditions of high salt concentrations and detergent, AChE is quantitatively extracted from rat brain, with 10S form being the predominant component (Reiger and Vigny 1976). After solubilization, the molecular forms may be separated according to sedimentation properties by density gradient centrifugation and molecular weight by gel filtration or by electrophoretic mobility.

Although most of these studies on molecular forms of AChE have focused on the physical differences, Chan et al. (1972) reported some difference in sensitivity of the low molecular weight form to physostigmine and fluoride ion, while Lenz and Maxwell (1981) showed differential sensitivity to soman of forms separated by isoelectric focusing. Studies showing selective increases in the 10S form during development (Muller et al. 1985) and selective loss in Alzheimer's disease (Atack et al. 1983) suggest that this form of the enzyme may be developmentally and functionally more important. Cortical and striatal areas show different patterns of cholinergic innervation: the cortex having primarily extrinsic innervation, while the striatal cholinergic pathways are predominantly intrinsic (Ceullo and Sofroniew 1984).

The purpose of this procedure is to determine the effects of various cholinesterase inhibitors on the two major molecular forms of acetylcholinesterase isolated from rat striatum and cerebral cortex.

Procedure

The procedure is divided into three main parts:

I. Preparation and isolation of molecular forms of AChE
II. Assays for the marker enzymes
III. Enzyme inhibition studies

I.a Preparation of molecular forms of AChE

Male Wistar rats (200–250 g) are sacrificed, their brains rapidly removed, and frontal cortices or corpora striata removed. The brain areas are weighed and homogenized in 5 volumes (wt/vol) of 10 mM phosphate buffer, pH 7.1, containing 1 M NaCl and 1 %Triton X-100, except were indicated. The homogenates are centrifuged at 20, 000 g for 20 min at 4 °C. The supernatant is aspirated, and marker enzymes for 16S (*E. coli* β-galactosidase), 11.3S (bovine catalase), and 4.8S (horse liver alcohol dehydrogenase) fractions are added. The supernatant is then centrifuged at 37, 000 rpm (140,000 g max) for 17.5 h in a Beckman L5–65 ultracentrifuge with a SW-60 rotor. 15-drop fractions are collected for each centrifuge tube and assayed for protein, β-galactosidase, catalase, alcohol dehydrogenase, and acetylcholinesterase activity. In addition, butyrylcholinesterase can be measured.

A 400 µl sample of the 20,000 g-supernatant is carefully layered on top of a 5–20 % continuous sucrose gradient. This gradient is made up in a centrifuge tube from 1.65 ml of 20 % sucrose and 1.65 ml of 5 % sucrose in homogenizing buffer by means of a gradient maker. A 50 % sucrose cushion (0.5 ml) is placed at the bottom of the tube.

Fractions are collected from the bottom of the tube, i.e., the densest fractions are collected first. Each fraction is 15 drops or approximately 24 fractions are collected per centrifuge tube.

I.b Analysis of fractions

β-Galactosidase, catalase, and alcohol dehydrogenase are determined by enzymatic activity (Hestrin et al. 1955; Chance and Maehly 1955; Bonnichsen and Brink 1955). Protein concentrations are determined by the method of Lowry et al. (1951). Acetylcholinesterase activity or butyrylcholinesterase activity is determined by a modification of the method of Ellman et al. (1961). Briefly, 10 µl aliquots of the fractions are added to

0.25 mM dithionitrobenzoic acid (DTNB) and 5 mM acetylthiocholine or 5 mM butyrylthiocholine in 0.05 M phosphate buffer, pH 7.2, and the absorbance is measured at 412 nm. Fractions of peak acetylcholinesterase activity are characterized by their sedimentation characteristics relative to the marker enzymes, and peak fractions are pooled for enzyme inhibition studies or determination of kinetic constants.

II. Assays for marker enzymes

(A) Equine liver alcohol dehydrogenase (ADH), sedimentation coefficient 4.8 S

1. Enzyme: alcohol dehydrogenase from equine liver, crystallized and lyophilized (Sigma Chem. Co.)
2. Reagents:
   (a) β-Nicotinamide adenine dinucleotide (NAD) (Sigma Chem. Co.)
   (b) 0.1 M glycine-NaOH buffer, pH9.6
   (c) Absolute *ethanol*
   (d) Buffer-*substrate*-NAD mixture:
   875 µl NAD + 875 µl ethanol + 18.75 ml
   0.1 M glycine-NaOH buffer, pH9.6
3. Assay
   10 µl enzyme fraction and
   850 µl mixture (reagent 2d)
   are incubated for 5 min at room temperature.
   The reaction is stopped by adding:
   300 µl 1.5 M $ZnSO_4$
   Absorbance is read at 340 nm, and enzyme units are determined from a standard curve using values of 1.25, 2.5, 5, 10, and 20 mU of ADH.

(B) Bovine liver catalase, sedimentation coefficient 11.3 S

1. Enzyme: catalase from bovine liver, purified powder (Sigma Chem. Co., C-10)
2. Reagents:
   (a) 30 % hydrogen peroxide
   (b) 0.05 M sodium phosphate buffer, pH7.0
   (c) Mixture: 111 µl 30 % hydrogen peroxide + 100 ml buffer, yielding 0.033 % peroxide
3. Assay
   10 µl enzyme fraction
   2,990 µl peroxide-buffer mixture (reagent 2c) wavelength is set to 240 nm; absorbance is adjusted to 0.480 units. The amount of time is recorded for absorbance to decrease from 0.450 to 0.400. This corresponds to 3.45 µmol of hydrogen peroxide in 3 ml solution. Total catalase activity in 3 ml is 3.45 µMol/min.

(C) *E. coli* β-galactosidase, sedimentation coefficient 16.0 S

1. Enzyme: β-galactosidase from *E. coli*, grade VI, partially purified, lyophilized (Sigma Chem. Co)
2. Reagents:
   (a) Substrate: 15 mg/ml *O*-nitrophenyl-β-D-galactopyranoside (Sigma Chem. Co.) in water
   (b) 0.6 M Na_2CO_3, pH7.25
   (c) 1 M $NaCO_3$
3. Assay
   10 µl enzyme fraction,
   150 µl 0.6 M phosphate buffer, pH7.25, and 50 µl *O*-Nitrophenyl-β-D-galactopyranoside (12 mM in assay) are incubated for 25 min at 30 °C. The reaction is stopped by adding:
   500 µl 1 M Na_2CO_3, 1.75 ml water
   Absorbance is read at 420 nm, and enzyme units are determined from a standard curve using values of 0.015, 0.030, 0.525, 0.125, and 0.250 U of β-galactosidase.

III. Enzyme inhibition studies

For the enzyme inhibition studies, 25 µl aliquots of the enzyme preparation are preincubated with varying concentrations of the inhibitor for 10 min at 25 °C, and acetylcholinesterase activity is determined as previously described.

Evaluation

Values for the IC_{50} are determined by log–probit analysis of the inhibition data using six to seven concentrations of the inhibitor and represent the means of three separate experiments.

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Release of [^{3}H]ACh and Other Transmitters from Rat Brain Slices

Purpose and Rationale

Electrically stimulated release of [^{3}H]ACh is used as a biochemical screen for agents which may possibly enhance or inhibit release of [^{3}H]ACh through a direct muscarinic interaction or other indirect interactions.

Muscarinic autoreceptors have been shown to have a role in the regulation of ACh release in several areas of the CNS (Hadhazy and Szerb 1977; DeBelleroche and Gardiner 1982; Strittmatter et al. 1982; James and Cubeddu 1984). Direct stimulation of muscarinic receptors with muscarinic agonists or indirect stimulation with acetylcholinesterase inhibitors decreases ACh release evoked by either increased potassium concentration or electrical stimulation. Muscarinic antagonists can either block their

inhibition or, under certain conditions, enhance ACh release (James and Cubeddu 1987; Sethy et al. 1988). Furthermore, other neurotransmitters, most notably 5-HT and DA, can also inhibit [^{3}H]ACh release via interaction with 5-T_2 and D_2 heteroreceptors (Robinson 1983; Jackson et al. 1988; Muramatsu et al. 1988; Drukarch et al. 1989), and this inhibition can be reversed by the appropriate receptor antagonists. A compound's effect on [^{3}H]ACh release may provide evidence for a wide variety of releasing activities.

The advantages of using the electrically stimulated release technique on tissue slices have been described by Zahniser et al. (1986). This technique measures only presynaptic effects of test compounds.

Procedure

This assay is based on the methods described by James and Cubeddu (1984, 1987).

Reagents

1. Krebs–Henseleit bicarbonate buffer, pH 7.4 (KHBB)

   Make a 2 l batch, containing the following salts:

NaCl	13.84 g	118.4 mM
KCl	0.70 g	4.7 mM
$MgSO_4 \cdot 7H_2O$	0.58 g	1.2 mM
KH_2PO_4	0.32 g	2.2 mM
$NaHCO_3$	4.20 g	24.9 mM
$CaCl_2$	0.28 g	1.3 mM
Prior to use, add:		
Dextrose	4.00 g	11.1 mM

   Aerate for 60 min with 95 % O_2/5 % CO_2 on ice and check pH.
2. [Methyl-^{3}H]-choline chloride (80–90 Ci/mmol) is obtained from New England Nuclear. The final desired concentration of [^{3}H]choline is 100 nM. Add 0.25 nmol of [^{3}H]choline to 2.5 ml KHBB.
3. For most assays, a 10 mM stock solution of the test compound is prepared in a suitable solvent and diluted such that the final concentration in the assay is 10 μM. Higher or lower concentrations may be used depending on the potency of the test compound.
4. Hemicholinium-3 or HC-3 (Sigma): Make a 10 mM stock solution in H_2O. Two milliliters of this stock are then diluted to one liter in KHBB to give a final concentration of 20 μM.

Tissue Preparation

Male Wistar rats (100–150 g) are decapitated; cortical, striatal, or hippocampal tissue are removed on ice; and 0.4 mm slices are prepared with a Mcllwain tissue slicer. The slices are made individually; placed in cold, oxygenated buffer (10–20 ml); and incubated at 35 °C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 2.5 ml of cold oxygenated buffer is added as well as enough [^{3}H]choline to bring the final concentration to 100 nM. This is then incubated and shaken for 60 min at 35 °C under oxygen. After this step, the buffer is decanted and the "loaded" slices are immediately placed on nylon mesh in the stimulation chambers.

Assay

Control buffer is pumped through the chamber for 42 min at a flow rate of 0.7 ml/min, to establish a stable baseline. Under these conditions released, [^{3}H]ACh is subject to hydrolysis by acetylcholinesterase. The perfusion buffer is changed to fresh KHBB containing 20 μM HC-3. The potent choline uptake inhibitor HC-3 is included to prevent the reuptake of any [^{3}H]choline formed from the hydrolysis of released [^{3}H]ACh. This maintains the stoichiometry of the stimulated release. The evoked release has been shown to be mostly [^{3}H]ACh rather than [^{3}H]choline, whereas spontaneous release under control, drug-free conditions is mostly [^{3}H]choline (Richardson and Szerb 1974; Szerb et al. 1977; Supavilai and Karobath 1985; Saijoh et al. 1985; Nishino et al. 1987).

Special Conditions

1. Stimulation parameters are set at 2 Hz (2 ms duration) for 120 s, with 1 ms delay and voltage setting of 750 SIU (250 Ω). Agonists are more potent modulators of [^{3}H]ACh release at low stimulation frequencies (5).
2. For striatal slices, 2 μM sulpiride is present in the buffer to prevent DA inhibition of ACh release.

3. In some experiments, 5 μM methysergide is present in the buffer to prevent the serotonergic inhibition of ACh release mediated by 5-HT_2 receptors.
4. In order to determine the muscarinic regulation of [^{3}H]ACh release, 10 μM atropine can be included in some experiments.
5. In some experiments, physostigmine is added to the perfusion buffer. This causes a marked inhibition of stimulated release via feedback at presynaptic receptors. Under these conditions, receptor antagonists enhance [^{3}H]ACh release.

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, 200 ml of 20 % methanol in distilled water, and again with distilled water for at least 20 min.

Evaluation

After conversion of dpm, percent fractional release is calculated for each fraction, using a Lotus program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue. "Spontaneous release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) values are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from the same animal.

Modifications of the Method

Release of Other Neurotransmitters

From Brain Tissue in Vitro

Several authors (Harms et al. 1979; de Belleroche and Gardiner 1982; James and Cubeddu 1984; Raiteri et al. 1984; Zahniser et al. 1986; Smith et al. 1984, 1994) studied the release of neurotransmitters from brain tissue in vitro.

Raiteri et al. (1974) described a simple apparatus for studying the release of neurotransmitters from synaptosomes.

De Boer et al. (1988) determined the release of noradrenaline and serotonin in synaptosomes from rat cerebral cortex.

Saijoh et al. (1985) studied the influence of hypoxia on release and uptake of the neurotransmitters dopamine and acetylcholine in guinea pig striatal slices.

Procedure

[^{3}H] Norepinephrine Release from Cortical Slices

Cortical slices (0.4 mm) from male Wistar rats are preincubated in Krebs buffer saturated with 95 % O_2/5 % CO_2, pH 7.4 for 30 min at 35 °C, and then incubated in fresh buffer containing 25 nM [^{3}H] NE (35 Ci/mmol) for 30 min at 35 °C. The slices are then placed in glass superfusion chambers containing platinum electrodes and perfused at 0.75 ml/min. Fractions are collected at 7 min intervals. Slices are electrically stimulated with unipolar pulses (15–30 mA) of 2 ms duration at 5 Hz for 60 s. Two rounds of stimuli are applied, separated by ten fractions. Test compound is applied at fraction 14 (28 min after the first stimulation).

The fractions collected are counted for tritium in 10 ml of Liquiscint scintillation fluid and corrected for quench. For measurement of remaining tritium, slices are dissolved overnight in 0.5 ml of Protosol, buffered with 1 ml of Tris HCl, and counted. Percent fractional release is defined as the ratio of tritium released versus the amount present in the tissue.

[^{3}H] Norepinephrine Release from Cortical Synaptosomes

Cortices from male Wistar rats are homogenized in 9 volumes of 0.32 M sucrose in a Potter–Elvehjem homogenizer and then centrifuged at 1,000 *g* for 10 min at 4 °C. The supernatant is recentrifuged at 17,000 *g* for 20 min, and the pellet is resuspended in 0.32 M sucrose at the original volume.

The freshly prepared synaptosomes are incubated with 50 nM [^{3}H]NE in Krebs buffer containing 10 mM pargyline for 10 min at 37 °C. The ratio of buffer to tissue suspension is 80:20. The [^{3}H]NE-loaded synaptosomes are then separated by centrifugation (17,000 *g* for 20 min), washed with buffer containing pargyline, recentrifuged, and then finally resuspended in 0.32 M sucrose at their original volume. The assay mixture consists of 900 ml Krebs buffer containing 10 mM pargyline, 100 ml of [^{3}H]NE-loaded synaptosomes, and 10 ml of vehicle or drug. This mixture is then vortexed and incubated for 5 min at either 37 °C or 0 °C (to define total versus nonspecific release). After a 10 min centrifugation (3,000 *g*), the pellets are solubilized in a Triton X-100/ethanol mixture and transferred to scintillation vials and counted in 10 ml of Liquiscint.

The net disintegrations for the 37 °C and 0 °C incubations are calculated, and % increase values determined as[(control–drug)/control] × 100.

Several chemical methods to measure acetylcholine (Israël and Lesbats 1982; Damsa et al. 1985; Stadler and Nesselhut 1986) or catecholamines and serotonin (Wagner et al. 1979; Magnusson et al. 1980; Nielsen and Johnston 1982; Wagner et al. 1982) are available.

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[^{3}H]-Oxotremorine-M Binding to Muscarinic Cholinergic Receptors in Rat Forebrain

Purpose and Rationale

The muscarinic receptors are members of the superfamily of G protein-coupled receptors. They are relatively abundant and mediate the diverse action of acetylcholine in the CNS, as well as throughout nonnervous tissues innervated by the parasympathetic nervous system. Five separate genes (m1–m5) encode muscarinic receptor proteins exhibiting the rhodopsin-like structural motif containing seven transmembrane domains. They show strong sequence homology with each other and with related G protein-coupled receptors within the transmembrane spanning domains, but each receptor has unique amino acid sequences located at the extracellular amino end and in the third intracellular loop (Caulfield 1993; Jones 1993; McKinney 1993; Wess 1996).

The purpose of this assay is to determine the binding affinity of potential cholinomimetic drugs for muscarinic receptors in brain, using an agonist ligand.

Oxotremorine is a potent centrally and peripherally acting muscarinic cholinergic agonist (Chao et al. 1962; Bebbington et al. 1966), which has been shown to be active in isolated tissue preparations as well as in vivo (Ringdahl and Jenden 1983). Both central and peripheral effects of oxotremorine are blocked by antimuscarinic drugs such as atropine (Chao et al. 1962;Bebbington et al. 1966). Structural modification of the oxotremorine molecular yields compounds which are full agonists, partial agonists, and antagonists at muscarinic receptors (Ringdahl and Jenden 1983). Oxotremorine-M (oxo-M), a quaternary nitrogen analog of oxotremorine, is a full agonist for the phosphatidylinositol response, while oxotremorine is a partial agonist (Fisher et al. 1984). Both oxotremorine and oxo-M are full agonists for inhibition of adenylate cyclase (Ehlert 1985; Olianas et al. 1983; Brown and Brown 1984). Of the muscarinic agonists, oxotremorine is the most potent inhibitor of [^{3}H]QNB binding; however, the IC_{50} is still only in the micromolar range. The apparent low affinity of agonist competition for [^{3}H]-antagonist binding sites is a common phenomenon and is due to the existence of multiple agonist affinity states of the receptor, as described by Birdsall et al. (1978). For this reason, it is desirable to use an agonist ligand to measure the binding affinities of potential agonists.

Molecular methods have disclosed the existence of five muscarinic receptors which are coupled to different second messenger systems. At least four of them are expressed as functional receptor proteins in the neocortex and hippocampus formation (McKinney and Coyle 1991).

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.4
   66.1 g of Tris HCl
   9.7 g of Tris base
   q.s. to 1 l with deionized water
2. 0.05 M Tris buffer, pH 7.4
   (tenfold dilution of reagent 1)
3. [Methyl-^{3}H]-oxotremorine acetate (specific activity 83–85 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations, [^{3}H]Oxotremorine-M is made up to a concentration of 100 nM. Fifty µl of this solution is added to each assay tube (yields a final concentration of 5 nM).

4. Atropine sulfate is obtained from Sigma Chemical Company. A 2 mM stock solution is made up in distilled water. Twenty µl is added to three tubes for determination of nonspecific binding (yields a final concentration of 40 µM).
5. A 0.5 % (w/v) solution of polyethyleneimine is prepared in distilled water. GF/C filters are soaked in this solution for at least three hours at room temperature. This is done to reduce the binding of the ligand to the filter strips.
6. Test compounds: For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-4} to 2×10^{-7} M.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The forebrains (all tissue forward of a vertical cut in front of the hypothalamus) are weighed (400–500 mg each) and homogenized in 10 volumes of 0.05 M Tris buffer, pH 7.4 (reagent 2), using a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 1,000 *g* for 10 min. The supernatant is then centrifuged at 50, 000 *g* for 60 min. The supernatant from this centrifugation is discarded and the pellet resuspended in the original volume of 0.05 M Tris buffer, pH 7.4, on the Polytron to 100 mg/ml (wet weight). Specific binding is roughly 1 % of total added and 50 % of total bound.

Binding Assay

50 µl	0.5 M Tris buffer, pH 7.4 (reagent 1)
380 µl	H_2O
20 µl	Drug or 2 mM atropine
50 µl	^{3}H-Oxotremorine-M (reagent 3)
500 µl	Tissue

Tubes are vortexed and incubated at 30 °C for 45 min (2). Bound [^{3}H]-oxotremorine-M is captured by filtration under reduced pressure. The incubation mixture is diluted with approximately 4 ml ice-cold 0.05 M Tris buffer, pH 7.4 (reagent 2), then exposed to vacuum, and tubes washed once more with approximately 5 ml of reagent 2. The filters (GFC in 0.5 % polyethyleneimine for more than 3 h, reagent 5) are then counted in 10 ml Liquiscint scintillation fluid.

Evaluation

Specific binding is the difference between total bound (in presence of vehicle) and that bound in the presence of 40 µM atropine. Percent inhibition of specific [^{3}H]-oxotremorine-M is calculated for each concentration of test drug and IC_{50} values determined by computer-derived log–probit analysis. The percent inhibition at each drug concentration is the mean of duplicate or triplicate determinations. Some day-to-day variability is present in this assay, and IC_{50} values should be confirmed by repeat analysis.

Modification of the Method

[^{3}H]Pirenzepine has been used to identify muscarinic receptor subtypes in the brain (Watson et al. 1983a, b).

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[^{3}H]*N*-Methylscopolamine Binding in the Presence and Absence of Gpp(NH) p

Purpose and Rationale

G protein-linked muscarinic receptors are converted by guanine nucleotides from a high-affinity binding state to a low-affinity binding state for muscarinic agonists (Gilman 1986), while the binding of muscarinic antagonists to the receptor is not affected. The effects of guanine nucleotides on muscarinic agonist affinity are brain region and temperature dependent (Aronstram and Narayanan 1988).

Therefore, incubation of cerebellar membranes with 50 μM 5′-guanylylimidophosphate (Gpp(NH) p), the non-hydrolyzable analog of GTP, causes a shift to the right (decreased affinity) of the muscarinic agonist inhibition curves when ^{3}H-NMS is used as the ligand.

The assay differentiates the interaction of muscarinic agonists and muscarinic antagonists with ^{3}H-N-me-thylscopolamine (^{3}H-NMS)-labeled receptors in cerebellar tissue based on the selective effect of guanine nucleotides on the affinity of muscarinic agonists for the receptor.

Procedure

The procedure is based on ^{3}H-NMS rat brain binding assay described by Aronstram and Narayanan (1988).

Reagents

1. 0.5 M Tris–HCl buffer, pH 7.4
2. 0.05 M Tris–HCl buffer, pH 7.4
3. 0.05 M Tris–HCl buffer, pH 7.4 + 2 mM $MgCl_2$
4. 0.05 M Tris–HCl buffer, pH 7.4 + 2 mM $MgCl_2$ + 100 μM phenylmethylsulfonyl fluoride
5. Atropine sulfate is made up to 1 mM in distilled water, and 20 μl is added to a 2 ml reaction mixture. This yields a final concentration of 10 μM. Atropine is used for nonspecific binding.
6. 5′-Guanylylimidodiphosphate (Gpp(NH) p) is made up to 2 mM in distilled water. The final concentration in the reaction mixture is 50 μM.
7. ^{3}H-N-Methylscopolamine (NMS) is obtained from Amersham and diluted to 4 nM in distilled water. The final concentration in the reaction mixture is 0.1 nM.
8. Test compounds

   For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-4} to 10^{-7} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cerebella are dissected, weighed, and homogenized in ten volumes of 0.05 M Tris–HCl buffer, pH 7.4 + 2 mM $MgCl_2$ + 100 μM phenylmethylsulfonyl fluoride (buffer 4), using a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 20, 000 *g* for 20 min. The pellet is resuspended in ten volumes of 0.05 M Tris–HCl buffer + 2 mM $MgCl_2$ (buffer 3).

Binding Assay

1,000 μl	0.05 M Tris buffer + 2 mM $MgCl_2$
780 μl	H_2O
20 μl	Vehicle or 1 mM atropine or appropriate drug concentration
50 μl	H_2O or Gpp(NH) p
50 μl	[^{3}H]NMS
100 μ	Tissue suspension

Tubes are incubated at 20 °C for 90 min. Bound [^{3}H]NMS is captured by vacuum filtration. The filters are washed three times with 5 ml aliquots of 0.05 M Tris buffer, pH 7.4. Filters are counted in 10 ml Liquiscint scintillation fluid.

Evaluation

Specific binding of [^{3}H]NMS is the difference between total bound (in the presence of vehicle) and that bound in the presence of 1 mM atropine. Percent inhibition of specific [^{3}H]NMS binding is calculated for each concentration of test drug, and IC_{50} values are determined by computer-derived log–probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

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Stimulation of Phosphatidylinositol Turnover in Rat Brain Slices

Purpose and Rationale

The purpose of this assay is to determine the ability of test compounds to stimulate the turnover of phosphatidylinositol (PI) in brain tissue. This assay can be used to determine agonist activity at a number of CNS receptors known to be linked to the PI response. A major interest is the evaluation of muscarinic cholinergic receptors.

Receptor-activated hydrolysis of inositol phospholipids is now recognized as an important second messenger system for muscarinic, alpha-adrenergic, histaminergic, serotonergic, excitatory amino acid and various neuropeptide receptors (Berridge and Irvine 1984; Nahorski et al. 1986; Fisher and Agranoff 1987). Furthermore, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) yields at least two important biologically active intermediates (Hirasawa and Nishizuka 1985; Berridge 1987). These include 1,4,5-inositol trisphosphate (IP_3), which acts to mobilize Ca^{2+} from the endoplasmic reticulum and diacylglycerol, which activates protein kinase C (PKC). These responses are associated with many cellular responses such as stimulus-secretion coupling, stimulus-contraction coupling, and cell proliferation. The exact mechanism for receptor-mediated turnover of cell membrane PI is not well understood, but it seems to involve coupling through a G protein (Cockcroft and Gomperts 1985) and requires extracellular Ca^{2+} to activate phospholipase C (Fisher et al. 1989).

Although the muscarinic receptor-PI link has been known for some time (Hokin and Hokin 1955), recent advances in knowledge of receptor mechanisms and increased interest in the muscarinic receptor have stimulated considerable research in this area. Even though muscarinic agonists can be shown to be weak partial agonists or full agonists for this response (Fisher et al. 1983), there are brain regional differences in sensitivity (Fisher and Bartus 1985); attempts to show receptor subtype selectivity for stimulation of PI turnover in either heart (Brown et al. 1985) or brain tissue (Fisher and Bartus 1985) have been disappointing. However, recent experiments using cells transfected with genomic clones for the various muscarinic receptors have had more success (Shapiro et al. 1988; Conklin et al. 1988). These studies show that the m_1, m_3, and the m_5 receptor subtypes are linked to PI turnover.

Stimulation of PI turnover by agents such as veratridine, batrachotoxin, and ouabain (Gusovsky et al. 1986) shows that there are also non-receptor mechanisms that can cause the stimulation of PI turnover.

Procedure

Equipment and Materials

1. McIlwain tissue slicer
2. Disposable columns (Kontes, 200 mm)
3. Column rack (Kontes)
4. Disposable screw-cap tubes (Pyrex, 16 × 100 mm)
5. Disposable culture tubes (Fisher, 16 × 125 mm)

Reagents

1. Modified Krebs bicarbonate buffer.

	g/l	mM
NaCl	8.30	142.0
KCl	0.42	5.6
$CaCl_2$	0.24	2.2
$NaHCO_3$	0.30	3.6
$MgCl_2 \cdot 6H_2O$	0.20	1.0
HEPES	7.15	30.0
Adjust pH to 7.4 with NaOH		
D-Glucose[a]	1.01	5.6

[a]Glucose added just before incubation

2. Concentrated Krebs buffer + LiCl (ninefold concentrated ions, tenfold concentrated Li^+), stock solution contains no glucose or Ca^{2+}.

	g/100 ml
NaCl	6.88 (amount adjusted to correct for Li^+)
KCl	0.38
$NaHCO_3$	0.27
$MgCl_2 \cdot 6H_2O$	0.18
HEPES	6.44
LiCl	0.42

CaCl2 (11 mg) and D-glucose (45 mg) are added to 5 ml of concentrated buffer before incubation.

3. Dowex AG-1-X8 (100–200 mesh) formate form (Biorad).
4. [^{3}H]Inositol (spec. act. 15 Ci/mmol) is obtained from American Radiolabeled Chemicals, Inc.
5. Myoinositol (M.Wt. 180.2) is obtained from Sigma Chemical Co. A 5 mM solution is made (0.9 g/l).
6. 1 M ammonium formate/0.1 M formic acid. Ammonium formate is obtained from Sigma Chemical Co.: 3.85 ml 99 % formic acid + 63.1 g ammonium formate to 1 l in H_2O.
7. $CHCl_3$/methanol (1:2, v/v).

Tissue Preparation

Male Wistar rats, approximately 6/assay.

1. Remove surface blood vessels by rolling brain on filter paper.
2. Remove cerebral cortex and gently scrape myelin layer off.
3. Prepare 350 × 350 μm tissue slices with the Mcllwain tissue chopper and place slices in buffer at 37 °C.

   Incubate for 10 min.
4. Disperse slices by aspirating into 1 ml pipette (cut-off tip).
5. Allow slices to settle, aspirate supernatant, add buffer, and repeat until the supernatant is clear.

Assay: Prelabeled Method

1. Add to screw-cap tubes:

50 μl	Tissue slices
350 μl	[^{3}H]Inositol

2. Incubate for 90 min at 37 °C under O2
3. Add:

50 μl	Drug
H_2O	To a final volume of 500 μl

4. Incubate for 30 min at 37 °C under O_2.
5. Stop the reaction by the addition of 1.5 ml of $CHCl_3$: MeOH. Place in ice bath.

Assay: Continuous Labeling Method

1. Add to screw-cap tubes:

50 μl	Tissue slices
350 μl	[^{3}H]Inositol
50 μl	Drug
H_2O	To a final volume of 500 μl

2. Incubate for 120 min. at 37 °C under O_2.
3. Stop the reaction by the addition of 1.5 ml of $CHCl_3$: MeOH. Place in ice bath.

For antagonist inhibition studies, the antagonist is usually preincubated with the slices before the agonist is added.

Extraction of Total [^{3}H]Inositol Phosphates

1. Add 1 ml of $CHCl_3$ and 0.5 ml of H_2O to each tube. Cap and vortex.
2. Centrifuge at 3,000 rpm for 10 min.
3. Aspirate the aqueous phase and place in culture tubes.
4. Add 1.7 ml of H_2O and heat in water bath at 55 °C for 20 min.
5. Place samples in cold room overnight.
6. Add 0.5 ml of Dowex 50 % slurry to each tube. Vortex 4 times.
7. Centrifuge at 3,000 rpm for 10 min.
8. Aspirate.
9. Add 2.5 ml of 5 mM myoinositol, let the resin settle, and aspirate. Repeat 5 times.
10. Centrifuge at 3,000 rpm for 10 min.
11. Add 1 ml of ammonium formate/formic acid, pH 4.8.
12. Put 0.7 ml of supernatant into scintillation vial, add 10 ml cocktail, and count.

Evaluation

The stimulation phosphatidylinositol turnover for each test compound is calculated as percent increase in total [^{3}H]inositol phosphates relative to the basal turnover rate of non-treated control brain slices. The EC_{50} values for agonists are determined by log–probit analysis of these data. IC_{50} values for antagonists are determined by log–probit analysis of the percent inhibition of stimulation by a full agonist.

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[^{3}H]*N*-Methylcarbamylcholine Binding to Nicotinic Cholinergic Receptors in Rat Frontal Cortex

Purpose and Rationale

Nicotinic acetylcholine receptors are a family of ligand-gated ion channels that are classified on the

basis of their activation by nicotine, although acetylcholine is the endogenous ligand. These conductance channels for Ca^{2+}, K^+, and Na^+ are pentameric in structure. They are members of a supergene family that also includes glycine, $GABA_A$, and 5-HT3 receptors. α, β, γ, and δ subunits constitute a pentameric neuronal receptor resulting in various receptor subtypes (Alkondon and Albuquerque 1993; Sargent 1993). Ten α ($\alpha 1 - \alpha 10$) and four β ($\beta 1 - \beta 4$) subunits have been cloned from mammalian and avian sources, each of which has a structural motif of four transmembrane spanning domains M1–M4 of which M2 lines the channel (Gotti et al. 1997; Jensen et al. 2005).

Nicotinic cholinergic receptors are classified as ligand-gated ion channels (Le Novere et al. 2002; Jensen et al. 2005) and are found in skeletal muscle, autonomic ganglia, and brain tissue. Nicotine itself has a variety of behavioral effects. Due to its rapid desensitization of the receptor, both stimulatory and depressant effects may result. Also, many of nicotine's effects are thought to be associated with release of neurotransmitter substances (Balfour 1982). Nicotine functions as a nicotinic cholinergic receptor agonist in the CNS and is thought to play a role in learning and memory (Clarke 1987; Levey 1996; Dajas-Bailador and Wonnacott 2004). Reductions in nicotinic binding sites were found in postmortem tissues from Alzheimer's patients by four separate groups of investigators (Whitehouse et al. 1988; Nordberg and Winblack 1986; Araujo et al. 1988; Shimohama et al. 1986). Neuronal nicotinic acetylcholine receptors play a role in acute and chronic neurodegeneration (O'Neill et al. 2002).

Unconventional ligands and modulation of nicotinic receptors are discussed by Pereira et al. (2002).

The structure and function of the acetylcholine-binding protein (ACHB), a homologue of the ligand-binding domain of the nicotinic acetylcholine receptor, are described by Smit et al. (2003).

Unwin (2003) explored structure and action of the nicotinic acetylcholine receptor by electron microscopy.

Cognitive improvement in Alzheimer patients 30 min after a nicotine infusion was reported (Sunderland et al. 1988). Therefore, nicotinic agonists may prove beneficial (Hogg and Bertrand 2004); however, clinical data are still quite limited. *N*-Methylcarbamylcholine (NMCC) is a nicotinic agonist which binds specifically and with high affinity to central nicotinic receptors and, like nicotine, causes an increase of acetylcholine release from certain cholinergic nerve terminals (Araujo et al. 1988; Lapchak et al. 1989). In addition, chronic nicotine treatment increases [^{3}H] NMCC binding sites in several rat brain regions, as it does with [^{3}H]nicotine- and [^{3}H]ACh-labeled sites (Lapchak et al. 1989). This is due to the loss of presynaptic nicotinic autoreceptor function (Lapchak et al. 1989). Pharmacological results reveal that along with its specificity and high affinity, [^{3}H]NMCC is selectively displaced by agonists (Araujo et al. 1988; Lapchak et al. 1989), making it a desirable ligand to screen for potential agonistic compounds.

The purpose of the following assay is to determine the binding affinity of potential nicotinic cholinergic agonists in brain, using an agonist ligand.

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.7.
   (a) 57.2 g Tris HCl
   16.2 g Tris Base
   q.s. to 1 l with distilled water
   (b) Make a 1:10 dilution in distilled H_2O (0.05 M Tris buffer, pH 7.7 at 25 °C).
2. Tris buffer containing physiological ions.
   (a) Stock buffer
   NaCl 7.014 g
   KCl 0.372 g
   $CaCl_2$ 0.222 g
   $MgCl_2$ 0.204 g q.s. to 100 ml with 0.5 M Tris buffer
   (b) Dilute 1:10 in distilled H_20.
   This yields 0.05 M Tris HCl, pH 7.7, containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (1 mM).
3. Methylcarbamoylcholine iodide ([*N*-Methyl-^{3}H]) is obtained from New England Nuclear.
   For IC_{50} determinations, [^{3}H]-NMCC is made up to a concentration of 100 nM in

distilled H_2O and 50 µl is added to each tube (yields a final concentration of 5 nM in the 1 ml assay).

4. (−) Nicotine ditartrate is obtained from Research Biochemicals Incorporated.

   A stock solution of (−) nicotine ditartrate is made up to a concentration of 0.5 mM in distilled H_2O. Twenty µl of stock is added to three tubes for the determination of nonspecific binding (yields a final concentration of 10 µM in the assay).
5. Test compounds. Most assays in 1 mM stock solution are made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay, and higher or lower concentrations may be used depending on the potency of the drug.
6. A 0.5 % (w/v) solution of polyethyleneimine is prepared in distilled H_2O. GF/B filters are soaked in this solution for at least four hours at 4 °C. This is done to reduce binding of the ligand to the filter strips.

Tissue Preparation

Male Wistar rats are decapitated and their frontal cortices removed, weighed, and homogenized in 40 volumes of ice-cold 0.05 M Tris buffer, pH 7.7 (1 b). The homogenate is centrifuged at 48, 000 *g* for 10 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 48,000 *g* for 10 min two more times. The final pellet is resuspended in the original volume of buffer, but with physiological salts (2b). This yields a final tissue concentration of 20 mg/ml in the assay.

Assay

130 µl	0.05 M Tris pH 7.7-physiological salts (2 b)
20 µl	Vehicle (for total binding) or 0.5 mM (−) nicotine ditartrate (for nonspecific binding) or appropriate drug concentration
50 µl	*N*-[^{3}H]Methylcarbamylcholine stock solution
800 µl	Tissue

The tubes are incubated at 0 °C for 60 min. The assay is stopped by rapid filtration through Whatman GF/B filters which are then washed four times with 3 ml of ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of the 10 µM (−) nicotine ditartrate. Specific binding is about 1 % of the total added ligand and 60–70 % of the total bound ligand. IC_{50} calculations are performed using log–probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

Modification of the Method

Pabreza et al. (1991) recommended [^{3}H]cytisine as a useful ligand for studying neuronal nicotinic receptors because of its high-affinity and low-affinity nonspecific binding.

Badio and Daly (1994) determined [^{3}H]nicotine receptor binding in rat cerebral cortex membrane preparations. The authors concluded that the analgesic activity of epibatidine, an alkaloid originally characterized from frog skin, is due to its activity as nicotinic agonist.

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Uncompetitive NMDA Receptor Antagonism

Purpose and Rationale

The uncompetitive NMDA antagonist memantine with moderate affinity to the (+) MK801-binding site has shown positive effects in the treatment of dementia (Möbius et al. 2004; Sonkusare et al. 2005). Several studies were performed to elucidate the mode of action (Frankiewicz and Parsons 1999; Parsons et al. 1999, 2004; Ikonomidou et al. 2000; Linden et al. 2001; Blanpied et al. 2005; Chen and Lipton 2005; Losi et al. 2006; Volbracht et al. 2006; Zoladz et al. 2006).

Sobolevsky and Koshelev (1998) and Sobolevsky et al. (1998, 1999) studied the blocking effects of memantine in open *N*-methyl-D-aspartate channels.

Procedure

Pyramidal neurons were acutely isolated from the CA-1 region of rat hippocampus using "vibrodissociation techniques" (Vorobjev 1991). The experiments began after 3 h of incubation of the hippocampal slices in a solution containing (in mM) NaCl, 124; KCl, 3;$CaCl_2$, 1.4; $MgCl_2$, 2; glucose, 10; and $NaHCO_3$, 26. The solution was bubbled with carbogen at 32 °C. During the whole period of isolation and current recording, nerve cells were washed with a Mg^{2+}-free solution containing 3 μM glycine (in mM: NaCl, 140; KCl, 5;$CaCl_2$, 2; glucose, 15; and HEPES, 10, pH 7.3). Fast replacement of superfusion solutions was achieved by using the concentration jump technique (Benveniste et al. 1990; Vorobjev 1991) with one application tube. This technique allows substitution of the tubular solution for the flowing solution with a time constant <30 ms but backward with the time constant of 30–100 ms (Sobolevsky 1999). Therefore, except where noted, the rate of the solution exchange was fast at the beginning of any application and slightly slower at its termination. The currents were recorded at 18 °C in the whole-cell configuration using micropipettes made from Pyrex tubes and filled with an "intracellular" solution (in mM: CsF, 140; NaCl, 4; and HEPES, 10; pH 7.2). Electrical resistance of the filled micropipettes was 3–7 MΩ. Analog current signals were digitized at 1 kHz frequency.

Evaluation

Statistical analysis was performed using the scientific and technical graphics computer program Microcal Origin (version 4.1 for Windows). The data presented are mean ± SE; comparison of the means was done by ANOVA, with $p < 0.05$ taken as significant.

Modifications of the Method

Maskell et al. (2003) reported inhibition of human α7 nicotine acetylcholine receptors by open channel blockers of *N*-methy-D-aspartate receptors. Human α7 nicotine acetylcholine receptors were expressed in *Xenopus* oocytes and the effects of the NMDA receptor open channel blockers memantine and cerestat on this receptor were examined using two-electrode voltage-clamp recordings and the ^{125}I-labeled α-bungarotoxin binding.

Aracava et al. (2005) found that memantine blocks α7* nicotinic acetylcholine receptors more potently than *N*-methyl-D-aspartate receptors in rat hippocampal neurons. Primary hippocampal cultures were from 16- to 29-day-old

fetal rats. Electrophysiological recordings were obtained from cultured neurons by means of the whole-cell mode of the patch-clamp technique. Atropine (1 μM) and tetrodotoxin (0.1–0.3 μM) were added to the external solution to block muscarinic receptors and voltage-gated Na^+ channels, respectively. The agonist solutions, with or without memantine, were applied to the neurons through a glass U-tube. Memantine was added at various concentrations to the bathing solution.

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Secretion of Nerve Growth Factor by Cultured Neurons/Astroglial Cells

Purpose and Rationale

Cultured brain cells can be used for many purposes such as investigation of synthesis and secretion of nerve growth factor or for testing neuroprotective drugs (Peruche and Krieglstein 1991). Nerve growth factor is required for the development and maintenance of peripheral and sensory neurons (Thoenen and Barde 1980). Nerve growth factor (NGF) prevents neuronal death after brain injury (Hefti 1986; Williams et al. 1986; Kromer 1987), especially in basal forebrain nuclei involved in memory processes. Drug-induced increase in nerve growth factor secretion may be beneficial in primary degenerative dementia.

NGF belongs to the family of neurotrophins which includes, besides the nerve growth factor, the neurotrophin 3 (NT3), neurotrophin4/5 (NT 4/5), and brain-derived neurotrophic factor (BDNF). Two types of neurotrophin transmembrane receptors are known: (1) a receptor termed p75, which is common to all neurotrophins, and (2) a family of neurotrophin receptor tyrosine kinases trkA, trkB, and trkC (Saragovi and Gehring 2000). TrkA is the receptor tyrosine kinase for NGF (Huang and Reichardt 2003).

NGF has a crucial role in the generation of pain and hyperalgesia in several acute and chronic pain states (Hefti et al. 2006).

Procedure

Whole brains of 8-day-old mice (ICR) are dissected out and cut into small pieces. The pieces are washed with calcium- and magnesium-free phosphate-buffered saline, treated with 0.25 % trypsin at 37 °C for 30 min, and triturated with a Pasteur pipette. The excess trypsin is removed by centrifugation at 200 *g* for 5 min. The cells or cell clumps from one brain are cultured in a culture bottle with Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10 % fetal calf serum (FCS), 50 milliunits/ml penicillin, and 50 mg/ml of streptomycin at 37 °C in a humid atmosphere of 5 % CO_2 for 1–2 weeks with medium changes every 3 days. After confluence is reached, the cells in each bottle are dissociated by trypsin treatment and recultured in new bottles. This procedure is repeated three times. The culture becomes composed of morphologically uniform cells.

Preparation of quiescent cells is performed by inoculating into 96-well plates and culturing in FCS-containing DMEM until confluence is reached. Then, the cells are cultured for an additional week in FCS-free DMEM containing 0.5 % BSA, with medium changes every 3 days. Because the cells never proliferate in FCS-free medium, most of the cells are arrested in the quiescent phase. Then, the medium is changed to DMEM containing 0.5 % BSA with or without drugs, and the cells are cultured for 24 h.

Nerve growth factor (NGF) content in the culture medium is determined by a two-site enzyme immunoassay (Furukawa et al. 1983; Lärkfors and Ebendal 1987). Mouse βNGF isolated from male mouse submaxillary glands is purified by CM-Sephadex C-50 chromatography. Antiserum to this mouse βNGF is produced in New Zealand white rabbits by repeated subcutaneous injections of an emulsion in complete Freund's adjuvant over 18 months. Immunoglobin G is prepared from anti-mouse βNGF antiserum by Sepharose chromatography. Antibody IgG is incubated with pepsin and chromatographed. Fab fragments are coupled to β-D-galactosidase. IgG-coated solid phase is prepared in polystyrene tubes. The IgG-coated polystyrene tubes are incubated with 0.25 ml buffer containing various amounts of NGF with gentle shaking. After incubation for

18–24 h at 4 °C, each tube is washed twice with 1 ml of buffer, and 0.13 milliunits of the Fab-β-D-galactosidase complex in 0.25 ml buffer is added. After incubation for 18–24 h at 4 °C with gentle shaking, each tube is washed as described above, and β-D-galactosidase activity bound to the tube is assayed. The enzyme reaction is started by addition of 60 mM 4-methylumbelliferyl-β-D-galactoside and 0.1 % Triton X-100 in 0.25 ml buffer. After 1 h incubation at room temperature, the enzyme reaction is stopped by the addition of 1.25 ml 0.1 M glycine-NaOH buffer (pH 10.3). The amounts of 4-methylumbelliferone formed are measured by fluorometry (excitation wavelength 360 nm, emission wavelength 450 nm).

Evaluation

Time–response curves of release of NGF into the medium are established after addition of drug and compared with controls. Dose–response curves can be prepared after addition of various amounts of test drug.

Modifications of the Method

Cultured neurons from chick embryo hemispheres were used for testing cerebroprotective drug effects in vitro and for testing antihypoxic drug effects by Krieglstein et al. (1988), Peruche et al. (1990), and Oberpichler-Schwenk and Krieglstein (1994).

Semkowa et al. (1996) found that clenbuterol protects mouse cerebral cortex and rat hippocampus from ischemic damage and attenuates glutamate neurotoxicity in cultured hippocampal neurons by induction of nerve growth factor.

Prehn et al. (1993, 1995) tested the prevention of glutamate neurotoxicity in neocortical cultures from rats. Mixed neuronal/glial primary cultures were derived from the cerebral cortices of neonatal Fischer 344 rats. Excitotoxic injury was induced after 14 days by L-glutamate following a procedure described by Choi et al. (1988) and Koh and Choi (1988).

Kinoshita et al. (1991) used primary cultured neurons from 17-day-old rat fetuses.

Qi et al. (1997) and Horton et al. (2001) described a novel catecholaminergic CAD CNS neuronal cell line in which neurotrophin-3 mediates the autocrine survival.

Shinpo et al. (1999) used cultured mesencephalic neurons from embryonic Sprague Dawley rats to study the protective effects of the TNF-ceramide pathway against glutamate neurotoxicity.

Matsumoto et al. (1990) described a method for quantifying the effects of neurotrophic factors on the number of surviving neurons and the total length of neurites in primary cultures from cerebral cortex and hippocampus of the brains from 2-week-old rats by using digital image processing techniques. Binary images of neuronal neurites were extracted from gray images of cultured neurons stained with Coomassie brilliant blue.

White et al. (1995) measured calcium transients in mouse cerebellar granule cells with the Ca^{2+}-sensitive probe indo-1/AM.

Beresini et al. (1997) developed two types of high-throughput assays to identify small molecules that interact with neurotrophin receptors. The first, the receptor binding assay, is a competitive binding assay that uses a recombinant receptor fusion protein and biotinylated neurotrophin. This assay detects compounds that inhibit neurotrophin binding to the receptor; these compounds may be either agonistic or antagonistic. The second assay, the kinase receptor activation ELISA, detects receptor autophosphorylation in response to sample or neurotrophin stimulation of receptor-transfected cells. Receptor autophosphorylation is evaluated by analyzing lysates of the stimulated cells in a receptor-specific ELISA for phosphotyrosine residues. This assay is bioactivity based and, consequently, has the power of detecting as well as distinguishing receptor agonists and antagonists.

Höglinger et al. (1998) used free-floating roller tube cultures prepared from embryonic day 14 rat ventral mesencephalon to study the influence of brain-derived neurotrophic factor treatment on dopamine neuron survival and function.

Nerve growth factor is crucial for survival of nociceptive neurons during development. Shu and Mendell (1999) investigated the acute effects of NGF on capsaicin responses of small-diameter dorsal root ganglion cells in culture.

For further studies with brain cell cultures, see ▶ Anti-Epileptic Activity.

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Inhibition of Respiratory Burst in Microglial Cells/Macrophages

Purpose and Rationale

Activated phagocytes can produce large amounts of oxygen intermediates that result from a process during which NADPH oxidase reduces O_2 to the superoxide anion (O_2^-), which subsequently dismutates together with H^+ to H_2O_2 and O_2. H_2O_2 is then reduced to hypochlorous acid by myeloperoxidase (Bellavite 1988). The cascade of metabolic steps is known as respiratory bursts. The intracellular formation of reactive oxygen intermediates can be determined by measuring the oxidation of the membrane permeable and no-fluorescent dihydrorhodamine 123 to the cationic and intracellularly trapped, green fluorescent rhodamine 123 in single viable cells. Microglial/brain macrophage-mediated damage in the central nervous system is accompanied by an increased production of free radicals, which also seems important in primary degenerative dementia (Alzheimer's disease) (Banati et al. (1993). Inhibition of this process by drugs may indicate therapeutic value in Alzheimer's disease.

Procedure

Cell Culture

Cultures of newborn rat brain are prepared as described by Guilian and Baker (1986); Frei et al. (1987). Isolated cerebral cortices from newborn albino rats are stripped of the meninges, minced in culture medium, and dissociated by trituration for 2 h in 0.25 % trypsin solution. Cells are plated in 75 cm^2 plastic culture flasks containing 10 ml medium with 10 % fetal bovine serum at a density of 85, 000 cells/ml. After 7 days, confluent cultures are vigorously agitated on a rotary shaker

at 37 °C for 15 h. Glial fibrillary acid protein-positive astroglia remain adherent to the flasks. The resulting cell suspension, rich in amoeboid microglia and oligodendroglia, is placed in plastic flasks and allowed to adhere at 37 °C. After a 1–3 h adhering interval, loosely adhering and suspended cells (most of which are oligodendroglia) are removed by gently shaking the flasks at room temperature. The strongly adherent microglia cells are then released by vigorous shaking in medium with 0.2 % trypsin. Once the majority of microglia is suspended, fetal bovine serum is added (15 % final volume), and the cell suspension added to new flasks. After a second 1–3 h interval to allow adhesion, the medium is removed, and adhering microglia are suspended using trypsin. Final preparation shows a nearly homogeneous population of nonspecific esterase-positive cells.

Peritoneal macrophages are obtained from 12-week-old male Wistar rats.

For flow cytometric measurement, cells are suspended (3–4 $\times$ 10^6 cells/ml) in Hank's buffered saline (HBS; Sigma Chemie, Deisenhofen, Germany) supplemented with N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (HEPES;5 mM, pH 7.35; Serva Feinbiochemica, Heidelberg, Germany) (HBS-HEPES) and stored at 4 °C for a maximum of 2 h.

Flow Cytometric Measurement of Respiratory Burst

Dihydrorhodamine 123 (DHR) is obtained from Molecular Probes (Eugene, OR, USA) and dissolved to obtain a 1 mM stock solution in *N*, *N*-dimethylformamide (DMF; Merck, Darmstadt, Germany).

The cellular suspensions of peritoneal macrophages and microglial cells (10 µl) are each further diluted with 1 ml HBS-HEPES and stained for 5 min at 37 °C with 10 µl of a 100 µM DHR solution in HBS (1 mM stock solution in DMF). The DHR-loaded cells are incubated with the test drug at various concentrations for 15, 25, 35, 45, and 60 min with and without Con A (100 µg/ml; Sigma Chemie) stimulation. The DNA of dead cells is counterstained with 10 µl of 3 mM propidium iodide (Serva Feinbiochemica) solution in HBS 3 min before the flow cytometric measurement. To exclude effects from a possible release of endogenous adenosine, control experiments with incubation medium containing adenosine deaminase (200 U/mg, 5 µg/ml Sigma Chemie) are performed.

The forward scatter, side scatter, and two fluorescences of at least 10,000 cells/sample are measured simultaneously on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Rhodamine 123 green fluorescence (515–545 nm) and propidium iodide red fluorescence (>650 nm) are measured with the light from an argon laser of 488-nm excitation wavelength.

Evaluation

The differences in respiratory burst activities caused by Con A stimulation and treatment with test drug are tested for significance by unpaired *t*-test. The *t*-test is performed for each time point including the data of at least four independent experiments. Each single fluorescence value of each experiment is based on the measurement of at least 10,000 cells. Before each experiment, the flow cytometer is calibrated with standardized yellow-green fluorescent microspheres of 4.3 µm diameter (Polysciences, St. Goar, Germany), thus ensuring the compatibility of the fluorescence values from different experimental series.

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Part VII

Effects on Peripheral Nervous System

Local Anesthetic Activity

Hans-Peter Hartung

Contents

Local Anesthetic Activity

General Considerations

One has to generally distinguish between conduction anesthesia, infiltration anesthesia, and surface anesthesia (Fromherz 1922; Schaumann 1938), and special pharmacological tests have been developed for each of these.

The mode of action of local anesthetics has been reviewed by Ritchie and Greengard (1966), Ritchie (1971), Borchard (1977), Steiner (1978), and Butterworth and Strichartz (1990).

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Conduction Anesthesia

Conduction Anesthesia in the Sciatic Nerve of the Frog

Purpose and Rationale

Based on earlier studies by Sollmann (1918), Fromherz (1922), Fußgänger and Schaumann (1931), and Bülbring and Wajda (1945) on plexus anesthesia in frogs, Ther (1953a, b, 1958) described a method for conduction anesthesia of the sciatic nerve in frogs.

Procedure

Frogs (*Rana temporaria*) of either sex are used and are kept at 4 °C. The frog is decapitated with a pair of scissors. The skin is incised in the thigh region at both sides and the sciatic nerves are carefully exposed in the thigh, avoiding any stretching and injury of the nerve. The frog is suspended on a vertical board. Small pieces of white cotton are soaked with different concentrations of the test preparations (between 0.05 % and 1 %) or the standard and placed gently around the sciatic nerve for 1 min. Then the cotton swab is removed and the frog is placed with its extremities into a bath with 0.65 % NaCl solution. This allows testing for duration and reversibility of the local anesthetic effect. One side is used for the test preparation and the other for the standard (e.g., 0.25 % butanilicaine). Every 3 min, the frog is removed from the bath and the toes of the legs or the ankle joint is pinched three times with a small forceps. The reflex contraction is abolished when conduction anesthesia is effective. The stimuli are repeated every 3 min until anesthesia vanishes. Two to five frogs are used for each concentration.

Evaluation

The time of onset and duration of anesthesia are recorded for each concentration. Time–response and dose–response curves can be established.

Modifications of the Method

In the original method by Bülbring and Wajda (1945), the frogs were decapitated and the upper part of the spinal cord was destroyed down to the level of the third vertebra. The viscera are removed exposing the lumbar plexus without damaging it. The frog was pinned to a vertical board and the solution of the local anesthetic dissolved in 0.7 % saline is put into the pocket formed by the lower abdomen. Different concentrations of HCl are used as stimuli into which the feet of the frog were immersed every minute.

Idänpään-Heikkilä and Guilbaud (1999) used a **rat** model of trigeminal neuropathic pain where the neuropathy is produced by a chronic constriction injury of the infraorbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain (see chapter "► Central Analgesic Activity").

For mechanical stimulation, a graded series of ten of von Frey filaments with a bending force between 0.217 and 12.5 g was used. The stimuli were applied within the infraorbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. Local injection of a local anesthetic (articaine)

into the rostral orbital cavity of the lesioned side, into the close proximity of the ligated infraorbital nerve, increased the mechanical threshold to the upper level. The duration of the effect was dose dependent.

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Conduction Anesthesia in the Sciatic Nerve of the Rat

Purpose and Rationale

Based on earlier studies (Truant 1958; Truant and Wiedling 1958/1959; Åström and Persson 1961), Camougis and Takman (1971) have written a detailed description for testing conduction anesthesia of the sciatic nerve in the rat.

Procedure

Male Wistar or Sprague–Dawley rats weighing 125–175 g are used. The animal is suspended in a prone position by grasping the base of the tail and thoracic cage. A hind limb is extended to its full length and the depression for needle insertion is located by palpation with the left index finger. The site of injection is the area under the skin at the junction of the biceps femoris and the gluteus maximus muscles. The sciatic nerve is blocked in the midthigh region with 0.2 ml of the drug solution administered by a 24–25-gauge needle attached to a 0.25-ml tuberculin syringe. Usually a 1 % solution of the test drug in 0.9 % NaCl is used as a test solution. The other leg is used for a control drug (e.g., procaine or lidocaine). Immediately after the injection, repeated checks of the digit of the foot and the walking behavior are performed. In the normal foot, the digits are wide apart, while in the blocked leg, the digits of the foot are close together. Also the successful block is evidenced by the dragging of the leg and an inability of the animal to use the leg in walking up the inclined wire mesh cover of the cage. After the time of block for each leg is noted, each animal is examined every 5–10 min in order to note the time of recovery.

Evaluation

From the data, averages for onset and duration of action are calculated, plus the frequency of blocks are noted. Using various doses of the test compound and standard, dose–response curves can be established and potency ratios calculated.

Modifications of the Method

With a similar technique, Lembeck (1953) tested the effect of the added vasoconstringent agents adrenaline, noradrenaline, and corbasil to procaine.

Sciatic nerve blockade in the **rat** was used by Feldman and Covino (1988) to study the comparative motor-blocking effects of bupivacaine and ropivacaine.

Grant et al. (1992) used a **rat** sciatic nerve model for independent assessment of sensory and motor block induced by local anesthetics. Motor block was assessed by measuring hind paw grip strength with a dynamometer. Sensory block was determined by measuring hind paw withdrawal latency from radiant heat.

Kohane et al. (1998) studied sciatic nerve blockade with ropivacaine and bupivacaine in infant, adolescent, and adult **rats**. Rats were anesthetized briefly with halothane by face mask. The sciatic block was conducted by introducing a 30-gauge needle posteromedially to the greater trochanter, pointing in an anteromedial direction. Once bone was contacted, the needle was withdrawn 1 mm and drug was injected. Nerve blockade was assessed by withdrawal of the hind leg after pinching the lateral aspect of the plantar surface, by a modified hot-plate test, by the positional placing response as a test of proprioception, by extensor postural thrust as a measure of motor strength, and by ability to hop. Thermal latency in the contralateral (uninjected) limb was determined as a measure of systemic toxicity.

Grant et al. (2001) tested perineural antinociceptive effects of opioids in a **rat** model. Analgesia was assessed using the hind paw latency response to radiant heat.

Luduena and Hoppe (1952) used **guinea pigs** to test the sciatic nerve block by local anesthetics. The anesthetic solutions were injected close to the sciatic nerve. Sensory paralysis was determined by pricking the posterior site of the thigh with a needle. The effect on motor nerve fibers was determined by observing the abnormal position of the injected leg in locomotion.

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebral anesthesia in man.

Åkerman et al. (1988) used **guinea pigs** to determine the brachial plexus block. The syringe was directed toward a line between the head of the humerus and the manubrium of the sternum and the first ribs. The needle was inserted about 1.5 cm into the pocket felt as a depression by palpation between the head of the humerus and sternum and associated structures. Following the retraction of the needle by a few millimeters, 0.2 ml of the solution was injected. The orientation of the needle was changed three to four times during the injection to enable all branches of the plexus to be reached. An assessment was made of motor and sensory blockade.

Leszczynska and Kau (1992) used a sciatic nerve blockade method in **mice** to differentiate drug-induced local anesthesia from neuromuscular blockade. The drugs were injected into the popliteal space of the right hind limb. A positive local anesthetic activity was recorded when a mouse was only able to walk using three limbs on an inverted wire mesh screen and the injected limb was hanging in the air. A positive neuromuscular blockade was recorded when a mouse could walk normally on the top of the wire mesh screen but was unable to stay on the inverted screen.

Thut et al. (1995) used the **rabbit** tooth-pulp assay (see chapter "► Central Analgesic Activity") to quantify the efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

Rosenberg and Heinonen (1983) found a differential sensitivity of A and C nerve fibers to long-acting local anesthetics.

The Local Anesthetics for Neuralgia Study Group (1994) designed a surgically implantable nerve irrigation system for intermittent delivery of local anesthetics and evaluated long-term performance and histocompatibility in rats.

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Conduction Anesthesia on the Mouse Tail

Purpose and Rationale

The radiant heat method as being used for the evaluation of systemic analgesic activity (D'Amour and Smith 1941; Ther et al. 1963; Grant et al. 1993) can also be used for the determination of conduction anesthesia by injecting the local anesthetic into the root of the tail.

Procedure

Groups of 10 mice (NMRI strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard, the normal reaction time is determined. The animal is placed into a small cage with an opening for the tail at the rear wall. The tail is held gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. After

about 6 s, the reaction of the animal is observed by the investigator. The mouse tries to pull the tail away and turns the head. The shutter is closed with a switch when the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test.

The test compounds and the standard are injected in a volume of 0.1 ml on both sides in the area of the tail root. The animals are submitted to the radiant heat again after 10 min. The area of heating is about 1.5 cm distal to the injection site. For each individual animal, the reaction time is noted.

Evaluation

There are two possibilities for evaluation:

1. The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
2. At each time interval, only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose and ED_{50} values are calculated according to Litchfield and Wilcoxon.

Modifications of the Method

Bianchi (1956) used the tail-clip method of Haffner (1929) as a simple quantitative method for testing local anesthetics. Fully grown albino **mice** of either sex were used. A small artery clip with its blades covered by a thin rubber tube was applied to the root of the tail. Those animals that did not show the pain reflex (the mice turn again and again trying to remove the clip) within 30 s were eliminated. The remainder received subcutaneously, about 1 cm from the root of the tail, 0.1 ml of the solution of drug. The pain reflex is tested 15 min after injection, applying the stimulus to the zone where the compound was injected. The proportion of animals that proved to be anesthetized was noted for each dose.

Saxen et al. (1993) and Smith (1997) described the mouse paw withdrawal assay: a method for determining the potency and duration of the effect of local anesthetics in mice. A standard rodent tail-flick apparatus was used to stimulate the dorsal skin of each hind paw in the mouse. The radiant heat intensity of the lamp was adjusted to yield hind paw withdrawal latencies of 6–8 s, with a 20-s cutoff time to prevent tissue damage. Testing was performed by gently restraining the animal in a small towel and passively placing one hind paw on the stage so that the animal was free to withdraw its paw from the stimulus without restriction. Paws were tested in an alternative order (right and then left, left and then right, etc.) to minimize procedural bias. The individual left and right paw withdrawal latency times were determined in each mouse and the difference between left and right latencies was calculated. Paw withdrawal time difference was calculated by subtracting the experimentally modified paw withdrawal latency from the control paw latency.

Grant et al. (1993) quantified the duration of the local anesthetic-induced conduction block in the mouse using the tail-flick test.

Grant et al. (1994) studied prolonged analgesia with liposomal bupivacaine in mice using the tail-flick test.

The tail-flick procedure using **rats** as test animals has been proposed by Herr et al. (1953).

Madan et al. (1970) determined conduction anesthesia by a tail-pinch technique in rats. The local anesthetic was injected subcutaneously bilaterally at the root of the tail. The test for local anesthesia was begun 15 min after injection by pinching with a polyethylene sheathed artery forceps and repeated every 15 min. The number of animals failing to remove the forceps by biting was recorded.

Kamerling et al. (1985) described a method for studying cutaneous pain perception and analgesia in **horses**. This method was used by Harkins et al. (1996, 1997) for determination of highest no-effect dose (HNED) for local anesthetic responses to procaine, cocaine, bupivacaine, benzocaine, and of a plant extract. A heat projection lamp was used as noxious stimulus directed onto the pastern to elicit a flexion-withdrawal reflex. Hoof-withdrawal latency was defined as the time between lamp illumination and withdrawal of the hoof. The local anesthetic drugs were injected into the area of the palmar nerve where it passes lateral to the sesamoid bone.

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Rabbit Tooth-Pulp Assay

Purpose and Rationale

The rabbit tooth-pulp assay (see chapter "► Central Analgesic Activity") has been used successfully to evaluate the potency, efficacy, and duration of the antinociception of analgesics (Yim et al. 1955; Piercey and Schroeder 1980; Wynn et al. 1984). Thut et al. (1995) described a method for administration of local anesthetic drugs to the maxillary arch of rabbits and subsequent measurement of antinociceptive action.

Procedure

Pulp chambers are exposed close to the facial gingival line of the two central incisors in New Zealand White male rabbits (1.5–2.0 kg) using a high-speed dental drill and 0.5-mm round burr immediately after the i.v. administration of 30 mg/kg ketamine. All experiments are conducted 48 h or more after pulp exposure. A linear ramp function stimulator is used as the direct current voltage source. The rabbit is slightly restrained, and the current is applied to the pulp via fine-wire platinum electrodes held in each of the cavities. Linearly rising voltage is applied at a rate of 0.33 V/s until a

patterned lick–chew response occurs. Mean threshold voltages are established for controls using an average of three determinations and then obtained after treatment using a single determination up to a maximum of 10 V. Rabbits having control values greater than 3 V are excluded from the study. The injection site of the test drug is 1.5 cm posterior to the central incisors and 4 mm below the roof of the maxillary buccal vestibule. The buccal vestibule is the area where the cheek and the top of the maxillary tissue join. The tip of a 27-Ga × 1/2″ syringe needle is inserted until it contacts the cancellous bone. The volume of injection is 0.4 ml. Injections are made bilaterally so that the total volume injected is 0.8 ml. The animals can be used chronically, but no rabbit should be exposed to the same drug or the same dose more than once, and none should be injected more frequently than every third day. One animal is used per dosage when analgesia is observed to be either 0 % or 100 %. Two or three animals are used for dosages with effects between 0 % and 100 %. When more than one animal is used, data are averaged and treated as a single observation. Pulp chambers have to be reexposed when the drilled openings disappear because of incisor growth (approximately after 3 weeks).

Evaluation

The percentage of maximum effect (*MPE*) for each observation time is calculated from the following equation:

$$MPE = (TV - CV)/(10V - CV) \times 100$$

TV is the voltage after treatment, *CV* is the control voltage, and 10 V are the maximum volts applied. The voltage that elicits the response is recorded at zero time and 5, 10, 15, 30, 60, 120, and 180 min after injection. The *MPE* is calculated for each observation time. Graphs of the *MPE* values versus time are constructed, and the maximal effect and time of the maximal effect are recorded. Further, the duration of each dosage is defined as the length of time that that dose achieved an *MPE* greater than 25 %. Data are regressed between observed time points to provide estimates of the time when 25 % *MPE* is first achieved and also the time when 25 % *MPE* is no longer observed. A computer program or the standard method of Litchfield and Wilcoxon (1949) can be used to calculate ED_{50}, ED_{90}, and ED_{95} values with 95 % confidence limits.

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Infraorbital Nerve Block in the Rat

Purpose and Rationale

Several authors have used the infraorbital nerve block in the rat to study the potency and duration of action of local anesthetics (Fink et al. 1975, 1978; Ready and Fink 1980; Buckley and Fink 1981; Buckley et al. 1985; Renck et al. 1988; Hassan et al. 1989, 1993).

Procedure

Male Sprague–Dawley rats weighing 500–600 g are used. The infraorbital nerve of the rat is 2–3 mm in diameter and supplies the upper lip and whisker area. It is homologous with the infraorbital nerve in humans. Since the rat lacks a closed orbit, the nerve runs forward beneath the eye and emerges from the maxilla through a deep

notch rather than a foramen. The animals are lightly anesthetized with intraperitoneal phenobarbitone 25 mg/kg. This degree of sedation abolishes the righting reflex but does not interfere with the generalized aversive response to pinching the upper lip with an artery forceps. Test solution (0.2 ml) is injected unilaterally on the right side. The intact left side serves as control for comparing the responses on the two sides. Only animals whose aversive response is abolished within 60 s of the time of injection are included in the study. The animals are tested at 5-min intervals thereafter until the first sign of return of the aversive response on the injected side. Ten animals are used for each concentration of each test compound.

Evaluation

The incidence of blocks at each concentration is recorded. The duration of the analgesic block (± SD) is calculated for each concentration and each test compound.

Modifications of the Method

Renck and Hassan (1992) tested analgesia of the upper lip by electrical stimulation using externally applied bipolar stimulatory electrodes (blunted 27-gauge hypodermic needles) connected to a constant current stimulator delivering one impulse per second of 0.24-ms duration.

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Retrobulbar Block in Dogs

Purpose and Rationale

Defalque and Stoelting (1976) published a method to determine the latency and duration of action of local anesthetics after retrobulbar injection in dogs.

Procedure

Young female mongrel dogs weighing 13–15 kg are used. Twenty-four hours before the test, 0.25 % eserine ointment is placed in each conjunctival sac of the dog. Pentobarbital (25 mg/kg) is administered intravenously and then repeated with 10 mg/kg at hourly intervals, thus maintaining the animal in light anesthesia (corneal reflex present). Ten minutes after induction, the dog is put into 30° head-down position, and 20 ml of 0.05 % tetracaine is forced into the epidural space through the interarcuate ligament. Horner's syndrome occurs within 5 min.

A 150-W surgical lamp is now focused upon the eye from a 1-m distance. Fifteen minutes later, a retrobulbar block is performed: the sclera is

seized with an ophthalmic forceps and the eyeball is pulled downward and medially; a 23-gauge needle is then introduced through the superior rectus muscle, tangentially to the globe, and is immobilized as soon as a click indicates penetration of the retrobulbar space; correct placement is confirmed by free motion of the needle tip and protrusion–rotation of the eyeball upon the injection of 1 ml of air.

After aspiration, 2 ml of the tested anesthetic is then injected at a rate of 0.5 ml per second. The pupil dilates and reaches its maximal diameter (6 mm) within a few minutes. This apparent diameter is estimated with a 2-cm-long ruler calibrated in millimeters, whose center is gently applied to the corneal center. The pupil is measured every 15 s for 5 min and then every 5 min until reappearance of maximal miosis (pinpoint and asymmetrical), a precise endpoint which generally coincides with corneal reflex and lacrimation.

Evaluation

Drug latency (in min) and duration (in 5-min units) are averaged for both eyes of each animal, and the mean and standard deviation are then calculated for all test animals. The analysis of variance is performed to find significant differences between various local anesthetics.

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Isolated Sciatic Nerve Preparation of the Frog

Purpose and Rationale

Isolated nerves are immersed between stimulating and recording electrodes in solutions containing local anesthetics which allows electrophysiological measurements (Paterson and Hamilton 1970).

Procedure

A large frog is sacrificed by decapitation with a pair of scissors, and the spinal cord is severed. The skin around the entire body in the region immediately posterior to the forelimbs is cut, and the abdomen and hind legs are skinned by peeling the skin in a posterior direction. The fat, muscles, and other tissues are cut at the posterior end of the body so as to expose the nerves extending posteriorly from the cord. The muscles of the thigh are cut and retracted so as to expose the sciatic nerve. Finally, the gastrocnemius muscle is removed and the peroneus muscle is extended laterally from the tibiofibula. The entire sciatic nerve with branches should now be exposed. A thread with a loop next to the nerve is tied around the nerve in the region of the sacral vertebra. The nerve is then cut anterior to the thread. During the isolation procedure, the nerve is kept moist with frog Ringer solution. The connective tissue and the nerve branches are cut carefully. The nerve is finally cut near the distal end of the tibiofibula and extended on a filter paper previously soaked with Ringer solution. With the nerve anchored by the thread, the remaining connective tissue is peeled back toward the distal end of the nerve. Any remaining branches are cut at the point of their bifurcation. The distal end of the nerve is tied with a length of thread. The sciatic-peroneal nerve preparation is mounted in a nerve chamber for recording.

Electrodes for stimulation are placed on the proximal end of the nerve while recording electrodes are placed at the distal end. The nerve is immersed in a trough containing frog Ringer solution as bathing fluid. The stimuli are applied with a commercial stimulator (e.g., Grass Model S4) with a duration of 5 ms, a frequency between 30/s, and 1.5–15 mV. The recording electrodes are connected with an amplification and display system. The stimulation of the nerve produces a display on the oscilloscope. Adjusting the voltage setting on the stimulator will cause the action potential to increase and decrease correspondingly on the scope. After pinching the nerve between the last two recording sites, the action potential should be monophasic and appear entirely above the baseline. The spike is observed periodically for several minutes to ascertain its stability.

The bathing trough is changed to a trough containing the local anesthetic dissolved in Ringer solution. At one- or 2-min intervals, the amplitude

of the spike is read and recorded on a polygraph. After 5-min treatment, the drug trough is removed and the nerve is immersed in the Ringer solution of the bathing trough. Amplitudes are measured in periodic intervals and thus the recovery process is recorded.

Evaluation

Block and recovery curves are achieved with various concentrations of the test compound and the standard. Dose–response curves can be obtained.

Modifications of the Method

Specialized techniques with isolated nerves (desheathed nerves, equilibrium blocks, single nerve fibers) have been described by Camougis and Takman (1971).

Den Hertog (1974) used the **desheathed cervical vagus of the rabbit** mounted in a single sucrose-gap apparatus and measured changes in the membrane potential and compound action potential. The ratio of the distance between stimulation and recording electrode (15 mm) and the time between stimulation of the nerve and the top of the action potential was taken as an index for the mean conduction velocity.

Salako et al. (1976) tested a new antiarrhythmic drug as a local anesthetic on desheathed **frog** nerve.

Lee-Son et al. (1992) used the sucrose-gap method on desheathed isolated frog peripheral nerves to study the stereoselective inhibition of neuronal sodium channels by local anesthetics.

Lambert et al. (1994) studied the reversibility of conduction blockade in desheathed bullfrog sciatic nerves, using the sucrose-gap method for recording compound action potentials, before and during exposure to local anesthetics and during drug washout.

Isolated nerves from other species have been used for the study of local anesthetics, such as those from rabbits by Ritchie et al. (1965a, b) or from rats by Condouris and Lagomarsino (1966) and Štolc and Mai (1993).

Gissen et al. (1980) observed differential sensitivities of mammalian nerve fibers to local anesthetic agents in rabbits. A fibers were blocked at the lowest drug concentrations, the intermediate B fibers were blocked at a higher drug concentration, and the smallest, slowest-conducting C fibers required the highest drug concentration for conduction blockade.

Fink and Cairns (1984) showed the differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons in rabbits.

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Isolated Mammalian Sciatic Nerve Preparation

Purpose and Rationale

Karlsson et al. (1994) tested the local anesthetic-like effects of an NK_1 receptor antagonist in a mammalian sciatic nerve preparation using guinea pigs.

Procedure

Male guinea pigs weighing 250–400 are sacrificed and the sciatic-tibial nerves immediately excised and cleaned in buffer equilibrated with 95 % O_2 + 5 % CO_2 under a dissecting microscope. The nerve is mounted in a Plexiglas chamber consisting of three connecting wells. The central well is perfused with a buffer at a rate of 5 ml/min and contained the grounding electrode (Ag/AgCl), the in- and outlet of the peristaltic pump-driven perfusion system, and a thermistor probe. The two lateral wells (one for stimulating, the other for recording) are filled with mineral oil. Stimulating and recording electrodes are made from bare platinum–iridium wire. The passages between the central and lateral wells are sealed with petroleum jelly. The length of the nerve exposed to the drug in the central well is 8 mm. The nerve chamber is kept at a temperature of 27.0 ± 0.5 °C.

A constant current supramaximal stimulus of 50-μs duration is used throughout the experiment. The intensity of the stimulus is ten times greater than the threshold intensity required to elicit a compound action potential. Throughout the experiment, the nerve is stimulated at the low frequency of 1/min to determine basal compound action potential amplitude. Once the response has stabilized, a single burst of pulses (pulse train duration 250 ms, at 40 Hz) is applied approximately 5 min before drug application (control train) and again after drug application to determine the extent of frequency-dependent block. The compound action potentials are digitized with a computer interface and a software program and recorded on a PC. A two-channel storage oscilloscope is used to follow the experiment and to automatically

sample and calculate the amplitudes of the compound action potentials.

The drugs are dissolved in a buffer and applied approximately 5 min after the control train. The development of basal block following drug application is followed with 0.0167-Hz stimulation. In the case of lidocaine, the maximal inhibition is reached 20–30 min after application. The basal block is allowed to reach equilibrium, which is defined as the condition in which the difference in amplitude between the first and the last of five consecutive responses is less than 1 % of the control compound action potential amplitude obtained prior to drug application. To assess the frequency-dependent block, a second stimulus is given after equilibrium is reached. Once the basal and frequency-dependent block has been determined, the nerves are washed with fresh buffer.

Evaluation

The magnitude of the basal block (%) is calculated by comparing the average peak amplitude of the last three compound action potentials with the average peak amplitude of the last three compound action potentials prior to drug application. Similarly, the frequency-dependent block is expressed in percent of the control train and is effectively the basal block plus the additional decrease in compound action potential amplitude. Dose–response curves for the standard and the test drugs are constructed by plotting the basal block and frequency-dependent block against drug concentration. The pIC_{50} values (the negative logarithm of the molar concentration of the drug producing 50 % inhibition of the compound action potential amplitude) are estimated by linear regression analysis of the results in the 10–90 % interval of the dose–response curves.

The dose–response data obtained with the standard and the test drugs and the effect of a single concentration (0.5 mM) of all compounds on the basal block and frequency-dependent block are analyzed by ANOVA. The values of latency to the peak of compound action potential are analyzed by paired *t*-test.

Modifications of the Method

Fink and Cairns (1984) studied the differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons.

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Effect of Local Anesthetics on Different Nerve Fibers

Purpose and Rationale

Wildsmith et al. (1989) examined the in vitro sensitivities of different types of fibers in rabbit vagus nerves to local anesthetic block with a range of local anesthetic drugs at high- and low-frequency stimulation. Rapidly conducting, myelinated (A) fibers are more sensitive than more slowly conducting, unmyelinated (C) fibers.

Procedure

The cervical portions of the vagus nerves of rabbits weighing 2.5 kg are removed, desheathed,

and mounted in an airtight chamber which is maintained at 37 °C by a water jacket. A central 1-cm section of the nerve chamber is perfused at 0.5 ml/min with carbonated Liley solution (pH 7.4 ± 0.02 after equilibration with 5 % carbon dioxide in oxygen). A tap system allows the perfusate to be changed to test solution without admission of air.

A square-wave generator is used to apply supramaximal electrical stimuli (10–15 V for 1 ms) at a rate of 0.0167 Hz during a 30-min period of stabilization. Two trains of stimuli (duration 0.25 s) are applied, one at 20 Hz and the other at 40 Hz. The preparation is considered valid only if there is less than 5 % decrement in height of the action potential during stimulation at 40 Hz. The signals from the nerve are amplified, digitized (Unilab 532.001 interface sampling at 40 μs intervals for 8 ms and then at 200 μs intervals for 50 ms), and recorded with a microcomputer-based system. The numeric derivative of the signal can be printed out immediately or stored on a disk for subsequent processing. Signals are monitored also on a storage oscilloscope.

A submaximal blocking concentration of the hydrochloride salt of a test drug dissolved in carbonated Liley solution is applied. Only one concentration of one drug is applied to each nerve. The stimulation rate remains at 0.0167 Hz until any changes in the compound action potential are complete (minimum period 30 min), when the trains of high-frequency stimulation are repeated. The drug effect is measured as the percent decreases in the height of the three components (A, B, and C fibers) of the compound action at each frequency of stimulation. At 20 and 40 Hz, the last signal produced by each train of stimulation is used for analysis. An experiment is considered valid only if the action potentials recover to more than 90 % of control height on washing the drug from the nerve.

Evaluation

From the data obtained, the ED_{50} (and its SE) for the effect of each drug on each fiber type at each rate of stimulation is determined by log-probit analysis. Plots of the development of block while the nerves are being stimulated at 0.0167 Hz are also made. From these, an index of the rate of development of A and C fiber block is derived ($T_{1/2B}$). This is defined as the time taken to develop 50 % of the eventual maximum degree of block for the particular fiber type in each experiment.

Modifications of the Method

Scurlock et al. (1975) examined the relative sensitivity of sympathetic preganglionic and postganglionic axons, B and C fibers, respectively, to structurally dissimilar local anesthetics. Cervical sympathetic trunks from adult rabbits were submerged in Krebs–Henseleit solution except during the brief recording periods, when they were stimulated electrically with twice maximal square-wave pulses of 0.15-ms (B fibers) or 0.5-ms (C fibers) duration. After control records, the bath chambers were filled with various concentrations of local anesthetics and the action potentials recorded at 5-min intervals. The decrease of action potentials of the B and C fibers was recorded separately.

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Measurement of Sodium and Potassium Conductance in Voltage Clamp Experiments

Purpose and Rationale

Voltage clamp experiments on single nodes of Ranvier can be performed with single myelinated nerve fibers from the sciatic nerve of the frog (Stämpfli 1954, 1968; Nonner 1969; Nonner et al. 1975; Stämpfli and Hille 1976; Borchard and Drouin 1978). Sodium, potassium, and leakage currents (I_{Na}, I_K, I_L) can be measured.

Descriptions of structure and function of voltage-gated sodium channels were given by Catterall (1988, 1992), Stühmer (1991), Narahashi and Herman (1992), and Cohen and Barchi (1993).

Procedure

Single myelinated nerve fibers are dissected from the sciatic nerve of the frog (*Rana esculenta*). The fibers are bathed in a Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 5.0 mM Tris, pH 7.3, at 15 °C. For measurement of sodium, potassium, and leakage currents (I_{Na}, I_K, I_L), the nodal membrane is clamped to a holding potential of −30 mV (relative to the resting potential of −70 mV). Hyperpolarizing prepulses (−40 mV, 50 ms) followed by 2-ms test pulses (60 mV) are applied every 5 s to record peak sodium currents, I_{Na}, and to avoid the influence of accumulation of frequency-dependent block. Every second pulse is followed by an afterpulse (120 mV, 20 ms) to measure the steady-state I_K. The leakage current, I_L, is measured at the end of the hyperpolarizing prepulse and recorded every 10 s.

After the superfusion of the nodal membrane with a local anesthetic, a quick and then a slow decrease of the peak sodium current (I_{Na}) occurs. When the application of the local anesthetic is stopped, I_{Na} increases quickly and then slowly within minutes indicating a reversible action of the local anesthetic on the nerve fiber. With local anesthetics, the potassium current (I_K) behaves similarly, whereas the leakage current (I_L) remains unchanged.

Dose-Dependent Action of Local Anesthetics on I_{Na}–V Relations

The holding potential is set at −30 mV. A hyperpolarizing prepulse (−40 mV, 50 ms) is followed by a depolarizing test pulse (30 ms) with varying amplitudes. Local anesthetics induce a dose-dependent reduction of sodium-inward and sodium-outward currents over the whole range of voltage clamp steps. The measurements are performed at extracellular pH 7.3.

Dose-Dependent Action of Local Anesthetics on I_K–V Relations

Potassium currents, I_K, are measured at the end of depolarizing test pulses of 30-ms duration. Local anesthetics induce a dose-dependent decrease of potassium currents at extracellular pH 7.3.

Evaluation

The effects on sodium, potassium, and leakage currents (I_{Na}, I_K, I_L), the dose dependence of action on I_{Na}–V relations, and the dose dependence of action on I_K–V relations of various local anesthetics are compared.

Modifications of the Method

Bräu et al. (1998) determined half-maximal blocking concentrations of local anesthetics for the tonic block of Na^+ and K^+ channels by using the axonal patch clamp method (Vogel and Schwarz 1995) to study potencies of clinically used local anesthetics in suppressing Na^+ and delayed-rectifier K^+ channels of peripheral nerve.

Sciatic nerves of the clawed **toad** *Xenopus laevis* were enzymatically prepared (Jonas et al. 1989). The nerves were dissected, desheathed, and treated with collagenase and protease to obtain single, partially demyelinated fibers. For recording axonal ionic currents, the outside-out configuration of the patch clamp method (Hamill et al. 1981) was applied to partially demyelinated axons. Patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) and coated with Sylgard 184 (Dow Corning, Seneffe, Belgium) and were fire-polished at the tip before use. The resistance of the pipettes after filling with internal solution was 15–25 MΩ.

The bath solution contained (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 2, and HEPES 10, adjusted to pH 7.4 with Tris. For Na^+ current recording, 10 mM tetraethylammonium chloride was added to suppress K^+ currents; for K^+ recording, 100 nM tetrodotoxin was added to suppress Na^+ currents. The pipette solution for Na^+ current recording contained (in mM): CsCl 110, NaCl 13, EGTA 2, and HEPES 10, pH adjusted to 7.2 with Tris. For the investigation of K^+ channels, CsCl was replaced by KCl.

Different concentrations of local anesthetics were applied to the patches using a multibarrel perfusion system that allowed solution changes within 1 s.

After forming an outside-out patch from the nodal membrane, the holding potential was adjusted to −90 mV in the voltage clamp mode using an EPC7 patch clamp amplifier (List, Darmstadt, Germany). The pulse protocol used to elicit Na^+ currents comprised a 50-ms prepulse to −130 mV to remove fast inactivation, followed by a 50-ms test pulse to −40 mV to activate Na^+ currents. Steady-state K^+ currents were activated by a 50-ms potential step to 60 mV and were measured as the mean current during the last 20 ms of the pulse. For analysis, currents were filtered with a four-pole Bessel filter, digitized with a Labmaster TM-40 AD/DA board (Scientific Solutions, Solon, Ohio, USA), and stored in the hard disk of a computer. To reduce the noise of single current traces that originated from individual channel openings, 20 successive traces, elicited every 5 s, were averaged to measure the peak Na^+ current or steady-state K^+ current. Blocking potencies of the local anesthetics were determined by analyzing concentration–inhibition curves, which were constructed by measuring the inhibition of the peak Na^+ current or steady-state K^+ current amplitude at different concentrations normalized to control.

Half-maximal inhibiting concentrations (IC_{50}) were obtained from nonlinear least-square fits of the function $F_1 = b_{max}/(1 + [IC_{50}/c]^p)$ to the data points. As first-order binding kinetics of the drug with the channel were assumed, the Hill coefficient (p) was set to 1 during fitting; b_{max} is the maximal achievable block. Data acquisition and evaluation were performed with pClamp 5.5.1 (Axon Instruments, Burlingame, Calif., USA); Fig. P 5.0 software (Biosoft, Cambridge, UK) was used for fitting procedures.

Olschewski et al. (1998) applied the patch clamp technique to intact lateral horn neurons from laminae I–III identified in 200-μm slices of spinal cord from newborn rats. Under voltage clamp conditions, the whole-cell Na^+ and K^+ currents activated by depolarization were recorded in the presence of different concentrations of anesthetics.

Olschewski et al. (2002) combined the patch clamp recordings in spinal cord slices of the rat with the "entire soma isolation" method, as described by Safronov et al. (1997) and Safronov (1999). In the whole-cell recording mode, the entire soma of the neuron was isolated from the slice by slow withdrawal of the recording pipette, leaving all or nearly all of its processes in the slice. The isolated structure was classified as *soma* if it had lost all its processes during isolation and preserved only 10–20 % of the original Na^+ current recorded from the neuron in the slice before its isolation. The isolated structure was considered as *soma* + *axon* complex if it contained one 10–100-μm process and preserved >85 % of the original Na^+ current recorded from the neuron in the slice before isolation. The structure was considered as *axon* + *dendrite* if it preserved one adjacent process but the amplitude of the Na^+ current was in the range of those typically seen in isolated *somata*. A successful isolation was usually accompanied by a considerable decrease in membrane leakage currents due probably to the loss of the large area of the dendritic membrane. The good physiological state of the isolated structures was confirmed by a considerable increase in their output resistances (reflecting a decrease in membrane leakage conductance) and by stable and even improved membrane resting potentials. Three types of tetrodotoxin-sensitive voltage-gated Na^+ channels on soma and processes of dorsal horn neurons of rat spinal cord were identified. Bupivacaine, lidocaine, and mepivacaine at low concentrations (1–100 μM) enhanced

delayed-rectifier potassium current in intact neurons within the spinal cord slice while exhibiting a partial blocking effect at higher concentrations (>100 μM).

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Inhibition of Fast Axonal Transport

Purpose and Rationale

Local anesthetics block not only nerve conduction but also fast axonal transport (Aasheim et al. 1974; Lavoie 1983; Lavoie et al. 1989). This mechanism may also be the way in which local anesthetics cause injury to the nerve and spinal cord (Kalichman 1993).

Procedure

The biological material consists of the eighth and ninth dorsal root ganglia of the bullfrog (*Rana catesbeiana*) and their respective spinal nerves. Each side of the animal provides one such set of ganglia and associated structures. The dissection is performed at room temperature with the preparation in an oxygenated medium with a pH of 7.1 and of the following composition (in mM): NaCl 114, KCl 2.0, $CaCl_2$ 1.8, dextrose 5.5, and HEPES 20. Once the dissection is completed, each spinal nerve is ligated at approximately 24 mm from the ganglion.

The ganglia are pulse-labeled with [^{3}H]leucine for 1 h. After the pulse-labeling, the preparations are incubated for 16–17 h at 18 °C in medium free of radioactivity; the two ganglia and spinal nerves from one side of the animal serve as control preparations and those from the other side as experimental preparations. The preparations are placed in a Lucite incubation chamber. The ganglion and the first 10 mm of the nerve trunk occupy one compartment, the next 3 mm is in the groove between two communicating compartments, and the rest of the nerve trunk (including the point of ligature) is placed in the second compartment. The two communicating compartments are then sealed from one another by filling the rest of each groove with silicone grease. The compartments containing the control ganglia and that containing the experimental ganglia are filled with medium for all experiments. A local anesthetic agent is added to the medium bathing the distal half of the experimental nerve trunks, whereas the same area of the control nerve trunks is in the corresponding medium without local anesthetic.

At the end of the incubation, the nerves are cut into 2-mm segments, and the protein-bound radioactivity of each ganglion and nerve segment is determined by liquid scintillation counting (Lavoie 1981). For each preparation, the total quantity of transported radioactivity is calculated by summing up the radioactivity present in the individual segments of the nerve starting at 6 mm from the ganglion and extending up to the point of ligature. The total quantity of [^{3}H]leucine incorporated into protein is calculated for each preparation as the sum of transported radioactivity and radioactivity remaining in the ganglion.

Evaluation

Mean values ± SEM for the ratio of radioactivity in the experimental preparation to radioactivity in the control preparation are computed in each pair of preparations for the total incorporated radioactivity, the total transported radioactivity, and the radioactivity at the ligature. The difference in the absolute amount of radioactivity at the ligature, the difference in total transported radioactivity, and the difference in total incorporated radioactivity are

calculated for each pair of preparations. A Student's *t*-test for paired samples is performed on the mean differences between control and experimental preparations. Using the individual ratios of radioactivity in experimental preparation to control preparation, an analysis of variance on parallel groups is done to compare the drugs; pairwise comparisons are obtained using Scheffé's critical *F* value.

Modifications of the Method

Anderson and Edström (1973) and Lavoie (1982) used frog sciatic nerves in vitro to study the effect of local anesthetics on fast axonal transport of proteins. Rabbit vagus nerves were used by Fink et al. (1972) and Aasheim et al. (1974).

Factors, such as kinesin and dynein, influencing fast axonal transport have been identified (Shea and Flanagan 2001).

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Infiltration Anesthesia

Infiltration Anesthesia in Guinea Pig's Wheals

Purpose and Rationale

Based on earlier work by McIntyre and Sievers (1937) and Sievers and McIntyre (1938), the use of intracutaneous wheals in guinea pigs was recommended by Bülbring and Wajda (1945). This method has become a standard operating procedure for testing local anesthetics.

Procedure

Adult guinea pigs of either sex weighing 250–300 g are chosen. On the day preceding the experiment, the hair on the back is clipped and two areas of 4–5-cm diameter are shaved. This produces a certain amount of irritation which disappears overnight. The sensitivity of the skin is greatest in the midline and slightly more so in the front than in the back area. For this reason, each concentration of a local anesthetic must be tested in both areas. Six tests using three guinea pigs can be performed simultaneously. The doses of local anesthetics are always injected intracutaneously in 0.1 ml saline. Three guinea pigs receive one dose in the front area and another dose in the back area; the size of the wheal is marked with ink. One side is used for the test preparation, the other side for the standard (e.g., 1 % butanilicaine). The reaction to pin prick is tested 5 min after injection in the following way. After observing the animal's normal reaction to a prick applied outside the wheal, six pricks are applied inside the wheal and the number of pricks is counted to which the guinea pig fails to react. The pricks are applied at intervals of about 3–5 s. Six pricks are applied every 5 min for 30 min. Having completed the test on three guinea pigs, the same solutions are injected into three other guinea pigs, but the solution which was used for the front is now used for the back area and vice versa.

Evaluation

The number of times the prick fails to elicit a response during the 30-min period is added up, and the sum, out of possible 36, gives an indication of the degree of anesthesia. Using various doses, dose–response curves can be established. For time–response curves, the prick tests are repeated every 10 min. Half-life times are calculated as the time when, after complete anesthesia, three out of six pricks elicit again a response (Ther 1953a, b).

Modifications of the Method

The test can also be used to study the influence of vasoconstrictors, such as adrenaline.

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Infiltration Anesthesia in Mice

Purpose and Rationale

Grant et al. (2000) described a mouse model for the evaluation of skin anesthesia after infiltration of local anesthetics.

Procedure

Male Swiss Webster mice weighing 23 ± 3 g are used. On the day preceding the experiment, the abdominal hair is shaved using electric clippers. On the day of the experiment, on either side of the abdomen (~0.5 cm lateral to the midline and ~1.0 cm below the thoracic cage), circular areas of ~0.7-cm diameter are marked with indelible ink for subcutaneous injections. For the generation of nociceptive electrical stimuli, a square-wave general-purpose generator (Model S48, Grass Instruments) coupled to an isolator unit (Model PSIU6 F, Grass Instruments) is used. The stimuli are delivered to the skin via blunt electrodes fashioned from 25-gauge needles. A thin layer of electrically conductive gel is applied to the area to be tested with a cotton-tipped applicator.

Mice are screened to determine their vocalization threshold, the current required to produce a vocalization (squeak). While nociceptive stimulus is applied, animals are restrained gently in the experimenter's palm by grasping the nape of their neck and their tail. The output of the stimulator is initially set at 5 mA and is progressively increased in steps of 1 mA. Mice are screened once, and those animals that vocalized at ≤8 mA are included in the study.

The local anesthetics are injected in various concentrations subcutaneously in a volume of 0.15 ml in the marked area of one side. After fixed time intervals (5, 10, 20, 30, 40, 50, 60, and 70 min), the skin overlying the injection site and the corresponding "control" side are stimulated using the threshold stimulus. If the animal does not vocalize after the threshold stimulus was

applied to the test site, the intensity of the stimulus is increased twice in 1-mA increments, to deliver a "maximal" stimulus 2 mA above the threshold. This is done to ensure the lack of vocalization response.

To assess the duration, analgesia is tested at 15-min intervals.

Evaluation

The all-or-none response data are subjected to statistical analysis using the survival curve procedure. The curves are generated using the method of Kaplan and Meier, and the 95 % confidence interval for fractional survival at any particular time is calculated using the Prism program (GraphPad Software). The curves are compared using the log-rank test. In this method of statistical analysis, the term "survival" refers to any well-defined endpoint and in the present study it is lack of analgesia. The two-tailed p value of <0.05 is considered significant.

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Surface Anesthesia

Surface Anesthesia on the Cornea of Rabbits

Purpose and Rationale

Following the pioneering work of Sollmann (1918), the block of the rabbit corneal reflex as described by Régnier (1923) has become a standard test method for evaluating local anesthetics (Quevauviller 1971).

Procedure

Albino rabbits of either sex weighing 2.5–3 kg are placed into rabbit holding cages. The upper and lower eyelashes are carefully clipped. The conjunctival sac of one eye is held open, thus forming a pocket. From a 1-ml syringe with a 22-gauge needle, 0.5 ml of a solution of the anesthetic is applied into the conjunctival sac for 30 s. Then the procedure is repeated, so that 1.0 ml is applied within 1 min. One ml of the standard (0.1 % solution of tetracaine hydrochloride) is applied to the other eye. Effective local anesthetics extinguish the corneal reflex (blinking) elicited by any touch of the cornea. For quantitative purposes, the irritation with a bristle according to von Frey (1894, 1896, 1922) has been recommended. An equine hair bending at a load of 230 mg is attached perpendicularly to a glass rod. Within 25 s, the cornea is touched 100 times. The summation of many stimuli applied this way gives better results than a single touch with a glass rod (Ther and Mügge 1953). The test is started 5 min after application of the drug and repeated every 5 min until anesthesia vanishes and blinking occurs again. The time between disappearance and reappearance of the corneal reflex is registered.

Evaluation

Using the time of loss of the corneal reflex as a parameter after the application of different doses, dose–response curves can be established and potency ratios versus the standard calculated.

Modifications of the Method

Chance and Lobstein (1944) used **guinea pigs** for testing surface anesthesia by the corneal reflex.

Bartsch and Knopf (1970) described an electrically operated stimulator with a bristle allowing variable frequencies to evaluate surface anesthesia on rabbit cornea.

Hotovy (1956) investigated the synergism between the local anesthetic compound dibucaine hydrochloride applied to the conjunctival sac of rabbits and intravenously administered analgesics using Régnier's method.

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Suppression of Sneezing Reflex in Rabbits

Purpose and Rationale

Nieschultz et al. (1958) and Åström and Persson (1960) used the sneezing reflex in rabbits to test local anesthetic activity.

Procedure

Groups of male rabbits weighing 3 kg are used. Using a cotton tampon, the test solution is applied to the mucous membrane of one nostril. The solution of a standard local anesthetic is administered to the nasal mucosa of the other nostril. After 2 min, the mucous membrane is stimulated by a fine pencil. Loss of the sneezing reflex is regarded

as sign of complete anesthesia. The stimulation is repeated after 3, 6, 10, and 15 min and continued every 5 min until the sneezing reflex reappears. Various concentrations of test compound and standard are applied.

Evaluation

Using the loss of the sneezing reflex as parameter after application of different doses, dose–response curves can be established and potency ratios versus the standard calculated. Furthermore, the duration of activity can be evaluated.

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Epidural Anesthesia

Epidural Anesthesia in Various Species

Purpose and Rationale

Activity and tolerability of new local anesthetics after epidural injection have to be studied in various animal species in order to predict both parameters in patients.

Procedure

Studies in Rats

Blomberg and Rickstein (1988) described a simple technique for cannulation of the thoracic epidural space in conscious and anesthetized rats. Male Wistar rats weighing 300–400 g were anesthetized with methohexital sodium 100 mg/kg intraperitoneally for insertion of PE-50 catheters into the tail artery and right jugular vein. Supplementary doses of methohexital were given during the cannulation of the epidural space. The catheters were exteriorized in the neck and the animals were placed in a lucite restraining cylinder where they were left to recover for 1–2 h from methohexital anesthesia and cannulation procedures. Arterial pressure, heart rate, and body temperature were recorded. A PE-50 catheter was inserted into the right common carotid artery for measurement of cardiac output with the cardiogreen dye-dilution technique and measurement of left ventricular end-diastolic pressure.

For the cannulation of the thoracic epidural space, the skin of the upper part of the back of the animal was shaved and a 3-mm-long midline incision was made over the spinous process from a point between the two scapulae. The fascia covering the superficial muscles of the back was opened and the muscles were dissected from the thoracic vertebrae and retracted laterally. The thick and protruding spinous process of the second thoracic vertebra was localized. The identification of the spinous process of Th3 to Th7 was easily made by the nail of the index finger. The interspinal muscles and ligaments between the spinous processes of Th6 to Th7 were cut and the spinous process of Th7 was removed. The distance between Th6 and Th7 was then maximized by a rostral traction of Th6 with a forceps attached to spinous process of Th6. The yellow ligament between Th6 and Th7 was identified by a dissecting microscope at $\times 16$ magnification and cut open to visualize the dura and the spinal cord. A PE-10 catheter, filled with the local anesthetic, was introduced cranially to a length of 1 cm. The area between Th6 and Th7 was covered and the catheter was firmly attached with cyanoacrylate, which completely prevented extradural leakage of the local anesthetic. The catheter tip was now located at the Th4–5 level. The superficial muscles were then sutured and the catheter was brought through the closed skin incisions. Incremental doses of 10 µl of bupivacaine were administered epidurally and the amount of the anesthetic bupivacaine (5 mg/ml) that caused no further heart rate decrease was assessed. The spread of the analgesia was assessed by a modified pin-prick technique. All hemodynamic

measurements were made before and then 10–15 min and 45–55 min after the induction of thoracic epidural anesthesia.

Studies in Guinea Pigs

A method to test epidural anesthesia in guinea pigs was described by Åkerman et al. (1988).

Male guinea pigs weighing 300–500 g are anesthetized by means of an intraperitoneal injection of an aqueous solution of chloral hydrate 42.5 g/l, ethanol 90 g/l, propylene glycol 428 g/l, sodium pentobarbitone 9.75 g/l, and magnesium chloride 21 g/l. A skin incision is made from the level of the lumbosacral fossa and approximately 1.5 cm down in order to expose the sacral area in the midline. With the vertebral column flexed, the lumbosacral intervertebral ligament is carefully incised. Through this small opening, a polyethylene catheter (PE-10) is inserted maximally 1.5 cm along the roof of the vertebral canal to the L4–L5 region. The catheter is sutured to the overlying lumbar fascia which is then closed. The catheter is tunneled under the skin and exteriorized through an incision in the neck region. After fixation of the catheter to the fascia of the neck muscles and suturations of the incisions, the catheter is filled with saline and sealed.

After a recovery period of at least 1 day, 0.1 ml of 2.0 % lidocaine is injected over a period of 1 min, and the motor and sensory blocks are assessed. The injection of lidocaine which results in a bilateral, reversible blockade indicates a successful preparation. A minimum of 8 animals are used in the further experiments for each test solution.

The mean time to onset of block and mean duration of block are calculated from number of legs blocked.

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebral anesthesia in man.

Studies in Rabbits

Chernyakova et al. (1994) studied the effects of azacaine during epidural anesthesia in **rabbits**.

Hughes et al. (1993) described a rabbit model for the evaluation of epidurally administered local anesthetics. A "loss-of-resistance technique" similar to that employed in caudal epidural injection in humans was used. The rabbit was carefully restrained by an assistant. The readily palpable cranial dorso-iliac spines, lying on either side of the prominent spinous process of the seventh lumbar vertebra, served as landmarks.

The thumb and the middle finger of the left hand were placed on the two crests and the left index finger was used to palpate the midline L7 spine and the depression over the lumbosacral fossa. With the index finger in position on the L7 spine to serve as a guide, a short-beveled 1.5-cm 20-gauge spinal needle was introduced at right angles to the skin in the midline with the bevel aligned longitudinally. After passage through the skin, only minor resistance was felt until the ligamentum flavum was reached. When passing through the ligament, a definite "pop" was felt and resistance to advancement of the needle was lost. When correctly placed, the needle was at a depth of approximately 0.75–1.0 cm and firmly held by the ligament. The stylet was then withdrawn and the hub inspected for the presence of blood or cerebrospinal fluid. If absent, the needle was rotated through 90° to direct the bevel caudally, a 1.0-ml syringe was attached and 0.1 ml of air injected. Accurate placement was indicated by the absence of resistance to injection and lack of subcutaneous crepitus. A syringe containing the desired dose of the local anesthetic was attached and the solution injected over a 5–10-s period. The pharmacodynamic responses were assessed by (1) sensory loss, (2) loss of weight-bearing ability, and (3) flaccid paresis.

Studies in Dogs

Kief and Bähr (1970, Epidural tolerance of articaine in dogs. Personal communication) studied the epidural tolerance of articaine with and without addition of Suprarenin in Beagle dogs weighing 9–12 kg. For preoperative sedation, the dogs received 0.03 ml/kg Combelen (= propionylpromazine) intravenously. The fur of the lumbosacral area was shaved and the skin disinfected. A single dose of 5 ml of a 2 % articaine solution was administered epidurally under sterile conditions. All dogs showed the typical symptoms of spinal anesthesia, which

subsided after a few hours. The dogs were sacrificed after 1 or 3 days. The portion of the vertebral column with the site of injection in the middle was removed and placed in 8 % buffered formalin. When semifixed, the vertebral arches were opened and the spinal cord and the roots of the spinal nerves with the adipose tissue of the epidural space were removed. After embedding in gelatin and Paraplast, the serial sections from the area of injection were stained with fast red 7B, hematoxylin–eosin, and myelin sheath staining after Olivecrona was performed. Furthermore, the PAS and iron reaction were performed on one section. The presence or absence of nerve damage and of inflammation was noted.

A technique for epidural administration in the **dog** was described by Feldman and Covino (1988).

Defalque and Stoelting (1966) used a standard veterinary epidural technique to study the latency and duration of action of some local anesthetic mixtures in **dogs**. The animals, prone and with their spread-out extremities attached to the table, were placed in 40° Trendelenburg position. Under sterile conditions, the epidural space was penetrated through the interarcuate ligament with a short-beveled no. 22 spinal needle. After identification of the epidural space (aspiration, air injection), 4 ml of the investigated solution was injected with a constant injection rate of 2 ml per sec. Absence to skin-twitch response to pinching with an Allis clamp was considered analgesia. This was tested on both flanks, along a line 2 cm off the spine. The disappearance of the contraction of the anal sphincter in response to stroking all quadrants of the anal margin closely corresponds to complete analgesia at the level of the interarcuate ligament; since this was an easily measurable parameter, the disappearance and recurrence of this reflex were chosen as the endpoint of latency and duration of action, respectively.

Raner et al. (1994) performed thoracic epidural anesthesia in **dogs** after general anesthesia with thiopental/chloralose and under artificial respiration. A thoracic epidural catheter (18G, Braun-Melsungen, Germany) was inserted percutaneously via the Th7–8 or Th8–9 interspace. Thoracic epidural anesthesia was established with a bolus injection of 10 mg/kg mepivacaine followed by a continuous infusion of 5 mg/kg per hour.

Kamibayachi et al. (1995) studied the halothane-induced myocardial sensitization to the dysrhythmogenic effect of epinephrine after thoracic epidural anesthesia in **dogs**. An epidural catheter was inserted under halothane anesthesia. The vertebral arches of T8 and T9 were surgically exposed and the catheter was introduced, via the T8–9 interspace, to the epidural space, which was identified by the loss-of-resistance technique. The catheter was advanced 5 cm in the cephalad direction and secured to the back. After the experiment, the position of the catheter was confirmed radiographically by injection of iopamidol (0.2 ml/kg).

Studies in Other Species

Ide et al. (2001) studied the effect of epidural anesthesia on respiratory distress induced by airway occlusion in isoflurane-anesthetized **cats**. The authors developed an animal model for the study of airway occlusion and proposed new concepts of minimum alveolar anesthetic concentration for airway occlusion and the duration from the start of airway occlusion to the onset of the positive motor response. Adult cats were anesthetized with isoflurane and an epidural catheter was placed after L5 laminectomy.

Richer et al. (1998) described sacrococcygeal and transsacral epidural anesthesia in the laboratory **pig**. The aim of the study was to assess epidural accessibility in 30–50-kg piglets, not in adult hogs. Each animal received general anesthesia combined with epidural anesthesia. The level of catheter insertion was the sacrococcygeal interspinous space or the adjacent space (S4–S5). The localization of the injection site was simple. The sacrococcygeal junction corresponds to the junction between the fixed portion (sacrum) and the mobile portion (coccyx) of the animal's tail. The catheter was inserted along the median line at this level or the level above. The bony structures were palpable beneath the superficial planes. The animals were placed on their sides under general anesthesia and disinfected under surgical conditions. Epidural space injection was performed using a 16-gauge needle, traversing the sacrococcygeal or transsacral

interspinous space. Entry to the epidural space was detected by a loss of resistance to puncture with absence of blood or cerebrospinal fluid on aspiration and by the ease of cephalic catheter progression within this space. A first injection of local anesthetic was performed 10 min prior to laparotomy. Epidural analgesia was maintained after this first injection by continuous administration of the same agent with a syringe. It was discontinued on awaking for a brief period in order to confirm the functional recovery of the lower limbs.

Lebeaux (1975) recommended the **sheep** as a model for testing spinal and epidural anesthetic agents. To ensure accurate and reproducible administration of drugs into these restricted anatomical regions, a wooden stock was built to restrain the sheep without stress but allow arching of the lumbosacral spine in order to open the intervertebral space. The stock consisted of a table with holes for the front feet, hinged at one end to a fixed stanchion. The hinges permitted tilt of the table. The sheep was placed prone on the table, the head secured in the stanchion and the front feet passed through the holes and tied. The lumbosacral interspace used for both spinal and epidural tap was separated by positioning and tying the rear limbs cephalad beside the abdomen, thereby extending the knee and flexing the hip. The wool was clipped from the sheep's back, the skin of the lumbosacral area was washed and swabbed with iodine, and the vertebral spines and interspaces identified by palpation were marked and numbered with a felt pen.

Epidural block was performed with a 19-gauge Crawford point needle, using the loss-of-resistance method, with a syringe containing saline to identify the epidural space. After the withdrawal of the stylet, a Luer-Lok syringe containing 10 ml of sterile saline solution with a final concentration of 1:200,000 epinephrine and 25.0 mg (0.25 %) bupivacaine was attached to the needle. After careful aspiration of blood or spinal fluid, the local anesthetic solution was injected within 30 s. The needle was withdrawn and the table kept horizontal.

Immediately after injection, the rear feet were untied and allowed to hang free on either side of the table for the evaluation of sensation and/or reflexes in the rear limbs. Then, 20 min after injection, the sheep was removed from the table and positioned in a sling fashioned from a heavy-duty 50-gal plastic barrel split in half longitudinally and mounted on a metal support. Holes cut in the barrel allowed the feet to drop through so that comfortable sternal recumbency was maintained while permitting anesthetic evaluation to continue. When complete sensation returned in the skin of the dorsum, the sheep was removed from the sling and placed on a straw-bedded floor so that weight support and full recovery could be assessed.

Feldman et al. (1997) compared anesthetic efficacy and pharmacokinetics of epidurally administered ropivacaine and bupivacaine in the **sheep**.

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Intrathecal (Spinal) Anesthesia

Spinal Anesthesia in Various Species

Purpose and Rationale

Transient neurological symptoms have been observed in patients after spinal anesthesia (Hampl et al. 1995). Myers and Sommer (1993) published a survey on methodology for spinal neurotoxicity studies.

The activity and tolerability of new local anesthetics after intrathecal injection were studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients.

Procedure

Studies in Rats

Intrathecal injections to **rats** are performed according to the method of Hylden and Wilcox (1980) or Åkerman (1985) as being used by Ossipov et al. (1988). Male Sprague–Dawley rats weighing 50–75 g are used. The rat is held firmly by the pelvic girdle. A 30-gauge needle is attached to a 25-µl Hamilton syringe is inserted into the tissue on one side of the L5 or L6 spinous process at an angle of about 20°. The needle is advanced to the groove between the spinous and transverse processes and then moved forward to the intervertebral space at an angle of about 10°. About 0.5 cm of the needle is then inserted in the vertebral column. Correct placement of the needle is indicated by an arching of the tail. Drugs are dissolved in saline or water and administered in a volume of 5 µl. Antinociception is determined in a modification of tail-flick assay in rats by placing the tail of the rat under a focused radiant heat source. The degree of antinociception is defined as the percentage of maximum possible effect. This percentage is determined for each dose at each time measured allowing to calculate ED_{50} values.

Omote et al. (1995) studied the effects of verapamil on spinal anesthesia induced by local anesthetics administered via a chronic intrathecal polyethylene catheter in **rats**. The catheter was inserted 15 mm cephalad into the lumbar subarachnoid space at the L4–L5 vertebrae with the tip located near the lumbar enlargement of the spinal cord. The catheter was tunneled subcutaneously and externalized through the skin in the neck region. At least 6 days of postsurgical recovery was allowed before animals were used in experiments. The tail-flick and the mechanical paw pressure tests were used to assess thermal and mechanical nociceptive thresholds.

Mestre et al. (1994) described a method for performing direct intrathecal injections in **rats** without introducing a spinal catheter.

Wang et al. (1991) described lumbar subarachnoid catheterization in rats. Male Wistar rats weighing 500–600 g were anesthetized with intraperitoneal ketamine 75–100 mg/kg. Following a

midline skin incision, the paravertebral muscles were detached from the spinous processes and retracted laterally. The intervertebral ligament between Th13 and L1 or between L1 and L2 was removed. After dissection, the inferior border of T13 or L1 was retracted cephalad, facilitating the application of rongeurs for partial laminectomy at the cephalic border of L1 or L2. This exposed the dura and the underlying spinal cord, which was easily identified by a midline blood vessel. Lidocaine (1.5 %) was applied topically to the dura to prevent the movement of the animal when the dura was picked up with fine forceps. The dura was perforated with a short-beveled no. 20-gauge needle, resulting in some leakage of cerebrospinal fluid. Under magnification, a PE-10 catheter was immediately inserted tangentially through the dural opening. It was directed caudally and maintained dorsal to the spinal cord. The catheter, 10 cm in length and containing a volume of 0.02 ml, was advanced slowly in the subarachnoid space to a mark 1.5 cm from the tip, while 0.01–0.02 ml of normal saline was simultaneously injected to open the way. The leakage of fluid through the dural opening during injection confirmed the patency of the catheter and also indicated that the catheter was properly placed in the subarachnoid space.

For the fixation of the catheter, the spinous process rostral to the laminectomy was denuded and perforated with a no. 18-gauge needle. The free end of the catheter was threaded through this lumen. On retraction of the needle, the PE-10 catheter was left in the perforation of the spinous process and anchored with cyanoacrylate glue. A connector was attached to the free end of the catheter for injection.

The wound was irrigated with saline and closed in layers leaving the catheter buried in the subcutaneous tissue. The hub of the connector was sutured to the skin with fine stainless steel wire. The injection port, covered by a removable metal cap, was brought out of the skin via a separate small opening lateral to the main skin incision.

One week after surgery, 0.03–0.05 ml of 1.5 % lidocaine was injected through the subarachnoid catheter. The correct positioning of the catheter was evidenced by prompt sensory and motor block of the hind limbs, developing in 1–5 min and exhibiting motor blockade for 20–30 min.

Dirksen et al. (1992) studied the dose–response and time–effect relationships of intrathecal bupivacaine in rats. The effect was a quantified drug-induced and graded reduction in the magnitude of the withdrawal reflexes elicited by transcutaneous stimulation.

Sakura et al. (1996) described an improved technique for the morphological analysis of drug-induced injury after intrathecal catheterization in the rat. Male Sprague–Dawley rats weighing 200–300 g were anesthetized by intraperitoneal injection of methohexital (40–60 mg/kg). Catheters were composed of 28-gauge polyurethane, 32-gauge polyimide, 32-gauge polyurethane, PE-10 polyethylene, or PE-10 polyethylene that has been stretched to twice its original length. They were passed through a slit in the atlantooccipital membrane and advanced 11 cm, to lie with the tip caudal to the conus medullaris. Animals were allowed to recover for 24 h before the study was started. The animals were placed in a horizontal acrylic restraint, and baseline tail-flick latency was assessed immediately before infusion. Infusions were administered via a mechanical infusion pump at a rate of 1 µl/min for 4 h. Animals were evaluated for alteration in tail-flick latency 7 days after infusion.

Animals were sacrificed by injecting an overdose of pentobarbital 7 days after infusion. They were perfused intracardially with a phosphate-buffered glutaraldehyde–paraformaldehyde fixative. The spinal cord and nerve roots were dissected out, immersed in the same glutaraldehyde solution for infusion fixation and embedded in glycol methacrylate. The embedded tissue was sectioned at the conus medullaris, 6 mm rostral and 6 and 12 mm caudal, using a Sorvall JB-4 microtome. The tissue was stained with hematoxylin–eosin or was treated with 4 % osmium tetroxide and stained with toluidine blue. Histologic evaluation was performed with light microscopy. Specimens obtained 12 mm caudal to the conus were used for quantitative comparison of catheter-induced damage. This region was chosen because cross sections obtained below the conus have a greater number

of fascicles. Each fascicle present in the cross section was assigned to injury scores of 1–3, where 0 = normal, 1 = mild, 2 = moderate, and 3 = severe injury. The injury score of each cross section was then calculated as the average score of all fascicles present in the cross section. The data were compared using the Kruskal–Wallis test and Dunnett test. For all comparisons, $P < 0.05$ was considered significant.

Grouls et al. (1997) compared the effects of *n*-butyl-*p*-aminobenzoate and bupivacaine after epidural and intrathecal solution in the **rat**. Anesthesia was measured by the latency of tail withdrawal from warm water (55 °C).

Kirihara et al. (2003) compared the **neurotoxicity** of intrathecal and epidural lidocaine in **rats**. Male Sprague–Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and 1.5 % halothane. A catheter of stretched polyethylene tubing PE-10 was introduced into the subarachnoid or epidural space using an aseptic technique. Catheters were passed via the L4–L5 intervertebral space and advanced 1.3 cm in the caudal direction. Rats were allowed 4 days to rest for recovery from the operation.

To measure the response to noxious heat stimulus, a tail-flick test was performed. A 100-W projector lamp was focused on the distal segment of the tail approximately 5 cm from the tip. The time at which rats withdrew the tail was defined as the tail-flick latency. A cutoff time of 10 s was used to avoid damaging the tail.

To measure the response of legs to a noxious mechanical stimulus, a paw pressure test was applied to the dorsal surface of both hind paws using a device capable of progressively increasing the pressure at a rate of 15 g/s. The pressure at which the rat withdrew the paw from the device was defined as the paw pressure threshold, and the mean for both paws was used for analysis. A cutoff pressure of 400 g was used to prevent damage to the paws.

Motor function in the lower limbs was assessed by grading: 0 = none, 1 = partially blocked, and 2 = completely blocked.

Various concentrations of local anesthetic or saline were injected intrathecally in a volume of 20 µl or epidurally in a volume of 100 µl followed by 10 µl saline to flush the catheter. Tail-flick test, paw pressure test, and motor function assessment were performed 10, 20, 30, 60, 120, 180, and 240 min after injection and continued daily for 4 days.

After the last experiments, the rats were euthanized by injection of an overdose of pentobarbital and then perfused intracardially with a phosphate-buffered 2.0 % paraformaldehyde/2.5 % glutaraldehyde fixative. Methyl green solution was injected to confirm the location of the catheter after the perfusion. The spinal cord and nerve roots were dissected out and immersed in the same fixative for 4 h. Two specimens (10 mm rostral and caudal to the conus medullaris from each rat) were postfixed with cacodylate-buffered 1 % osmium tetroxide, dehydrated in a series of graded alcohol solutions, and embedded in epoxy resin. From the embedded tissue, 1-µm transverse sections were obtained and stained with toluidine blue dyes. Sections obtained from 10 mm rostral to the conus (caudal spinal cord) were used for qualitative evaluation. Quantitative analysis of nerve injury was performed using the sections obtained from 10 mm caudal to the conus. Each fascicle present in the cross section was assigned an injury score of 0–3. The injury score for each cross section was then calculated as the average score of all fascicles present in the cross section.

Data are presented as mean ± SEM. Tail-flick latencies and paw pressure thresholds were converted to the percentage of the maximal possible effect. The area under the time–effect curve was calculated by accumulating the effect measured at discrete time intervals using the trapezoidal integration method. The results were analyzed by ANOVA with repeated measures followed by Scheffe and Dunnett tests. The injury score for each technique and each solution was compared using two-way ANOVA followed by the Scheffe test. The frequency (i.e., the number of rats with lesions) in each group was analyzed by the chi-square test.

De la Calle and Paíno (2002) described a procedure for direct **lumbar puncture in rats**. Female Sprague–Dawley rats weighing 180–250 g were used. The rats were placed in a transparent plastic box and anesthetized with a

mixture of 4 % isoflurane in O_2:N_2O (30:70 v:v). Then, a mask was placed over the rat's nose and mouth, and the isoflurane concentration was lowered to 1.5–2 % for the remainder of the procedure. The lower half of the animal's back was shaved and scrubbed with povidone-iodine. The animals were then placed in a prone position on a Styrofoam board (25 cm × 13 cm × 6 cm), with the mask opening fixed 12 cm from the end of the board. The rat's forelimbs were extended to the front and fixed to the board with tape, taking care not to force the neck. The hind limbs were left to hang off the board, lying on the table. In that way, the animal's vertebral column was flexed around the L3–L5 level, widening these intervertebral spaces. Using the anterior part of the iliac crest as a tactile landmark for the L5–L6 intervertebral level, a 2-cm longitudinal incision was made with a scalpel rostral to this point. The fascia of the paravertebral muscles was excised and withdrawn to the sides with the help of sterile cotton plugs. A neonatal lumbar puncture needle (25 G × 1 TW) was introduced perpendicular to the surface through the widest intervertebral space and lowered until it came into contact with the vertebral body. Occasionally, a short flicking of the tail or of a limb was observed. The moment of penetration into the intrathecal space could be detected by a change of resistance to the introduction of the needle. Normally, the bevel of the needle was oriented rostrally although, if the puncture was not initially successful, the needle was tentatively rotated. A few seconds after withdrawal of the inner needle filament, the cerebrospinal fluid (CSF) could be observed to spontaneously flow into the needle cup. Triggering or accelerating liquid flow could be achieved by: (1) pressing the neck of the animal with a finger (thereby increasing the intrathecal pressure), (2) holding the head of the rat secure with one hand and pulling its tail with the other (the mechanical traction accommodates the needle tip in the intrathecal space), and (3) changing the inclination of the board to about 45° (also increases intrathecal pressure).

The procedure can also be used for the delivery of chemical products or cell suspensions to the intrathecal space. After collecting 20–50 µl CSF, 50 µl of 2 % mepivacaine was injected. Hind limb paresis and anesthesia were produced that lasted for 30 min.

Bahar et al. (1984) described the chronic cannulation of the intradural and extradural space in the **rat**. Under anesthesia, a hole was drilled in the penultimate lumbar vertebra of male rats and the appropriate space cannulated. The catheter was tunneled subcutaneously to emerge at the neck.

Using this technique, Chanimov et al. (1997) studied neurotoxicity after spinal anesthesia induced by serial intrathecal injections of magnesium sulfate. Male Wistar rats were given intrathecal injections of 0.02 ml of 6.3 % or 12.6 % magnesium sulfate or 2 % lignocaine or 0.9 % sodium chloride as a series of 15 injections on alternate days for a period of 1 month.

Yaksh and Rudy (1976) described a procedure of chronic catheterization of the spinal subarachnoid space in **rats** and **rabbits**. A polyethylene catheter (PE-10) was inserted through a puncture of the atlantooccipital membrane into the spinal channel in anesthesia and secured to the skull. In this way, drugs could be administrated into the spinal subarachnoidal space of unanesthetized animals.

Cole et al. (1990) used this model to determine the influence of spinal tetracaine on central nervous system metabolism during nociceptive stimulation in **rats**.

Studies in Mice

Åkerman et al. (1988a, b) used **mice** to study spinal morphine antinociception potentiation by local anesthetics.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized **mice** was described by Hylden and Wilcox (1980).

This model has been used by Langerman et al. (1994) to evaluate the potency of various local anesthetics. Adult Swiss Webster male mice were slightly anesthetized with halothane and the skin overlying the dorsal lumbar spine was opened using a transverse incision 8–10 mm. Animals were allowed to recover for 1 h before the evaluation of the baseline nociceptive response latency. Thirty minutes after baseline testing, intrathecal local injections were

performed with an automatic syringe fitted with a 30-gauge needle. The fourth spinal space was identified and the needle was inserted between the two spinal processes in a cephalad direction at a 20° angle. Resistance encountered at approximately 5–6 mm from the skin indicated proper location of the needle in the spinal canal. A fixed 10 µl volume of injectate was used. Analgesia was measured using the tail-flick test based on tail withdrawal in response to heat generated by a light beam focused on the ventral tail surface.

Using this model, Åkerman (1985) obtained dose–response curves for lignocaine, mepivacaine, bupivacaine, amethocaine, and cinchocaine after a single intrathecal injection in **mice**.

Studies in Rabbits

Bieter et al. (1936a) described a method of inducing spinal anesthesia in the **rabbit** and determined threshold anesthetic and lethal concentrations. Injections were performed at the lumbosacral union between the spinous process of the last lumbar vertebra and the first sacral spinous process. A dose of 0.02 ml per centimeter of spinal length was chosen as standard volume for determining minimal anesthetic and minimal lethal doses. These authors (Bieter et al. 1936b) determined the duration of spinal anesthesia in the rabbit after applying an electrical stimulus to the skin of the animal and observing the changes in respiration.

This method was also used by Luduena et al. (1960).

Langerman et al. (1991) studied the duration of anesthesia after a single subarachnoid injection of a local anesthetic in **rabbits**. Tetracaine 1 % 0.5 mg/kg was administered in 10 % glucose or in lipid solution via catheters chronically implanted in the subarachnoid space. The pharmacologic effect was assessed by evaluating the intensity and duration of motor blockade according to a three-stage scale.

Wakamatsu et al. (1999), Ohtake et al. (2000), and Oka et al. (2001) studied the effects of intrathecally administered local anesthetics on glutamate release and neuronal injury in **rabbits**. New Zealand White rabbits were anesthetized with isoflurane. With the rabbits in prone position, midline skin and subcutaneous fascia were incised between the third lumbar and the first sacral spinous process after infiltration with 0.25 % bupivacaine. Muscles were dissected; the third to seventh processes, ligamentum flavum, and epidural fat were sequentially removed; and the underlying dura was exposed. Using an operating microscope, a small slit was made in the dura and arachnoid membrane at the L3–L4 interlaminar space. A loop-type dialysis probe was then implanted. A PE-10 catheter for the administration of saline or the local anesthetic to be tested was implanted intrathecally through the slit made at the L6–L7 interlaminar space so that the tip of the catheter was located at the level of the cauda equina. The implanted dialysis probe was perfused with artificial cerebrospinal fluid bubbled with 95 % oxygen and 5 % CO_2 at pH 7.2. Samples were collected before and after administration of the test substance and analyzed for glutamate.

After collecting the last sample (90 min after intrathecal administration of test substance), the catheters were removed, and all incisions were sutured. Isoflurane was discontinued, and the lungs were ventilated with 100 % oxygen. Extubation of the trachea was performed when adequate spontaneous ventilation occurred. The animals were allowed to recover with infusion of Ringer solution and antibiotic treatment.

The animals were neurologically assessed daily until 1 week after test drug administration by an observer unaware of the treatment group. Sensory function was evaluated by seeking an aversive response to pinprick stimulation with a 23-gauge needle, progressing from sacral to thoracic dermatomes. The score of the sensory function was assessed by a three-point grading scale. The hind limb motor function was assessed by a five-point grading scale.

After completion of the neurologic function scoring at 1 week, the animals were re-anesthetized, and transcardiac perfusion and fixation were performed. The spinal cord was removed and refrigerated in phosphate-buffered formalin 10 % for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. The

coronal sections of the spinal cord at L3, L4, and L5 levels were cut at a thickness of 8 μm and stained with hematoxylin and eosin. The degree of spinal cord damage was assessed in terms of vacuolation of the dorsal funiculus with a four-point grading scale and chromatolytic changes in the motor neuron. The neurons with chromatolytic appearance were identified by round-shaped cytoplasm with the loss of Nissl substance from the central part of the cell and eccentric nuclei. The number of motor neurons with a chromatolytic appearance was counted in two sections for each animal and averaged.

Parametric data were presented as mean ± SD. To determine the differences in glutamate concentrations, a repeated-measure analysis of variance was performed. The cutaneous sensation, hind limb motor function, and morphological changes of the spinal cord were analyzed with a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney *U*-test.

Studies in Dogs

Wagner et al. (1940) performed spinal anesthesia in anesthetized **dogs** by puncture at the fourth lumbar interspace and measured the duration of anesthesia after the application of various local anesthetics.

Muschaweck R, Kief H, Baehr H (1971, Comparative intrathecal tolerance of articaine in dogs. Personal communication) performed comparative intrathecal tolerance studies in dogs. **Beagle dogs** weighing 8–12 kg were anesthetized with 30 mg/kg sodium pentobarbital intravenously. The animals were intubated and submitted to artificial respiration. The fur on the neck was shaved and the skin disinfected. All further procedures were carried out under sterile conditions. The spinal canal was punctured through the foramen magnum at the atlantooccipital joint. Successful entry of the spinal cervical canal was checked by withdrawal of cerebrospinal fluid. Then 5 ml of cerebrospinal fluid was withdrawn and used as the solvent for the tested local anesthetics. The same volume was injected intrathecally either as a solution of local anesthetics in concentrations used in therapy or as control (saline solution). Artificial respiration was continued until spontaneous breathing resumed. Motor performance was checked for 24 h. Two days later, the animals were sacrificed under anesthesia. Dissection included the cerebellum, medulla oblongata, and sections from the cervical, thoracic, and lumbar cord, carefully observing that the same segments were always taken, including the injection site. Segments were semifixed in 8 % buffered formaldehyde for 2 days. When semifixed, the vertebral arches were opened, and the spinal cord and the roots of the spinal nerves were removed and completely fixed. After embedding in gelatin and Paraplast, a fast red 7B and myelin sheath staining according to Olivecrona were performed on serial sections from the site of injection as well as from cervical, thoracic and lumbar marrow. Hematoxylin–eosin staining as well as periodic acid–Schiff (PAS) staining and the iron reaction were all performed in one section. The presence or absence of nerve damage and of inflammation was noted.

A chronic model for investigation of experimental spinal anesthesia in the **dog** was described by Feldman and Covino (1981).

Dohi et al. (1987) inserted a polyethylene catheter into the lumbar subarachnoid space in **dogs** through a small hole in the dura for administration of drugs. The tip of the catheter was placed approximately 2–3 cm cephalad to the lumbar electrode introduced for the recording of hydrogen clearance to measure spinal cord blood flow.

Kozody et al. (1985) measured spinal cord and spinal dural blood flow in the cervical, thoracic, and lumbosacral regions in **dogs** using the microsphere technique. Measurements were taken 20 and 40 min after lumbar subarachnoidal injection through the L5–L6 or L6–L7 interspace.

Yaksh et al. (1995) studied the safety of chronically administered neostigmine methylsulfate in rats and **dogs**. Adult beagle dogs weighing 13–17 kg were adapted for 5 days to experimental protocols and placement of a nylon vest. For placement of the spinal catheter, the dogs were sedated (atropine 0.04 mg/kg and xylazine (Rompun) 1–2 mg/kg i.m.) given an i.m. injection of penicillin G and procaine. Anesthesia was induced by mask administration of halothane (3–5 %) and then the trachea was intubated. The dog was maintained under

spontaneous ventilation with 1–2 % halothane and 50 % N_2/50%O_2. Surgical areas on the back of the neck and head were shaved and prepared with alcohol and a povidone-iodine scrub, and the dog was placed in a stereotaxic head holder. After draping and using sterile technique, the cisterna magna was exposed, and a small incision (1–2 mm) was made. The intrathecal catheter (polyethylene rubbing PE-50 stretched by 30 %, making the nominal diameter 0.6 mm) was inserted and passed caudally at a distance of 40 cm, to a level corresponding approximately to the L3–L4 segment. The presence of the catheter in the intrathecal space was confirmed by free withdrawal of cerebrospinal fluid. A small stainless steel screw was placed in the skull and the catheter fixed to the screw. The catheter was tunneled subcutaneously and caudally to exit on the upper left back at the level of the scapula. The incision was closed by sutures, the halothane turned off, and the animal allowed to recover. An analgesic was administered for postoperative pain medication. At this time, the catheter was connected to the infusion pump placed into a vest side pocket, and an infusion of sterile saline (2 ml/day) was started.

For a 28-day infusion study, dogs were randomly assigned to receive saline or neostigmine (4 mg/4 ml). After 28 days of infusion, the dogs were sacrificed. After induction of deep anesthesia, the animal was manually ventilated to maintain adequate oxygenation. A percutaneous puncture of the cisterna magna was performed and cerebrospinal fluid withdrawn for analysis. The chest was opened and a large-bore cannula placed in the aortic arch through which was perfused saline followed by 10 % formalin. After fixation, the dura was exposed by an extensive laminectomy of the spinal canal and the lower brainstem, being careful to leave the catheter and the dura undisturbed. Dye was injected through the catheter to determine its integrity, visualize the position of the intrathecal catheter, and determine the spread of dye around the catheter. The spinal cord was removed in four blocks (cervical, thoracic, caudal, and rostral from the catheter tip), taking care to keep the dura intact, and placed in formalin. After fixation, tissue blocks were embedded in paraffin and then decalcified overnight, embedded in paraffin, sectioned at a thickness of 6–7 μm, and stained with hematoxylin and eosin. Particular attention was given to the presence or absence of fibrosis and other reactions around the catheter, dural thickening, or other reactions such as inflammation in the epidural space, leptomeninges/subarachnoid space, or spinal cord parenchyma, microglial nodules, demyelinization, or gliosis. The degree of chronic and/or acute inflammation was graded as normal, mild, moderate, or severe.

Studies in Other Species

Bahar et al. (1984b) performed chronic implantations of nylon catheters into the subarachnoid space of Wistar **rats** and **marmosets** and tested the effects of local anesthetics.

The technique of evaluation of spinal anesthesia by a local anesthetic in the **rhesus monkey** was described by Denson et al. (1981).

Lebeaux (1975) recommended the **sheep** as a model for testing spinal and epidural anesthetic agents. To ensure accurate and reproducible administration of drugs into these restricted anatomical regions, a wooden stock was built to restrain the sheep without stress but to allow arching of the lumbosacral spine in order to open the intervertebral space. The stock consisted of a table with holes for the front feet, hinged at one end to a fixed stanchion. The hinges permitted tilt of the table. The sheep was placed prone on the table, the head secured in the stanchion, and the front feet passed through the holes and tied. The lumbosacral interspace used for both spinal and epidural tap was separated by positioning and tying the rear limbs cephalad beside the abdomen, thereby extending the knee and flexing the hip. The wool was clipped from the sheep's back, the skin of the lumbosacral area was washed and swabbed with iodine, and the vertebral spines and interspaces identified by palpation were marked and numbered with a felt pen.

Spinal block was performed using either the midline or lateral approach for tapping the subarachnoid space. A 22-gauge disposable spinal needle was inserted subarachnoidally, and proper position was verified by the spontaneous flow of cerebrospinal fluid upon removal of the stylet.

With the table top and sheep parallel to the floor, the Luer-Lok syringe holding 1 ml of sterile 5.0 % dextrose solution containing 2.5 mg (0.25 %) tetracaine was attached to the needle. After aspiration of spinal fluid, the solution was injected within 5 s. At completion of the injection, the spinal fluid was again aspirated to confirm the injection into the subarachnoid space. The needle was withdrawn and the table tilted 10° caudally to restrict the spread of the hyperbaric solution to the lumbosacral area. The sheep was held in this position for 20 min.

Immediately after injection, the rear feet were untied and allowed to hang free on either side of the table for evaluation of sensation and/or reflexes in the rear limbs. Then, 20 min after injection, the sheep was removed from the table and positioned in a sling fashioned from a heavy-duty 50-gal plastic barrel split in half longitudinally and mounted on a metal support. Holes cut in the barrel allowed the feet to drop through so that comfortable sternal recumbency was maintained while permitting anesthetic evaluation to continue. When complete sensation returned in the skin of the dorsum, the sheep was removed from the sling and placed on a straw-bedded floor so that weight support and full recovery could be assessed.

Kyles et al. (1992) described a simple and noninvasive method for the chronic implantation of intrathecal catheters in the **sheep**.

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Blockade of Urethral Reflex in Rabbits

Purpose and Rationale

For evaluation of spinal anesthetics, the test substance has to be injected into the spine. For the study of local anesthetics for which conduction anesthesia has been verified, intraspinal injection

provides more complete information. Spinal anesthesia has been studied in rabbits (Bieter et al. 1936a, b; Luduena and Hoppe 1951; Luduena 1957; Gonzales and Luduena 1961; Turner 1965), cats (Sechzer 1965), and dogs (Dvorak and Manson 1930). If the solution of test substance is injected intrathecally to male rabbits, the presence of anesthesia may be determined by the urethral reflex (Luduena 1957).

Procedure

Male Chinchilla rabbits weighing 3.0–3.5 kg are used. The volume administered intrathecally is 0.02 ml per centimeter of spinal length. It is injected at a rate of 1 ml per min with a 22-gauge needle that is 3.8-cm long. The needle is introduced between the sixth and seventh lumbar vertebrae and not through the lumbosacral space. The needle, held as lightly as possible between the thumb and forefinger, is introduced slowly until a typical sharp and sudden twitch occurs. This indicates penetration into the subarachnoidal space. A 1-ml tuberculin syringe is then attached to the needle. It is not possible to aspirate fluid after insertion of the needle. However, it is possible to aspirate a portion of the injected solution. Very little pressure is required for injection of 0.4–0.6 ml of anesthetic solution while the animal is restrained in a canvas hammock. A catheter is inserted into the urethra, where it is kept without digital pressure. At 15- or 20-s intervals, 2–3 ml of water, at room temperature, is injected rapidly into the catheter. This causes the "urethral reflex," consisting of the retraction of the penis and contraction of the anal sphincter. The water runs out of the urethra around the catheter. Without medium or high concentrations of the anesthetics, the reflex is absent on the first reading. After loss of the urethral reflex, the test is repeated every minute or two for 15 min, and thereafter at longer intervals. The duration of urethral areflexia is taken as the time of the first positive reading (reflex absent) to the middle of the interval between the last positive and the first negative reading. This duration varies linearly with the logarithm of concentration.

Evaluation

The threshold anesthetic concentration in grams per 100 ml for abolition of the urethral reflex for 5 min, the TAC_5, is a standard for comparison. There is a correlation between activity and systemic toxicity. The ratio of the activity to the irritancy is the most important parameter.

Modifications of the Method

Burdyga and Magura (1986) studied the effects of local anesthetics on the electrical and mechanical activity of the guinea pig's ureter.

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Endoanesthetic Effect

Purpose and Rationale

Endoanesthesia has been described as the effect of local anesthetics on viscero-afferent receptors, located predominantly in the lung as distension receptors (Meier and Bein 1950; Zipf 1953, 1957, 1959, 1966, 1968; Zipf et al. 1955; Zipf and Oehler 1955; Reichertz et al. 1957; Zipf and Reichertz 1957; Zipf et al. 1963; Wellhöner and Conrad 1965; Siemoneit et al. 1966; Dittmann and Zipf 1973; Borchard 1979). The endoanesthetic effect has been claimed to be the basis of an antitussive action of local anesthetics (Bucher 1956; Bein and Bucher 1957; Kraushaar et al. 1964).

Procedure

Male guinea pigs weighing 300–400 g are anesthetized by the intraperitoneal injection of 1.25 g/kg urethane. The studies are carried out on spontaneously breathing or artificially respirated monovagotomized or bivagotomized animals. Intravenous injections are given via a catheter into the jugular vein. The flow rate of the respiratory air is measured with a Fleisch tube connected to a pneumotachograph. In experiments with positive pressure respiration, the open end of the Fleisch tube is attached to a respiratory pump (40 strokes per min). A cannula is inserted into the carotid artery and connected to a pressure transducer.

The left nervus vagus is exposed from the thorax aperture until its entry into the foramen jugulare and cut in the middle of the distance. Both ends are placed over three wires of Ag/AgCl 6-way electrodes. The edges of the skin wound are pulled upward with clamps, and the space around the trachea is filled with paraffin to prevent the nerve from drying out. The afferent and efferent vagus potentials and also their integration curve (Zipf and Reichertz 1957) are recorded on a direct recorder. In addition, the ECG in the second lead is recorded continuously. The animal is shielded electrically with the aid of a Faraday cage.

The nervus vagus conducts not only afferences from the lung but also from other organs to the brain. The base points of the integral curve, which correspond to the vagus action potentials being synchronous with inspiration and issued by the pulmonary distension receptors, therefore lie above the baseline of the recorder tracing. The vagus afferences which remain after subtraction of the vagus activity synchronous with respiration ("inspiratorial activity") are called "residual activity." The level of inspiratorial activity depends both on the bronchial width (degree of inflation of the lungs) and on the functional state of the pulmonary receptors and of the afferent pathway. The occurrence of a bronchospasm is detectable from the decrease in the pneumotachogram, if the respiratory rate is not reduced at the same time.

Evaluation

The following parameters are evaluated:

Afferent and efferent inspiratorial vagal activity, maximum flow rate of the respiratory air during inspiration and expiration, tidal volume, duration of the respiratory phases (inspiration $= t_i$, expiration $= t_e$, respiratory pause $= t_p$), heart rate, and blood pressure.

The decrease of electrical activity after various doses of standard and test drugs is calculated at different time intervals after drug administration. Maximum of activity and decay with time are registered.

Statistical analysis is performed using the paired and non-paired *t*-test, after the applicability has been first checked with the F-test.

Critical Assessment of the Method

The correlation between antitussive and local anesthetic activity has been challenged by Sell et al. (1958) and by Ther and Lindner (1961).

Modifications of the Method

Bein and Bucher (1967) and Wellhöner and Conrad (1955) tested various compounds for their anesthetic effect on pulmonary stretch receptors of **rabbits**.

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Effect on Electroretinogram

Purpose and Rationale

The retina, which belongs in ontogenesis to the central nervous system, can be prepared without damaging the parenchyma. The transretinal potential which can be induced by a light flash

can be used as an objective criterion for the functional state (Sickel et al. 1960). The multi-phase potential path of the electroretinogram consists of an initial a-wave, a subsequent b-wave, and an e-wave which starts with a delay after the end of the stimulus. While the amplitudes of the a- and e-wave are in the order of 50 μV, the amplitude of the b-wave (Φ_b) is 500–1,000 μV. The electroretinogram can be used for various purposes, such as the evaluation of local anesthetics (Borchard 1979).

Procedure

Frogs (*Rana esculenta*) are kept in dark for 1 h before beginning of the preparation. The frog is sacrificed by decapitation, and the eyeball is excised in dim red light and cut into two halves with the aid of a rotating blade. The retina together with the pigmented epithelium and choroid is removed from the rear half of the eye and transferred to a dish with Tyrode solution. The retina is then detached from the pigmented epithelium by careful shaking and spread out on a Monodur net. The net is fixed to a round carrier with a narrow ring and the chamber is transferred to a flow-through apparatus. An electrical pump is used for the superfusion of the retina with Tyrode solution.

In order to apply the local anesthetic only to the receptor or vitreous body side of the retina, an apparatus is used in which the tissue on a silk net is stretched as the partition between two chamber halves which can perfused separately (Borchard and Erasmi 1974).

The electroretinogram is recorded with Ag/AgCl electrodes. After amplification, the signal is registered on a recorder or an oscillograph. Light stimuli of 10 ms are generated. The light from a low-voltage light source is focused on a electromagnetic shutter with a stimulus frequency of 0.1 Hz. Light stimuli of 1 s duration are used at intervals of 3 min to measure the influence of the local anesthetics on the electroretinogram as a function of light intensity.

All experiments are performed after adaptation to darkness and at room temperature. The amplitude of the b-waves (Φ_b) which is measured from the lowest point of the a-wave to the maximum of the b-wave is used for evaluation. In order to obtain stationary test conditions, the experiment is started after a 45-min adaptation period. The stimulus signal is recorded by means of a photocell inserted in the light path.

Various concentrations of the local anesthetics are applied and various light intensities are used. The decrease of the b-wave starts immediately and then progresses slowly and continuously. After changing over to Tyrode solution, the decrease in the exposure potential is completely reversible.

Evaluation

After various doses, time–response curves can be drawn, and for a given time interval, ED_{50} values can be calculated. Moreover, the influence of local anesthetics on the b-wave at various light intensities can be estimated.

Modifications of the Method

Isolated retina for electroretinogram has been used not only from **frogs** but also from **rats** (Huang et al. 1991; Doly et al. 1993), from **rabbits** (Mochizuki et al. 1992; Maynard et al. 1998), and from **cows** (Gosbell et al. 1996; Walter et al. 1999).

Moreover, the electroretinogram has been used as parameter for many in vivo studies with different purposes, e.g., on the effect of drugs after retinal ischemia, in various animal species, such as **rats** (Sugimoto et al. 1994; Hotta et al. 1997; Biró et al. 1998; Block and Schwarz 1998; Estrade et al. 1998; Ettaiche et al. 1999; Li et al. 1999), **rabbits** (Takei et al. 1993; Zemel et al. 1995; Horiguchi et al. 1998; Jarkman et al. 1998; Liang et al. 1998), **cats** (Imai et al. 1991; Ostwald et al. 1997; Kim et al. 1998), **dogs** (Jones et al. 1995; Yanase and Ogawa 1997), or **monkeys** (Tagliati et al. 1994).

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Neuromuscular Blocking Activity

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General Considerations

Neuromuscular transmission is mediated by nicotinic acetylcholine receptors for which various subtypes are described (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

Neuromuscular blocking agents are distinguished by whether or not they cause depolarization of the motor end plate. They are classified either as competitive (stabilizing) agents, of which D-tubocurarine is the classical example, or as depolarizing, desensitizing agents such as succinylcholine.

The **rabbit head-drop method** was described by Varney et al. (1948, 1949), Burn et al. (1952), and Levis et al. (1953). Rabbits were given an interrupted intravenous injection at a rate of 0.1 ml every 15 s until the muscles supporting the head become sufficiently relaxed to prevent the head to be raised when the back is stimulated. This method has been replaced by the rabbit sciatic nerve-gastrocnemius muscle preparation, the isolated phrenic nerve diaphragm preparation of the rat, and the chick sciatic nerve-tibialis anticus muscle preparation.

The method described by Allmark and Bachinski (1949) used an inclined screen for testing curare-like activity in **rats**.

Skinner and Young (1947) placed **mice** weighing 15–17 g in sloping rotating cylinders. Mice falling away during 20 min after subcutaneous injection were considered as reactors. Dose-response curves and potency ratios could be calculated from the logarithmic dose-response curves using different doses of test compound and the standard tubocurarine chloride.

Collier et al. (1949) used a rotating drum to assess the activities of paralysant, convulsant, and anesthetic drugs in mice.

Fatt and Katz (1951) performed an extensive study on neuromuscular junction by recording end-plate potentials from curarized frog sartorius motor end-plates, using a KCl-filled microelectrode inserted into the muscle fiber in the region of the motor end-plate.

Electrophysiological analysis of transmission at the skeletal neuromuscular junction was reviewed by Prior et al. (1993).

A review on new neuromuscular blocking drugs was given by Hunter (1995).

Savarese et al. (1975) discussed the potential uses of short-acting non-depolarizing neuromuscular-blocking agents as predicted from animal experiments.

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Isolated Phrenic Nerve Diaphragm Preparation of the Rat

Purpose and Rationale

The isolated phrenic nerve diaphragm preparation of the rat was originally described by Bülbring (1946) to study the influence of adrenaline on tissue functions normally elicited by acetylcholine. The method has been modified and is widely used by many investigators for studying drugs affecting the neuromuscular transmission.

Procedure

Adult male Wistar rats are used. The animal is sacrificed and the blood is drained. The skin is removed from the middle of the chest. The thorax is opened and the front part of the left thoracic wall is removed. The phrenic nerve can be seen quite distinctly. The nerve is cut just below the thymus and a thread is attached to the cut end. The nerve is then freed carefully from the attached tissue. However, no attempt is made to clean the nerve completely from all attached tissue. An incision is made in the left abdominal wall just below the diaphragm. Two converging cuts are made through the diaphragm and the ribs towards the tendinous part of the diaphragm with the phrenic nerve attached to the center of the diaphragm. The fan-like preparation is about 3 mm wide at the tendinous end and is about 15 mm wide at the costal margin. A thread is attached to the tendinous part of the diaphragm.

The preparation is fixed by a stainless steel rod with a pair of pins hooked to the rib. It is lowered into an organ bath and the thread from the muscle is attached to a force transducer. The nerve is stimulated with a pair of electrodes with a hole about 1 mm wide. The right phrenic nerve-diaphragm preparation is isolated in the same manner. The organ bath containing Tyrode solution with 2.0 g/l glucose is oxygenated at 37 °C with 95 % O_2 and 5 % CO_2. The nerve is stimulated 12 times per min by rectangular-wave pulses of 0.5 ms duration at 3–5 V. Contractions are isometrically recorded through a transducer on a polygraph. The test drugs are left in the organ bath either for short periods of time (3–8 min) or for as long as the maximum effect can be observed. After a wash-out period of 3–5 min, the next dose can be added.

Evaluation

The force of contractions after the addition of various doses of the test drug is compared with the effect seen prior to drug application. Dose-response curves can be established.

Critical Assessment of the Method

The isolated phrenic nerve diaphragm preparation of the rat is an excellent method for determining the potency of a drug to block or facilitate neuromuscular transmission. However, it is not a good preparation for differentiating between depolarizing and non-depolarizing neuromuscular blocking agents because, in many cases,

depolarizing blocking agents fail to demonstrate initial facilitation and fail to reverse the effect of non-depolarizing blocking agents. For differentiating between depolarizing and non-depolarizing blocking agents, the chick sciatic nerve-tibialis anticus muscle preparation is preferred. In this preparation, the curare-like drugs produce a neuromuscular blockade whereas the decamethonium-like drugs induce a contracture of the slow fiber when the neuromuscular transmission is blocked.

Modifications of the Method

Colbert et al. (1990) studied the effects of temperature on the experimental reliability of the isolated rat phrenic nerve/diaphragm preparation.

Vizi et al. (2003) used in vitro isolated phrenic nerve-hemidiaphragm preparations of mice, rats, and guinea pigs and in vivo sciatic nerve-anterior tibialis muscle preparations from anesthetized rats, guinea pigs, and cats to characterize a new short-acting non-depolarizing muscle relaxant.

Van Riezen (1968a, b) described the sciatic nerve-tibialis anticus muscle preparation in **chicks**. Three- to eight-day old chicks are decapitated, and the skin of the legs is rapidly removed. The leg is separated from the body by cutting through the hip joint. The muscles of the thigh are dissected and the sciatic nerve with the superficial peroneal branch is freed from the upper leg tissue. The fascia is removed from the lower leg and the tibialis anticus tendon identified. A thread is attached to the tendon of the muscle, and the tibialis anticus muscle is freed towards but not up to the knee joint attachment where the nerve enters into this region. The upper and lower leg bones are then cut off leaving the muscle with its nerve attached to the knee joint. The tendon is fasted by a hook to a ring in the bottom of the organ bath. A thin steel rod is attached to the knee joint. The contractions of the muscle are recorded isometrically. The nerve is passed through an electrode similar to that used in the rat phrenic nerve-diaphragm method. The nerve is stimulated six times per min for 0.5 ms duration at supramaximal voltage.

Jenden et al. (1954) described the isolated lumbrical muscle of the **rabbit** as a preparation which is sensitive to competitive (D-tubocurarine-like) and depolarizing (decamethonium-like) blocking agents. This muscle is cylindrical in shape, about 18 mm long and 1 mm in diameter and has a wet weight of about 15 mg.

Jenden (1955) used the isolated diaphragm of **guinea pigs** to study the effect of drugs upon neuromuscular transmission.

Hoppe (1955) reviewed the potency of neuromuscular blocking agents in various species.

Birmingham and Hussain (1980) used the phrenic nerve-diaphragm and the hypogastric nerve-vas deferens preparation of the guinea pig for comparison of the skeletal neuromuscular and autonomic ganglion-blocking potencies of non-depolarizing relaxants.

Wessler and Kilbinger (1986), Wessler and Steinlein (1987), and Wessler et al. (1992) described a modified **rat** phrenic nerve-hemidiaphragm preparation whereby most of the muscle was cut off (end-plate preparation).

Muir et al. (1989) used biventer cervicis nerve-muscle preparations from **young chickens** and phrenic nerve-hemidiaphragm preparations from **rats** to evaluate neuromuscular blocking agents. Micro-electrode recordings were obtained from the nerve-hemidiaphragm preparation of the rat and from the costocutaneous nerve-muscle preparation of the North American **garter snake** (*Thamnophis sirtalis*). The later preparation is particularly suitable for voltage clamp recording from the neuromuscular junction because it possesses large diameter fibers which aid visualization and penetration of endplates. This, coupled with compact endplates, allows good control of membrane voltage over the entire endplate region when using the two-microelectrode voltage clamp technique.

In order to record evoked endplate currents without accompanying muscle contraction, cut fiber preparations were used. Dissection and cutting of muscle fibers was performed at low K^+ (2 mmol/L) physiological solution perfused for approximately 30 min. Snake nerve-muscle preparations were mounted in a physiological salt solution of pH 7.1–7.2 containing (mmol/l) NaCl

159, KCl 4.2, $CaCl_2$ 1.5, $MgCl_2$ 4.2, and HEPES 10. Rat nerve-muscle preparations were mounted in Krebs solution of the same composition as that used for tension experiments. The muscles were mounted in a Sylgard-coated Perspex dish and endplates were voltage clamped using glass capillary microelectrodes (resistance 2–10 mΩ). Voltage recording electrodes were filled with potassium chloride 3 mol/l and current passing electrodes were filled with potassium sulfate 0.6 mol/l.

The nerves were stimulated through platinum electrodes at a frequency of 0.5 Hz with rectangular pulses of 0.05 ms duration and of strength sufficient to produce evoked endplate currents. The evoked endplate currents were filtered by a 5-kHz low pass filter and recorded on magnetic tape. The currents were amplified and digitized by a laboratory interface connected to a computer at a digitization rate of 25 kHz. Ten to 20 evoked endplate currents were collected and averaged after alignment at the middle of their rising phase. Evoked endplate currents decayed as a single exponential function according to the following relationship:

$$I(t) = I(0)\exp^{-t/\tau}$$

where $I(t)$ is the current amplitude at time t after the peak, $I(0)$ is the peak current amplitude, and τ is the decay constant.

Experiments were carried out at room temperature. Drug solutions were perfused through the tissue bath for 10 min by peristaltic pump, ensuring a complete exchange of solution.

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Sciatic Nerve-Gastrocnemius Muscle Preparation in the Rabbit

Purpose and Rationale

Levis et al. (1953) and Long and coworkers (1959, 1967, 1969) described the sciatic nerve-gastrocnemius muscle preparation in the rabbit as an in vivo model for testing neuromuscular blocking agents.

Procedure

Dutch rabbits, weighing 1–2 kg, are anesthetized with 200 mg/kg of phenobarbital administered slowly into the marginal ear vein. The sciatic nerve is ligated and cut, and a shielded electrode is placed on the peripheral portion of the nerve. The gastrocnemius muscle is freed as completely as possible from surrounding muscles and a thread is attached to the tendon of the muscle. The twitches of the muscle are elicited by supramaximal stimulation and are recorded through a force transducer. The parameters for interrupted tetanic stimulation are 250 c.p.s. with pulse durations of 1 ms at 15 V applied for 0.2 s every 10 s. The test drugs are administered intravenously into the marginal ear vein.

Evaluation

The force of contraction after injection of various doses of the test drug is compared to the values obtained prior drug administration.

Critical Assessment of the Method

The preparation has the advantage of studying the drug effects under conditions similar to clinical use.

Modifications of the Method

There are two other preparations, the **cat soleus muscle** preparation and the **cat tibialis anticus muscle** preparation that very useful. They can be prepared in a method similar to that described for gastrocnemius muscle except that close arterial injection can be made using the tibialis anticus preparation. Also, soleus muscle consists primarily of slow muscle, whereas tibialis anticus is fast muscle. The experimental procedures for these

two preparations are described in detail by Brown (1938), Bowman et al. (1962), and Salafsky (1968).

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Evaluation of Neuromuscular Blockade in Cats, Pigs, Dogs and Monkeys

Purpose and Rationale

Neuromuscular blocking drugs have been evaluated in various animal species. Muir et al. (1991) studied the effects of an analog of vecuronium in anesthetized cats, pigs, dogs and monkeys.

Procedure

Anesthetized Cats

Experiments were carried out on cats of either sex anesthetized with a mixture of α-chloralose (80 mg/kg) and pentobarbitone (5 mg/kg) injected intraperitoneally. Animals were ventilated with air at a rate of 26 breaths per minute using a tidal volume of 13 ml/kg. The right hind limb was immobilized and the contractile responses of the tibialis anterior and soleus muscles to single shock stimulation of the sciatic nerve were recorded. The sciatic nerve was stimulated at a rate of 0.1 Hz using rectangular pulses of 0.2 ms duration and of a strength greater than that required to produce a maximal twitch. Contractions of the nictitating membrane were evoked in response to preganglionic stimulation of the cervical sympathetic nerve with 10 s duration trains at a frequency of 5 Hz and a strength sufficient to produce maximal contractions of the nictitating membrane. Arterial blood pressure was recorded from the carotid artery using a Statham PC45 pressure transducer. The blood pressure pulse

triggered a cardiotachograph to display the heart rate. Both vagus nerves were ligated, and at 100-s intervals, the right vagus nerve was stimulated with 10 s duration trains at a frequency of 2–5 Hz and with pulses of 0.5 ms duration and strength greater than that required to produce a maximal reduction of heart rate. Contractile responses of muscles were recorded using Grass FTO3C and FT10C force displacement transducers. All responses were displayed on a Grass model 5 in. writing oscillograph.

Savarese (1979) determined not only the potencies of metocurine and D-tubocurarine but also the autonomic margins of safety in anesthetized cats.

Procedure

Adult cats of either sex are anesthetized with α-chloralose, 80 mg/kg, and pentobarbital 7 mg/kg, given intraperitoneally. Cannulas were placed in the left femoral vein and artery for drug injection and recording blood pressure and heart rate. The lungs were mechanically ventilated through a tracheostomy and a small animal ventilator set to deliver 15 ml/kg tidal volume and 20 breaths/min.

The right vagus nerve and the right sympathetic trunk were exposed and divided in the neck. The distal ends were placed on the same shielded platinum wire electrode to permit preganglionic stimulation of both nerve trunks. The left sympathetic trunk was also dissected along its postganglionic portion at the base of the skull, and cut distal to the superior cervical ganglion to permit postganglionic stimulation through another electrode. Trains of square wave pulses (20 Hz for 10 s) were delivered at supramaximal voltage every 4 min simultaneously to all three autonomic nerve trunks. The resulting bradycardia and hypotension (the vagal response) were measured. Contractions of both nictitating membranes, one (the right) elicited pregangionically and the other (the left) elicited postganglionically, were recorded. The maximal vagal response (i.e., cardiac arrest for 10 s) was achieved by stimulation of the right vagus nerve or of both vagus nerves.

Twitches of the right tibialis anterior muscle were elicited at 0.15 Hz via the peroneal branch of the right sciatic nerve, to which square-wave shocks of 0.2 ms were applied at supramaximal voltage. Twitch recording was done via a transducer. All nerves and tendons were kept moist in small pools of mineral oil or in cotton pledges soaked in mineral oil. Tibialis anterior and esophageal muscle temperatures were monitored and kept between 35 °C and 38 °C by heat lamps.

Simultaneous recordings of heart rate, arterial pressure, pre- and postganglionic elicited contractions of the nictitating membrane, and twitches of the tibialis anterior muscle were made on a polygraph. Cumulative dose–response curves for the inhibition of neuromuscular, vagal (parasympathetic), and sympathetic functions were determined simultaneously for each animal. The mechanism of vagal inhibition was localized at parasympathetic ganglia or cardiac muscarinic receptors by determining whether the bradycardic response to methacholine (20 μg/kg) was blocked as well as the neurally elicited bradycardia.

A single-bolus dose of the neuromuscular relaxants producing the delayed depressor response plus tachycardia (Paton 1957) was determined in each animal. This response, being pathognomonic for histamine release, is defined as sudden hypotension to less than 80 % of the control arterial pressure within 2 min of relaxant injection and with tachycardia to more than 25 % above the baseline value.

Test drugs were given intravenously.

Evaluation

Data analysis was done by the method of Litchfield and Wilcoxon. Mean dose–response curves were plotted on log-probit paper. Best fit to straight lines on these scales was determined by computerized regression. The cumulative ED_{50} values for vagal and sympathetic inhibition and the cumulative ED_{95} values for neuromuscular blockade were determined from the lines and 95 % confidence limits were calculated. Differences in potency were considered significant when $P < 0.05$.

The occurrence of histamine release was also treated as an all-or-none response to permit

log-probit plotting. The delayed depressor response plus tachycardia was judged to have or have not occurred after each single bolus injection of the drugs. The percentage of animals responding at each dose level was then determined and the data handled by the Litchfield–Wilcoxon method.

The autonomic margins of safety of the test drugs were calculated as the ratios of cumulative doses producing 50 % block (ED_{50}) of vagal (parasympathetic) and sympathetic transmission and the ED_{50} for histamine release, each divided by the ED_{50} of neuromuscular blockade.

Anesthetized Dogs

Anesthesia was induced in beagle dogs using intravenous pentobarbitone sodium (3 mg/kg). Animals were intubated, without the use of a muscle relaxant, and artificially ventilated. Anesthesia was maintained with halothane (1.2 % inspired concentration) and oxygen. The electrocardiogram was recorded continuously and the signal used to integrate heart rate throughout the experiment. Catheters were inserted percutaneously into the right femoral artery and vein for the recording of arterial blood pressure and the injection of drugs, respectively.

A "boomerang" type transducer, originally constructed for recording adductor human pollicis tension (Walts 1973), was fitted to the lower left leg for recording muscle contractions. Submaximal rectangular pulses of 0.1 ms duration at a frequency of 0.1 Hz were delivered to the sciatic nerve through electrodes inserted percutaneously, and the resultant twitches of the foot recorded.

Clutton et al. (1992) studied the autonomic and cardiovascular effects of neuromuscular blockade antagonism in the **dog**. Neuromuscular blockade was antagonized with various anticholinesterase-antimuscarinic drug combinations including atropine, neostigmine, and glycopyrrolate.

Anesthetized Pigs

Domestic pigs of either sex (10–15 kg) were administered a tranquillizing dose (approximately 2 mg/kg) of azaperone about 20 min before the induction of anesthesia with 3–4 % halothane in oxygen. Following induction, anesthesia was maintained with α-chloralose (200 mg/kg) dissolved in polyethylene glycol 300 given slowly into a jugular vein. Approximately 1 h later, anesthesia was supplemented with additional chloralose given by slow intravenous infusion which was continued throughout the investigation. The lungs were mechanically ventilated with room air via a tracheal cannula at a rate of 28 breaths per minute and a tidal volume of 12–14 ml/kg. Arterial pressure was recorded through a polyethylene catheter placed in the right carotid artery and connected to a Gould-Statham pressure transducer. Heart rate was monitored continuously by using the arterial pulse pressure to trigger a Grass 7P4F cardiotachometer. Drugs were administered through a catheter in the contralateral vein. Contractions of the tibialis anterior and soleus muscle were recorded by force displacement transducers. A resting tension of 30 g was applied to each muscle. Twitches were evoked every 10 s by stimulation of the two branches of the sciatic nerve supplying the lower leg, immediately distal to the point where the nerve divides, using square wave pulses of 0.25 ms duration and at twice the voltage required to produce maximum contraction.

Anesthetized Rhesus Monkeys

Rhesus monkeys were anesthetized with intramuscular ketamine (10 mg/kg). Endotracheal intubation was performed without the use of muscle relaxants. Anesthesia was maintained using a single bolus of pentobarbitone sodium (4 mg/kg) given intravenously followed by infusion, which was adjusted to maintain a steady state of anesthesia. Heart rate was measured continuously by integration of the electrocardiogram. Atropine sulfate (0.25–0.5 mg) was administered i.m. to prevent excessive salivation. Artificial ventilation was supplied and blood pressure recorded continuously. The ulnar nerve was stimulated with rectangular electrical pulses via bipolar subcutaneous needle electrodes. The twitch responses of the adductor pollicis muscle were recorded via a Statham transducer attached by a wire to a small U-clamp, which was fixed firmly to the basal phalanx of the thumb.

Evaluation

Experiments were performed in the various animal species by injecting different doses at hourly intervals to obtain a range of neuromuscular paralysis, i.e., between 10 % and 95 % inhibition of single-twitch tension. From the data, dose-inhibition lines were constructed using the Levenberg-Marquardt non-linear interactive curve-fitting routine (Brown and Dennis 1972). From these data, doses producing 50 % inhibition of induced contractions of the tibialis anterior muscle (cat and pig), hind limb (dog), and adductor pollicis muscle (monkey) were calculated. Time course measurements were made with doses which produced between 85 % and 95 % neuromuscular block. Onset time was measured as the time from injection to the first maximally depressed contraction. The recovery time was the time from 75 % block to 25 % block and the duration of action was the time elapsing from injection to 90 % spontaneous recovery compared to pre-drug control twitch tension.

Modifications of the Method

Bowman et al. (1988) investigated structure-action relationships among some desacetoxy analogues of pancuronium and vecuronium in the anesthetized **cat**. Blockade of sciatic nerve-induced contraction of the tibialis and soleus muscles, as well as the effects on vagal-induced bradycardia and on sympathetically induced contractions of the nictitating membrane, were studied.

Khuenl-Brady et al. (1990) used anesthetized **cats** to study the effects of two new non-depolarizing neuromuscular blocking drugs. The indirectly evoked twitch tension of the anterior tibialis muscle elicited by supramaximal square-wave stimuli applied to the peroneal nerve was continuously quantitated by means of a force-displacement transducer and recorded. Onset time (from injection of muscle relaxant to maximum depression of twitch tension), duration of action, and recovery index were determined.

Muir et al. (1989) used tibialis anterior and soleus muscle/sciatic nerve preparations in anesthetized **cats** and **pigs** to evaluate neuromuscular blocking agents.

Hoppe (1950) used a nerve-muscle preparation in **dogs** to evaluate curarimimetic drugs. Diaphragmatic respiration was recorded directly by means of a light thread from a suture imbedded in the peritoneal aspect of the right hemi-diaphragm. Stimulation of the peripheral end of the sectioned tibial nerve was accomplished by an induced current once every 10 s. Muscle contraction was recorded from the Achilles tendon being severed just proximal to the calcaneus.

Hughes and Chapple (1976a, b, 1981; Hughes 1984) used nerve-muscle preparations of **cats**, **dogs**, and **rhesus monkeys**.

Ono et al. (1990) recorded the twitch tension of the gastrocnemius-soleus muscle in **rats** after stimulation of the distal stump of the tibial nerve under the influence of a centrally acting muscle relaxant.

Cullen et al. (1980) described two mechanical techniques to measure neuromuscular activity in the intact, anesthetized **dog**. Simultaneous stimulation of the dorsal buccal branch of the facial nerve and ulnar nerve was performed and the evoked mechanical muscle responses measured.

Keesey (1988) discussed the use of single-fiber electromyography (SFEMG) by the AAEE minimonograph 33 as an electrodiagnostic approach to defects of neuromuscular transmission.

Marshall et al. (1994) gave an overview of the pharmacology of rocuronium bromide in experimental animals.

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Evaluation of Neuromuscular Blockade in Anesthetized Mice

Purpose and Rationale

Electromyographic investigations of the neuromuscular junction are relatively easy to perform in large animal species such as the dog (Cullen et al. 1980). A simple in vivo method for the quantitative evaluation of neuromuscular blockade in anesthetized mice has been described by Lefebvre et al. (1992).

Procedure

Female BALB/c mice, 7–9 weeks old, are anesthetized by intraperitoneal administration of 50 mg/kg etomidate or 250 mg/kg mephenesin. Hair is removed from the sciatic area and hind leg by application of a depilatory cream. A lamp is placed 30 cm above the anesthetized animal to maintain a constant body temperature. Two monopolar needle-stimulating electrodes are subcutaneously inserted into the sciatic notch area and two monopolar recording electrodes

subcutaneously over the gastrocnemicus belly and in the vicinity of the tendo calcaneus communis, respectively. A ground electrode is inserted under the skin between recording and stimulating electrodes. Stimulation is carried out using a square signal of 0.2 ms duration. The supramaximal stimulation of the sciatic nerve consists of a train of 10 stimuli, lasting 3.3 s with a frequency of 3 Hz. Stimulation intensity is fixed to a value 50 % higher than that required to attain the maximal evoked potential response. Two control trains of stimulation are applied before test-drugs administration and then repeated at 1 min intervals until the end of anesthesia which is assessed by reflex movement after pinching the toes or the tail.

Muscle action potentials are recorded using an electromyograph. The low and high cut-off frequencies are set to 16 Hz and 10 kHz, respectively. The electromyogram signal is amplified and plotted on an oscilloscope screen and printed. Evoked active potentials are analyzed using an 8 bits digitizer, and the area under the response wave is evaluated for each stimulus (S).

The neuromuscular blocking agents are administered by intraperitoneal route.

Evaluation

The effects of the test drugs are quantified by measuring the ratio of the fifth response (S5) to the first one (S1) in a given train (S5/S1 response), according to Keesey (1989). These values are almost not influenced by etomidate anesthesia. Neuroblocking agents of competitive type, such as alcuronium, or depolarizing type, such as suxamethonium, decrease this ratio significantly. These effects can be blocked by neostigmine, but not the effect of the snake venom alpha-bungarotoxin.

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See Vogel (2006).

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Part VIII

Analgesic, Anti-Inflammatory and Anti-Pyretic Activity

Central Analgesic Activity

Vino Daniel

Contents

General Considerations

Many diseases have pain as a classical symptom requiring treatment with analgesics. Primary cancer and secondary metastases can lead to severe pain, which needs to be managed with strong analgesics, like opioid drugs. Opioids usually are safe when they are used correctly, but people who misuse opioids can become addicted. The addiction liability of opioids led to intensive research for compounds without this side effect. Many approaches have been used to differentiate the various types of actions of strong analgesics by developing animal models not only to study for analgesic activity but also to understand addiction liability. Several types of opioid receptors have been identified in the brain allowing in vitro binding tests. However, the in vitro tests can only partially substitute for animal experiments involving pain. Pain is a common phenomenon seen in all animals, at least in vertebral animals, similar to that felt by man. Analgesic effects in animals are comparable with the therapeutic effects in man. Needless to say, in every instance, painful stimuli to animals must be restricted as much as possible. Painful stimuli can be elicited due to direct stimulation of the efferent sensory nerves or stimulation of pain receptors by various means such as heat or pressure. The role of endogenous peptides such as enkephalins and endorphins gives more

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insight into how do brain processes pain and the action of central analgesics. Endorphin and enkephalin are the body's natural painkillers. We need to consider how an understanding of the opioid system affects clinical responses to opiate administration and what the future may hold for improved pain relief.

One of the salient features of inflammatory states is that normally innocuous stimuli can produce pain. Considerable progress has been made in elucidating the role of various endogenous substances such as prostaglandins and peptides in the inflammatory process. Most of the so-called non-steroidal anti-inflammatory agents have also analgesic activity. Lim and Guzman (1968) differentiated between antipyretic analgesics causing analgesia by blocking impulse generation at pain receptors in the periphery and narcotic analgesics that act by blocking synaptic transmission of impulses signaling pain in the central nervous system. An old but excellent survey on methods being used to test compounds for analgesic activity has been provided by Collier (1964). Today, the classification into central and peripheral analgesics is definitively too simplified (Bannwarth et al. 1993) but provides a guide for differentiation by pharmacological methods.

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In Vitro Methods for Central Analgesic Activity

Survey

High-affinity stereospecific binding of radiolabeled opiate compounds using CNS membrane preparations was reported in 1973 (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). The in vivo pharmacological potency of opiate agonists and antagonists parallels the in vitro displacement of ^{3}H-naloxone, a potent narcotic antagonist. Based on these findings, the ^{3}H-naloxone binding assay was introduced for evaluation of potential analgesics with opiate-like properties. Based on the different pharmacological profiles of opiates, several receptor types have been identified designated as μ-, κ-, δ-, and σ-receptors (μ for morphine = MOP receptor, κ for ketocyclazocine = KOP receptor, δ for deferens because it was first identified in mouse vas deferens = DOP receptor). The σ-receptor (σ for SKF10047) was only initially classified as an opioid receptor (see below). Several reviews on opioid receptors have been published: Knappe et al. (1995), Mansour et al. (1995), Satoh and Minami (1995), Dhawan et al. (1996), Singh et al. (1997), Standifer and Pasternak (1997), Law et al. (2000), Snyder and Pasternak (2003), Janecka et al. (2004), Eguchi (2004), and Waldhoer et al. (2004).

The four major subtypes of opioid receptors were reclassified according to recommendations of the International Union of Physiological Sciences and the International Union of Pharmacology (IUPHAR; Dhawan et al. 1996, 1998; Alexander and Peters 2000). This nomenclature applies an abbreviation of the generic term for the family (OP for opioid) and a subscript number. **OP_1 stands for δ-, OP_2 for κ-, and OP_3 for μ-receptor**.

For the **μ-receptor, subtypes named μ_1 and μ_2** have been described (Fowler and Fraser 1994; Traynor 1994; Pasternak 2001). Analgesia is thought to involve activation of μ-receptors (largely at supraspinal sites) and κ-receptors

(principally within the spinal cord); δ-receptors may also be involved at the spinal and supraspinal level. Other consequences of μ-activation include respiratory depression, miosis, reduced gastrointestinal motility, and euphoria. The μ_1-receptors are postulated to mediate the supraspinal analgesic action, the μ_2-receptors to mediate respiratory depression and suppression of gastrointestinal motility, and the μ_3 for possible vasodilation. Moreover, different effects on heart rate were described (Paakkari et al. 1992). Two endogenous peptides were described, named **endomorphins**, as agonists with high specific affinity for the μ-receptor (Hackler et al. 1997; Zadina et al. 1997, 1999; Horvath 2000).

Several studies provide evidence for the existence of **δ-opioid receptor subtypes** (Sofuoglu et al. 1991; Porreca et al. 1992; Horan et al. 1993; Miyamoto et al. 1993; Tiseo and Yaksh 1993; Burkey et al. 1998). Binding studies with δ-opioid receptors have been performed by Mosberg et al. (1983). Simonin et al. (1994) reported the genomic organization, cDNA cloning, the functional expression in COS cells, and the distribution in human brain of the human δ-opioid receptor.

Endogenous ligands for δ-receptors are **enkephalins**. An enkephalin is a pentapeptide involved in regulating nociception in the body. The enkephalins are termed endogenous ligands. They are internally derived and bind to the body's opioid receptors.

A rat **κ-opioid receptor** has been cloned (Meng et al. 1993). Evidence for different subtypes of the κ-opioid receptor is available (Zukin et al. 1988; Clark et al. 1989; Rothman et al. 1989, 1992, 1993; Wollemann et al. 1993). Simonin et al. (1995) described cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system of the δ-opioid receptor in humans.

Salvinorin A – derived from *Salvia divinorum*, a hallucinogenic plant used by Mazatec Indians of Mexico for traditional spiritual ceremonies – is a highly selective κ-opioid receptor agonist with antinociceptive effects (Yan and Roth 2004; John et al. 2006; Rothman et al. 2006; Stewart et al. 2006; Vortherms and Roth 2006).

Endogenous ligands for κ-receptors are **dynorphins**.

With the development of highly selective ligands, it has become possible to label selectively each of the μ-, δ-, and κ-opioid binding sites.

The **μ-binding sites** are labeled with [^{3}H]-[Tyr-D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (Kosterlitz and Paterson 1981), ^{125}I-FK 33-824 (Moyse et al. 1986), [^{3}H]-Tyr-Pro-MePhe-D-Pro-NH_2 (PL O17; Hawkins et al. 1987), or [^{3}H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2] (CTOP) (Hawkins et al. 1989); the **δ-binding sites** with [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (Akiyama et al. 1985; Cotton et al. 1985; Mosberg et al. 1987), [^{3}H]-[D-Ser^2 (O-*tert*-butyl), Leu^5]enkephalyl-Thr^6 (Delay-Goyet et al. 1988), [^{3}H]-[D-Pen^2-pClPhe^4,D-Pen^5] enkephalin (Vaughn et al. 1989), or [^{3}H]TIPP (Nevin et al. 1993); and the **κ-sites** with [^{3}H]-U-69593 (Lahti et al. 1985; Maguire et al. 1992), [^{3}H]-PD117302 (Clark et al. 1988), or [^{3}H]-CI-977 (Boyle et al. 1990) or [^{3}H]norBNI (Marki et al. 1995).

Cloning and molecular biology of opioid receptors has been reviewed (Reisine and Bell 1993).

Advances in research on non-peptide opioid receptor ligands were published by Kaczor and Matusiuk (2002).

Exploring the opioid system by gene knockout was described by Kieffer and Gaveriaux-Ruff (2002).

Oligomerization of opioid receptors and the generation of novel signaling units was discussed by Levac et al. (2003).

Other Receptors

There is some evidence that other opioid receptors may exist, such as a β-endorphin-sensitive **ε-receptor** (Wüster et al. 1981). The **ζ-receptor** (Zagon et al. 1991) and a high-affinity binding site referred to as the **λ-site** (Grevel et al. 1985) may also be part of the opioid receptor system.

The existence of a **σ-receptor** was first postulated by Martin et al. (1976) to account for the psychotomimetic effects of *N*-allylnormetazocine (SKF 10,047) in the chronic spinal dog. σ-Binding sites were proposed to be identical to phencyclidine binding sites based on the finding that phencyclidine was generalized to (+)-SKF 10,047 in drug discrimination tests. Further work led to the application of the **term σ to a unique class of non-opiate, non-phencyclidine sites** that may serve as receptors for an as yet unidentified neuromodulator or neurotransmitter (Monnet et al. 1994). At least two subtypes of binding sites, σ_1 and σ_2, are proposed (Bowen et al. 1989; Itzhak and Stein 1991; Karbon et al. 1991; Knight et al. 1991; Connick et al. 1992; Quirion et al. 1992; Leitner et al. 1994). Radioligands for σ-receptors (Weber et al. 1986; de Costa et al. 1989) and for subtype σ_1 (Matsuno et al. 1996) and subtype σ_2 (Mach et al. 1999) were described. Pharmacological studies indicate a role of σ-receptors not only in analgesia (Mach et al. 1999) but also in motor function (Walker et al. 1993), schizophrenia (Debonnel and de Montigny 1996; Guitard et al. 1998; Takahashi et al. 1999), and learning and memory (Maurice et al. 1999).

Isolated tissue preparations in which neurotransmission are sensitive to inhibition by opioids were used to study the heterogeneity of opioid receptors. The relative potencies of opioid agonists are assessed by their ability to inhibit the electrically evoked contractions of isolated tissue preparations from five different species: the contractions of the mouse vas deferens are inhibited by μ-, δ-, and κ-agonists (Maguire et al. 1992), those of **the guinea pig myenteric plexus-longitudinal muscle** preparation by μ- and κ-agonists (Berzetei-Gurske and Troll 1992), those of the **rabbit vas deferens** by κ-agonists, and those of the **hamster vas deferens** by δ-agonists (Sheehan et al. 1986), and the contractions of the **rat vas deferens** are inhibited mainly, but not exclusively, by δ-agonists. The actions of β-endorphin in the rat vas deferens are mediated by a further type of opioid receptors, termed **ε-receptor** (Wüster et al. 1981; Corbett et al. 1992; Smith and Leslie 1992).

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^{3}H-Naloxone Binding Assay

Purpose and Rationale

A good correlation between the in vivo pharmacological potency of opiate agonists and antagonists with their ability to displace radiolabeled naloxone has been reported. It was later discovered that Na^+ (100 mM) enhances the binding of antagonists and reduces the binding of agonists, and this has subsequently led to the development of an assay, used to classify compounds as opiate agonists, mixed agonist–antagonists, and antagonists by determining the IC_{50} values for ^{3}H-naloxone in the presence or absence of Na^+.

Procedure

Reagents

[N-allyl-2,3-^{3}H] Naloxone (38–58 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations, ^{3}H-naloxone is made up to a concentration of 100 nM and 50 µl is added to each tube yielding a final concentration of 5 nM in the assay.

Levorphanol tartrate is obtained from Hoffmann–La Roche. A stock solution of 1 mM levorphanol is made up in distilled water. This stock is diluted 1:200 in distilled water, and 20 µl is added to 3 tubes to determine stereospecific binding yielding a final concentration of 0.1 µM in the assay.

Dextrorphan tartrate is obtained from Hoffmann–La Roche. A stock solution of 1 mM dextrophan is made up in distilled water. This stock is diluted 1:200 in distilled water, and 20 µl is added to the tubes containing the various concentrations of test drug and the tubes for total binding.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. At least 7 concentrations are used for each assay. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. Whole brains minus cerebella are weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer with a Tekmar tissue homogenizer. The homogenate is centrifuged at 40,000 g for 15 min, the supernatant is decanted, and the pellet resuspended in fresh buffer and recentrifuged at 40,000 g. The final pellet is resuspended in the original volume of fresh 0.05 M Tris buffer. This yields a tissue concentration in the assay of 10 mg/ml.

Assay

310 µl	H_2O
20 µl	5 µM dextrophan (total binding) or 5 µM levorphanol (nonspecific binding)
50 µl	2 M NaCl or H_2O
50 µl	0.5 M Tris buffer, pH 7.7
20 µl	Drug or vehicle
50 µl	^{3}H-Naloxone
500 µl	Tissue suspension

The tubes are incubated for 30 min at 37 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed three times with ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint liquid scintillation cocktail. Stereospecific binding is defined as the difference between binding in the presence of 0.1 µM dextrophan and 0.1 µM levorphanol. Specific binding is roughly 1 % of the total added ligand and 50 % of the total bound in the absence of Na^+ and 2 % of the total added ligand and 65 % of the total bound ligand in the presence of Na^+ (100 mM).

The increase in binding is due to an increase in specific binding.

Evaluation

Data are converted into % stereospecific ^{3}H-naloxone binding displaced by the test drug. IC_{50} values are determined from computer-derived log-probit analysis. The sodium shift is calculated from IC_{50} values with and without NaCl. High sodium shifts are found with agonists, low values with antagonists, and medium values with mixed agonists–antagonists.

Data can be analyzed using a computer program as described by McPherson (1985).

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^{3}H-Dihydromorphine Binding to μ-Opiate Receptors in Rat Brain

Purpose and Rationale

μ-Receptors are considered to mediate the supraspinal activity of opioids. ^{3}H-Dihydromorphine (^{3}H-DHM) exhibits some selectivity for the μ-receptor, a high-affinity opiate binding site. The test is used to detect compounds that inhibit binding of ^{3}H-DHM in a synaptic membrane preparation obtained from rat brain.

Procedure

Reagents

[1,7,8-^{3}H]Dihydromorphine (^{3}H-DHM) (specific activity 69 Ci/mmol) is obtained from Amersham.

For IC_{50} determinations, a 20 nM stock solution is made up. Fifty µl is added to each test tube to yield a final concentration of 0.5 nM in the 2 ml assay.

Levallorphan tartrate is used for the determination of nonspecific binding. A 0.1 mM stock solution is prepared in deionized water. Twenty µl is added to each of the 3 tubes to yield a final concentration of 0.1 µM in the 2 ml assay.

A 1 mM stock solution is made up of the test compounds in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-6} to 10^{-9} M. At least seven concentrations are used for each assay.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebella are removed, weighed, and homogenized in 30 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 15 min, the supernatant decanted, and the pellet resuspended in the same volume of buffer. This homogenate is then incubated for 30 min at 37 °C to remove the endogenous opiate peptides and centrifuged again as before. The final pellet is resuspended in 50 volumes of 0.05 M Tris buffer, pH 7.7.

Assay

1,850 µl	Tissue suspension
80 µl	Distilled water
20 µl	Vehicle, or levallorphan, or appropriate concentration of drug
50 µl	[^{3}H]DHM

Tubes are incubated for 30 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed twice with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 0.1 mM levallorphan. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The K_D value for [^{3}H]DHM binding was found to be 0.38 nM by Scatchard analysis of a receptor saturation experiment. The K_i value may be calculated from the IC_{50} by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D.$$

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^{3}H-Bremazocine Binding to κ-Opiate Receptors in Guinea Pig Cerebellum

Purpose and Rationale

κ-Receptors are thought to be involved in the analgesic activity of opiates mainly within the spinal cord, whereas μ-receptors are

predominately located at supraspinal sites. The pharmacological effects of κ-agonists differ from the μ-agonists in various analgesic tests, with effects on diuresis, sensitivity to naloxone, and propensity to cause respiratory depression. κ-Agonists may induce water diuresis (Salas et al. 1992). The receptor subtype selectivity can be determined by testing the affinity of new compounds for the κ-opiate receptor and comparing these results with the data from the μ-receptor assay.

Although the benzomorphanes, such as ethylketocyclazocine and bremazocine, are potent κ-agonists, they are not selective for this receptor subtype. To demonstrate specific binding of these ligands to κ-receptors, the assay must be done in a tissue where the κ-subtype predominates, such as the guinea pig cerebellum. Moreover, binding to μ- and δ-receptors is prevented by inclusion of the peptide DAGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) to mask the μ-receptor and of [D-Pen^{2,5}]-enkephalin (Tyr-D-Pen-Gly-Phe-D-Pen) to mask the δ-receptor.

Procedure

Reagents

Bremazocine(−)-[9-^{3}H] (specific activity 21–28 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations, a 24 nM stock solution is made up. Fifty μl is added to each tube to yield a final concentration of 0.6 nM in the 2 ml assay.

U50,488H is made up to a 500 μM stock solution in deionized water. Twenty ml is added to each of the 3 tubes for the determination of unspecific binding yielding a final concentration of 5.0 μM in the 2 ml assay.

Opiate peptides of the μ- and δ-type are included in the assay to prevent binding of the radioligand and the test drug to these receptors. DAGO and [D-Pen^{2,5}]-enkephalin are obtained from Peninsula Laboratories. Concentrated stock solutions of 10^{-3} M are made up in deionized water and further diluted to 10^{-5} M. Twenty μl of this solution is added to each tube to result in a final concentration of 100 nM of each in the 2 ml assay.

For the assays, a 1 mM stock solution of test compounds is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. At least seven concentrations are used for each assay.

Tissue Preparation

Male guinea pigs are sacrificed, and cerebella are removed, weighed, and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.4. The homogenate is centrifuged at 48,000 g for 10 min, the supernatant decanted, and the pellet resuspended in 20 volumes of buffer. This homogenate is then incubated for 45 min at 37 °C to remove endogenous opiate peptides and centrifuged again as before. This pellet is resuspended in 200 volumes of 0.05 M Tris buffer, pH 7.4.

Assay

1,850 μl	Tissue suspension
60 μl	Distilled water
20 μl	Peptide solution
20 μl	Vehicle, or U50,488H, or test drug
50 μl	[^{3}H]Bremazocine

Tubes are incubated for 40 min at 25 °C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed three times with 5 ml of 0.05 M Tris buffer. The filters are then placed in scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 5.0 μM U50, 488H. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The K_D value for [^{3}H] bremazocine binding was found to be 0.14 nM by Scatchard analysis of a receptor saturation experiment. The K_i value may be calculated from IC_{50} by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D.$$

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Inhibition of Enkephalinase

Purpose and Rationale

Since the discovery of brain peptides with pharmacological properties similar to morphine (Hughes 1975), the metabolic breakdown of enkephalins has been studied (Malfroy et al. 1978; Llorens and Schwartz 1981; Mumford et al. 1981; Malfroy and Schwartz 1982; Roques et al. 1982; Schwartz 1983). Roques et al. (1980) and Costentin et al. (1986) found that the enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. A highly sensitive fluorometric assay for "enkephalinase," a neutral metalloendopeptidase that releases tyrosine–glycine–glycine from enkephalins, has been developed by Florentin et al. (1984). A fluorogenic peptide, dansyl-D-Ala-Gly-Phe (pNO$_2$)-Gly (DAGNPG), was synthesized as a selective substrate for the neutral metalloendopeptidase involved in enkephalin metabolism.

This enzyme, designated "enkephalinase," cleaves the Gly-Phe(pNO_2) peptide bond of DAGNPG leading to a fluorescence increase related to the disappearance of intramolecular quenching of the dansyl fluorescence by the nitrophenyl residue.

Enkephalinase induces inactivation of atrial natriuretic factor (ANF). The protection of endogenous ANF against inactivation may result in therapeutic applications (Schwartz et al. 1990).

Procedure

Fresh rat kidney is homogenized in 10 vol of cold 0.05 M Tris–HCl buffer, pH 7.4, using a Polytron homogenizer. The homogenate is centrifuged for 5 min at 1,000 g. The pellet is discarded and the supernatant centrifuged at 60,000 g for 60 min. The resulting pellet is resuspended in 50 mM Tris–HCl buffer, pH 7.4, and used as the enzyme source.

Standard assays for "enkephalinase" activity using DAGNPG are carried out at 37 °C in hemolysis tubes. A 0.1 ml amount of 50 mM Tris–HCl buffer, pH 7.4, containing 50 μM DAGNPG is preincubated 15 min at 37 °C. The reaction is initiated by the addition of 50 μl of the enzyme preparation together with 0.5 μM captopril. The tubes are incubated for 30 min in a water bath with constant shaking. The enzymatic reaction is stopped by boiling at 100 °C for 5 min. The samples are then diluted with 1.35 ml of Tris–HCl buffer and centrifuged at 500 g for 30 min. An aliquot of 1 ml of the supernatant is transferred to thermostated cells of a spectrofluorometer. Readings are performed at 562 nm with an excitation wavelength of 342 nm. A calibration curve is prepared by adding increasing concentrations of DNS-D-Ala-Gly and decreasing concentrations of the substrate in Tris–HCl buffer containing the denaturated enzymatic preparation. For the assay of "enkephalinase" inhibition, the test compound or the standard thiorphan = [(R-,S-)-3-mercapto-2-benzylpropanoyl]glycine is added in various concentrations.

Evaluation

The inhibitory potencies of test compounds are compared with the standard.

Modifications of the Method

Ksander et al. (1989) incubated synaptic membranes from rat striatum with ^{3}H-Tyr-Leu-enkephalin for 15 min at 30 °C, pH 6.5, in the presence of 10^{-6} M bestatin. The reaction was stopped by the addition of 30 % acetic acid, and the reaction product ^{3}H-Tyr-Gly-Gly was separated from unreacted ^{3}H-Tyr-Leu-enkephalin on a Porapak Q column followed by a Cu^{2+} chelex column. The ^{3}H-Tyr-Gly-Gly was counted by liquid scintillation.

The antinociceptive effects of intrathecally administered SCH32615, an enkephalinase inhibitor, were studied in the rat by Oshita et al. (1990).

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Nociceptin

General Considerations on Nociceptin

Purpose and Rationale

A heptadekapeptide (nociceptin or orphanin FQ) has been isolated as endogenous agonist of the **opioid receptor-like ORL_1 receptor** (Reinscheid et al. 1995; Meunier et al. 1995; Barlocco et al. 2000; Mollereau and Mouledous 2000; Mogil and Pasternak 2001; Witta et al. 2004) which shows high structural homology with opioid peptides, especially dynorphin A (Calò et al. 2000). Nociceptin is an opioid-related peptide and is a potent anti-analgesic. Its action is different from the classic opioid receptors mu, kappa, and delta. Nociceptin actions are not antagonized by the opioid antagonist naloxone. Nociceptin is widely distributed in the CNS, and it is found in many regions of the hypothalamus, brainstem, and forebrain as well as in the ventral horn and dorsal horn of the spinal cord. Nociceptin binds to its own specific receptors called NOP receptors and does not bind well to other opioid receptors. Nociceptin activates a specific receptor, which has been cloned in man and animals and has been shown to be structurally similar to opioid receptors (Mollereau et al. 1994; Calò et al. 2000; Hawkinson et al. 2000). At the cellular level, the nociceptin receptor has been shown to act through the same mechanisms as classical opioid receptors, namely, the inhibition of adenylyl cyclase, the activation of potassium channels, and the inhibition of calcium channels (Connor et al. 1996a, b). In vitro and in vivo studies have demonstrated that nociceptin mediates a variety of biological actions (Civelli et al. 1998; Darland et al. 1998). Nociceptin induces analgesia when administered intrathecally (Stanfa et al. 1996; Xu et al. 1996), while it causes hyperalgesia and reversal of opioid-induced analgesia when given intracerebroventricularly; nociceptin stimulates food intake (Polidori et al. 2000) and produces anxiolysis. Depending on the dose, nociceptin stimulates or inhibits locomotor activity. Nociceptin inhibits long term potentiation in the hippocampal area, memory processes, induces bradycardia, hypotension, and diuresis. In addition, nociceptin inhibits neurotransmitter release both at central and peripheral sites. Intracavernosal injection of nociceptin induces a potent and relatively long-lasting erectile response in the cat (Champion et al. 1997, 1998). Intrathecal injection of nociceptin elicits scratching, licking, and biting in mice (Sakurada et al. 1999, 2000). Synthetic agonists and antagonists of the nociceptin

receptor have been reported (Guerrini et al. 1998; Salvadori et al. 1999; Calò et al. 2000; Hashimoto et al. 2000; Meunier 2000; Ozaki et al. 2000).

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Receptor Binding of Nociceptin

Purpose and Rationale

The nociceptin receptor has been termed by different groups of investigators as ORL1, LC132, and ROR (see Meunier 1997). Based on the structural and transductional similarities between receptors for nociceptin and those for opioids, Hamon (1998) proposed to include the nociceptin receptor in the opioid receptor family with the name OP_4.

Varani et al. (1999) tested synthetic nociceptin analogues for their displacement at the nociceptin and at classical opioid receptors. The displacement of [^{3}H]$NCNH_2$ ([^{3}H]nociceptin amide, ORL1 site) and of the selective opioid receptor ligands [^{3}H]DAMGO (μ-site), [^{3}H]deltorphin II (δ-site), and [^{3}H]U69593 (κ-site) was studied.

Wisner et al. (2006) described human opiorphin, a natural antinociceptive modulator of opioid-dependent pathways inhibiting enkephalin-inactivating zinc ectopeptidases.

Procedure

Membrane Preparation

Guinea pigs are decapitated and the whole brain (without cerebellum) rapidly removed. The tissue is disrupted in a Polytron homogenizer (setting 5) in 50 mM Tris–HCl, pH 7.4, to prepare membranes for the classic opioid receptor studies. The homogenate is centrifuged at 4,000 g for 10 min, and the pellet is resuspended with a Polytron PTA 10 probe (setting 5) in the same ice-cold buffer. To study the binding to ORL1 receptor, the tissue is homogenized in 50 mM Tris–HCl, 2 mM EDTA, and 100 μM phenylmethylsulphonylfluoride HCl (PMSF) at pH 7.4. The suspension is centrifuged at 40,000 g for 10 min and the pellet is resuspended in the same buffer. After 30 min of incubation at 37 °C, the membranes are centrifuged at 40,000 g for 10 min and the pellets are stored at −70 °C. The protein concentration is determined with bovine albumin as standard.

Binding Assays

Classic opioid receptors, μ, δ, and κ, are studied according to Bhargava and Zhao (1996). Saturation binding experiment is carried out using 8–10 different concentrations of [^{3}H]DAMGO ranging from 0.15 to 15 nM, [^{3}H]deltorphin II from 0.1 to 10 nM, and [^{3}H]U69593 from 0.15 to 15 nM, respectively. Inhibition experiments are carried out in duplicate in a final volume of 250 μl in test tubes containing either 1.5 nM [^{3}H]DAMGO or 1.0 nM [^{3}H]deltorphin II or 1.5 nM [^{3}H]

U69593, 50 mM Tris–HCl at pH 7.4, guinea pig brain membranes (150–200 µg of protein/assay), and at least 8–10 different concentrations of the ligands under study. Binding assays to the ORL1 receptor are carried out according to Varani et al. (1998). In saturation studies, membranes are incubated with 8–10 different concentrations of [^{3}H]$NCNH_2$ ([^{3}H]nociceptin amide) ranging from 0.1 to 10 mM. Inhibition experiments are carried out in duplicate in a final volume of 250 µl in test tubes containing 1 mM [^{3}H]$NCNH_2$, 50 mM Tris–HCl, 2 mM EDTA, 100 µM phenylmethylsulphonylfluoride HCl (PMSF) at pH 7.4, guinea pig membranes, and at least 8–10 different concentrations of the compound under examination. The incubation time is 1 h for [^{3}H]DAMGO and [^{3}H]U69593 and 2 h for [^{3}H]deltorphin II and [^{3}H]$NCNH_2$. Nonspecific binding is defined as the binding measured in the presence of 100 µM bremazocine for classic opioid receptors and 10 µM $NCNH_2$ for ORL1 receptors.

Bound and free radioactivity are separated by filtering the assay mixture through Whatman GF/B glass-fiber filters, previously treated with PEI 0.1 %; the incubation mixture is diluted with 3 ml of ice-cold incubation buffer, rapidly filtered by vacuum, and the filter is washed three times with 3 ml of incubation buffer. The filter-bound radioactivity is measured in a Beckman LS-1800 spectrometer.

Evaluation

The inhibitory binding constant (K_i) values are calculated from the IC_{50} values according to the Cheng and Prusoff equation. The weighted nonlinear least-squares curved fitting program LIGAND (Munson and Rodbard 1980) is used for computer analysis of saturation and inhibition experiments.

Modifications of the Method

Ardati et al. (1997) developed two radioligands for the orphanin FQ receptor: a tritiated OFQ peptide ([^{3}H]orphanin FQ) and radioiodinated form in which Leu^{14} is substituted by tyrosine (^{125}I-Tyr^{14}-orphanin FQ). Both exhibit virtually identical characteristics.

Seki et al. (1999) analyzed the pharmacological properties of κ-opioid receptor-selective agonist TRK-820 using Chinese hamster ovary cells expressing cloned rat μ-, δ-, and κ-opioid receptors and human nociceptin receptor.

Mouledous et al. (2000) reported a site-directed mutagenesis study of the ORL1 receptor transmembrane-binding domain.

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Bioassays for Nociceptin

Purpose and Rationale

Nociceptin receptors in the periphery can be characterized by studies in isolated organs (Guerrini et al. 1998; Bigoni et al. 1999): the guinea pig ileum according to Paton (1957) (see chapter "▶ Pharmacological Effects on Intestinal Functions"), the mouse vas deferens according to Hughes et al. (1974), the rabbit vas deferens according to Oka et al. (1980) (see chapter "▶ Cardiovascular Analysis In Vivo"), and the guinea pig renal pelvis according to Giuliani and Maggi (1996) (see chapter "▶ Uricosuric and Hypo-Uricemic Activity").

Procedure

Tissues are taken from male Swiss mice (25–30 g), guinea pigs (300–350 g), Sprague–Dawley rats (300–350 g), and New Zealand albino rabbits (1.5–1.8 kg). They are suspended in 10 ml organ baths containing Krebs solution oxygenated with 95 % O_2 and 5 % CO_2. The temperature is set at 33 °C for the mouse vas deferens and at 37 °C for the other tissues. A resting tension of 0.3 g is applied to the mouse deferens; 1 g to the guinea pig ileum, rats vas deferens, and rabbit vas deferens; and 0.15 g to the guinea pig renal pelvis. For experiments at the mouse vas deferens, a Mg^{2+}-free Krebs solution is used and for rat vas deferens experiments a Krebs solution containing 1.8 mM $CaCl_2$. Guinea pig renal pelvis experiments are performed in the presence of indomethacin (3 μM).

The mouse vas deferens, guinea pig ileum, rat vas deferens, and rabbit vas deferens are continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions are measured isotonically with a strain gauge transducer and recorded on a multichannel chart recorder. After an equilibration period of about 60 min, the contractions induced by electrical field stimulation are stable; at this time, cumulative concentration response curves to nociceptin or opioid peptides are performed (0.5 log unit steps).

The guinea pig renal pelvis is stimulated through two platinum ring electrodes with 100 V square wave pulses of 1 ms duration at a frequency of 5 Hz for 10 s. The spontaneous activity and the positive inotropic responses to electrical field stimulation are measured by an isotonic transducer and recorded by a two channel recorder. The experiments are started following a 60 min equilibration period. Four electrical field stimulations are performed with each tissue at 30 min intervals. Agonists are added to the bath 5 min and antagonists 15 min before the next stimulus. The contractile responses to electrical field stimulation are expressed as % increment of the spontaneous activity of the tissue; the biological effects of the application of agonists or antagonists are expressed as % inhibition of electrical field stimulation-induced contraction.

Evaluation

Data are expressed as means ± SEM of *n* experiments and statistically analyzed with Student's two-tailed *t*-test of one-way ANOVA plus Dunnett test. The agonist potencies are given as pE_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50 % of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given preparation. Antagonist

potencies are expressed in terms of pA_2, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response.

Modifications of the Method

Rizzi et al. (1999) studied nociceptin and nociceptin analogues in the isolated mouse colon.

Bigoni et al. (1999) used nociceptin, a series of nociceptin fragments, naloxone, as well as [Phe$^1\Psi$ (CH_2-NH)Gly^2]nociceptin(1–13)NH_2 and [Nphe^1]nociceptin(1–13)NH_2 to characterize nociceptin receptors in peripheral organs, such as mouse and rat vas deferens (noradrenergic nerve terminals), in the guinea pig ileum (cholinergic nerves), and renal pelvis (sensory nerves) and in vivo by measuring the blood pressure and heart rate in anesthetized rats.

Menzies et al. (1999) described the agonist effects of nociceptin and [Phe$^1\Psi$ (CH_2-NH)Gly^2] nociceptin(1–13)NH_2 in the mouse and rat colon and in the mouse vas deferens.

Kolesnikov and Pasternak (1999) found an ED_{50} of 16.3 μg after peripheral administration of nociceptin in the tail-flick test in mice.

Bertorelli et al. (1999) found anti-opioid effects of nociceptin and the ORL1 ligand [Phe$^1\Psi$ (CH_2-NH)Gly^2]nociceptin(1–13)NH_2 in the Freund's adjuvant-induced arthritic rat model of chronic pain.

Yamamoto and Sakashita (1999) studied the effect of nocistatin, a 17 amino acid peptide which is processed from prepronociceptin and its interaction with nociceptin in the rat formalin test.

Hashiba et al. (2003) measured the effects of nociceptin/orphanin FQ receptor ligands on blood pressure, heart rate, and plasma catecholamine concentrations in guinea pigs.

Using the forced swimming test and the tail suspension test in rats and mice, Gavioli et al. (2004) demonstrated antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101.

Varty et al. (2005) characterized a nociceptin receptor (ORL-1) agonist in tests of anxiety across three species: rat, guinea pig, and mouse.

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Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

Purpose and Rationale

Several peptides are considered to play a role in the altered transmission of sensory information in neuropathic conditions, such as neuropathic pain arising from trauma or compression injury of peripheral nerves (Zhang et al. 1998).

Vasoactive intestinal polypeptide (VIP), isolated by Nakajima et al. (1970), is a neuropeptide of 28 amino acids with widespread distribution in both the central and peripheral nervous system (Fahrenkrug 1979; Gafvelin 1990). VIP is produced in many tissues including the hypothalamus and suprachiasmatic nuclei of the brain, gut, and pancreas. Vasoactive intestinal polypeptide (VIP) stimulates contractility in the heart, lowers arterial blood pressure, causes vasodilation, increases glycogenolysis, and relaxes the smooth muscle of the trachea, stomach, and gall bladder. VIP has a half-life ($t_{½}$) in the blood of about two minutes. The vasoactive intestinal peptide is encoded by the *VIP* gene.

Together with the structurally related pituitary adenylate cyclase-activating peptide (PACAP), this peptide is considered to play an important role in the somatosensory processing of pain (Dickinson and Fleetwood-Walker 1999). PACAP-38 (a 38 amino acid polypeptide) and the C-terminally truncated form PACAP-37 share 68 % amino acid homology at their N-terminal domain with VIP. A shorter peptide with 27 amino acids, named as PACAP27, was described by Miyata et al. (1990). These peptides are members of a superfamily of hormones that include glucagon, glucagon-like peptide, secretin, and growth hormone-releasing factor.

Three G protein-coupled receptors are described: the $VPAC_1$ receptor, originally described as the VIP receptor and subsequently designated as VIP_1 receptor; the $VPAC_2$ receptor, previously designated VIP_2; and the PAC_1 receptor, previously known as PACAP type I receptor (Buscail et al. 1990; Guijarro et al. 1991; Felley et al. 1992; Calvo et al. 1994; Van Rampelbergh et al. 1996; Harmar et al. 1998; Robberecht et al. 1999).

Many peripheral activities of VIP/PACAP are described, such as stimulation of pancreatic secretion (Onaga et al. 1997; Ito et al. 1998; Soo Tek Lee et al. 1998); stimulation of duodenal bicarbonate secretion (Takeuchi et al. 1998); relaxation of smooth muscle cells in the intestinal tract, e.g., gall bladder (Pang and Kline 1998), cecal circular smooth muscle (Motomura et al. 1998), and internal anal sphincter (Rattan and Chakder 1997); duodenal motility (Onaga et al. 1998); enhancement of insulin secretion (Yada et al. 1997; Filipsson et al. 1998); and bronchodilation (Linden et al. 1998; Shigyo et al. 1998; Okazawa

et al. 1998). Centrally administered PACAP showed an anorectic effect (Mizuno et al. 1998).

Agonists (Gourlet et al. 1997a, b) and antagonists (Gozes et al. 1991; Gourlet et al. 1997c) for VIP were described. Further studies are aimed to develop drugs for neuropathic analgesia, ultimately of non-peptide nature, using $VPAC_1$, $VPAC_2$, and PAC_1 receptors as drug targets (Dickinson and Fleetwood-Walker 1999).

Procedure

CHO cell lines expressing the rat VIP_1 receptor (Ciccarelli et al. 1994), the human VIP_2 receptor (Sreedharan et al. 1993), the rat PACAP I receptor (Ciccarelli et al. 1995), and the rat secretin receptor (Ishihara et al. 1991) are used.

Transfected CHO cells are harvested with a rubber policeman and pelleted by low speed centrifugation. The supernatant is discarded and the cell lysed in mM $NaHCO_3$ solution with immediate freezing in liquid nitrogen. After thawing, the lysate is first centrifuged at 4 °C for 10 min at 400 g and the supernatant is further centrifuged at 20,000 g for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$, is used immediately as a crude membrane fraction.

Binding is performed using [^{125}I]VIP (specific radioactivity of 0.5 Ci/nmol), [^{125}I]Tyr^{25} secretin (specific radioactivity of 1.0 Ci/nmol), and [^{125}I-Ac-His^{1}]PACAP-27 (specific radioactivity of 0.7 Ci/nmol) as tracers. In all cases, nonspecific binding is defined as the residual binding in the presence of 1 μM of the unlabeled peptide corresponding to the tracer. Binding is performed at 37 °C in a 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, and 1 % bovine serum albumin (pH 7.4) buffer. Bound radioactivity is separated from free radioactivity by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.1 % polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1 % bovine serum albumin.

Evaluation

The IC_{50} values (in mM) for each peptide on each receptor are calculated from complete dose–effect curves performed on three different membrane preparations using the LIGAND program.

Modifications of the Method

Schmidt et al. (1993) studied the binding of PAPAC, VIP and analogues of VIP, and PAPAC in rat AR 4-2J pancreatic carcinoma cells and isolated pancreatic acini to the PAPAC-1 receptor, abundantly expressed in AR 4-2J pancreatic carcinoma cells, and to the VIP/PAPAC-2 receptor. Simultaneously, biological effects (lipase secretion and cAMP production) in pancreatic acini were determined. PAPAC was regarded as a potent ligand for both receptor types and as a potent VIP-like secretagogue.

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Cannabinoid Activity

General Considerations on Cannabinoids

In the centuries since hashish and marijuana (*Cannabis sativa*) were used as psychoactive drugs, the most significant discoveries in regard to the mechanism of action were made with the isolation of (−)-trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) as the principal active ingredient (Mechoulam et al. 1970), the characterization and localization of the cannabinoid receptor in the brain (Devane et al. 1988), the cloning of its gene (Matsuda et al. 1990), and the identification of an endogenous ligand (Devane et al. 1992). Most cannabinoid effects occur receptor mediated in the CNS (Martin 1986; Herkenham et al. 1990; Porter and Felder 2001; Howlett et al. 2002). The recognized CNS responses to cannabinoids include alterations in cognition and memory, euphoria, and sedation (Howlett 1995). Ranganathan and D'Souza (2006) reviewed the acute effects of cannabinoids on memory in humans.

The two main types of cannabinoid receptors in the body are CB1 and CB2. Cannabinoid receptors are present on the surface of cells. These receptors are found in different parts of the central nervous system. In 1992, a naturally occurring substance in the brain that binds to CB1 was discovered, called anandamide. This cannabinoid-like chemical and others that were later discovered are referred to as endocannabinoids. The effects of cannabinoids depend on the brain area involved. Effects on the limbic system may alter the memory, cognition, and psychomotor performance; effects on the mesolimbic pathway may affect the reward and pleasure responses, and pain perception may also be altered. The study of cannabinoid receptors, endocannabinoids, and synthetic agonists and

antagonists has helped for studying the mechanism of cannabinoid-induced analgesia. The CB1 receptor which is found in both the central nervous system and in peripheral nerve terminals regulates the nociceptive processing. CB2 receptors are located predominantly in peripheral tissue, and they do exist at very low levels in the CNS. Cannabinoids may also contribute to pain modulation through an anti-inflammatory mechanism, a CB2 effect with cannabinoids acting on mast cell receptors which in turn cause to attenuate the release of inflammatory agents, such as histamine and serotonin, and on keratinocytes resulting in the enhanced release of analgesic opioids.

Cannabinoids have been shown to produce analgesia without the respiratory problems associated with opioid analgesics (Buxbaum 1972; Martin 1985; Dewey 1986; Razdan 1986; Compton et al. 1992; Meng et al. 1998; Strangman et al. 1998) which may be of value for therapeutic applications (Hollister 1986; Izzo et al. 2000a; Pertwee 2000). Simultaneous administration of cannabinoid receptor agonists and μ- or κ-receptor agonists indicates a cannabinoid–opioid interaction in antinociception (Manzanares et al. 2000). Baker et al. (1990, 2000) described experimental allergic encephalomyelitis with relapsing-remitting episodes, spasticity, and tremor similar to multiple sclerosis in human beings in Biozzi AB/H mice. These symptoms could be antagonized by cannabinoids.

A multiple-evaluation paradigm of in vivo mouse assays is employed to test for cannabimimetic effects. This paradigm includes assays for reduction in spontaneous activity and the production of hypothermia, catalepsy, and antinociception measured by tail-flick assay (Compton et al. 1992; Welch et al. 1998). The behavioral effects of Δ^9-THC and related cannabinoids in mice have been termed the "popcorn" effect. That is, groups of mice are in a sedated state with little or no movement until a stimulus causes one mouse to jump (hyper-reflexia). This animal falls on another mouse which in turn jumps so that this repeated hyperreflexic jumping looks like corn popping in a machine. Subsequently, all mice will be sedated until another stimulus reinitiates the process (Dewey 1986). Like the opioids, cannabinoids inhibit electrically evoked contractions of the mouse vas deferens and the guinea pig ileum, but unlike the opioids, these effects are not antagonized by naloxone (Pertwee et al. 1992; Hillard et al. 1999).

In addition to the effects in the CNS (Chaperon and Thiebot 1999), peripheral effects of cannabinoids are known (Lynn and Herkenham 1994) including actions on the **endocrine system** (Patra and Wadsworth 1990; Block et al. 1991; Wenger et al. 2000), on the **digestive tract** (Rosell and Agurell 1975; Izzo et al. 1999a, b, 2000; Coutts et al. 2000; Massa et al. 2005), on **ingestive behavior** (Giuliani et al. 2000), on the **pulmonary and cardiovascular system** (Stengel et al. 1998; White and Hiley 1998; Niederhoffer and Szabo 1999; Liu et al. 2000; Niederhoffer et al. 2003), and on **immune modulation** (Kaminski et al. 1992; Lynn and Herkenham 1994; Achiron et al. 2000).

An endogenous cannabinoid was isolated from porcine brain by Devane et al. (1992) and found to be an unsaturated fatty acid ethanolamide, arachidonylethanolamide, also called **anandamide**, which activates CB1 receptors (Devane et al. 1992; Hillard and Jarrahian 2005) and produces similar effects as Δ^9-tetrahydrocannabinol including antinociception, hypothermia, hypomotility, and catalepsy in mice (Smith et al. 1994). The brain enzyme hydrolyzing and synthesizing anandamide has been characterized by Ueda et al. (1995). The human brain fatty acid amide hydrolase was characterized by Maccarrone et al. (1998) as a single protein, which hydrolyses anandamide to arachidonate and ethanolamine.

Similar effects are produced by other polyunsaturated *N*-acetylethanolamines, such as *N*-palmitoylethanolamine, which activates the CB-2-like receptor subtype (Hanu et al. 1993; Facci et al. 1995). Both endogenous cannabinoids (called endocannabinoids) derive from the cleavage of a precursor phospholipid, *N*-acylphosphatidylethanolamine, catalyzed by Ca^{2+}-activated D-type phosphodiesterase activity (Cadas et al. 1996). 2-Arachidonylglycerol was described as a further endogenous ligand for cannabinoid receptors (Ameri and Simmet 2000; Sigiura et al. 2000).

Numerous synthetic analogues and cannabimimetic compounds have been evaluated as agonists and antagonists by in vitro and in vivo pharmacological methods (Martin et al. 1991; D'Ambra et al. 1992; Melvin et al. 1993; Barth and Rinaldi-Carmona 1999; Hillard et al. 1999; Palmer et al. 2000; Piomelli et al. 2000; De Petrocellis et al. 2004; Costa et al. 2005; Griebel et al. 2005; Lange et al. 2005; Makriyannis et al. 2005; Pertwee 2006).

Costa et al. (2004) described oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw.

For studies on the effects of cannabinoids and cannabinoid antagonists on digestive system and obesity, see chapter "▶ Assays of Anti-Obesity Activity."

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Receptor Binding of Cannabinoids

Purpose and Rationale

After the discovery of cannabinoid receptors in the brain (Howlett et al. 1988; Devane et al. 1988; Pertwee 1993), two cannabinoid receptor subtypes were identified: CB1 and CB2. Cannabinoid receptors were reviewed by Felder and Glass (1998) and Pertwee (1999, 2001).

CB1 has an amino acid sequence consistent with a tertiary structure typical of the seven transmembrane-spanning proteins that are coupled to G proteins (Gerard et al. 1990, 1991; Howlett et al. 1990; Matsuda et al. 1990). The CNS responses to cannabinoid compounds are apparently mediated exclusively by CB1, since CB2 transcripts could not be found in brain tissue. CB1 transduces signals in response to CNS-active constituents of *Cannabis sativa*, as well as synthetic bicyclic and tricyclic analogues, aminoalkylindole, and eicosanoid cannabimimetic compounds. CB1 is coupled to G_1 to inhibit adenylate cyclase activity and to a pertussis-sensitive G protein to regulate Ca^{2+} currents. Zimmer et al. (1999) produced a mouse strain with a disrupted CB1 gene. These CB1 knockout mice had a significantly increased mortality rat and displayed reduced locomotor activity, increased ring catalepsy, and hypoalgesia in hot plate and formalin tests.

CB2, the second cannabinoid-binding seven-transmembrane spanning receptor, exhibits 68 % identity to CB1 within the helical regions and 44 % identity throughout the total protein. The CB2 clone was derived from a human promyelocytic leukemia cell line HL60 cDNA library (Munro et al. 1993), also expressed in human leukocytes (Bouaboula et al. 1993). The gene for the rat CB2 receptor was cloned and expressed and its properties compared with those of mouse and human CB2 receptors (Griffin et al. 2000).

Receptor binding of cannabinoids in correlation to in vivo activities was described by Compton et al. (1993).

Procedure

Membrane Preparation

Male Sprague–Dawley rats weighing 150–200 g are decapitated and the brain rapidly removed. The cortex is dissected free using visual landmarks following reflection of cortical material from the midline and immersed in 30 ml of ice-cold centrifugation solution (320 mM sucrose, 2 mM Tris-EDTA, 5 mM $MgCl_2$). The process is repeated until the cortices of five rats are combined. The cortical material is homogenized with a Potter–Elvehjem glass-Teflon grinding system. The homogenate is centrifuged at 1,600 g for 15 min, the supernatant saved and combined with the two subsequent supernatants obtained from washing and 1,600 g centrifugation of the P_1 pellet. The combined supernatant fractions are centrifuged at 39,000 g for 15 min. The P_2 pellet is resuspended in 50 ml buffer (50 mM Tris–HCl, 2 mM Tris-EDTA, 5 mM $MgCl_2$, pH 7.0), incubated for 10 min at 37 °C, and then centrifuged at 23,000 g for 10 min. The P_2 membrane is resuspended in 50 ml of buffer A, incubated again except at 30 °C for 40 min, and then centrifuged at 11,000 g for 15 min. The final wash-treated P_2 pellet is resuspended in assay buffer B (50 mM Tris–HCl, 1 mM Tris-EDTA, 3 mM $MgCl_2$, pH 7.4) to a protein concentration of approximately 2 mg/ml. The membrane preparation is divided into four aliquots and quickly frozen in a bath solution of dry ice and 2-methylbutane and then stored at −80 °C.

Binding Assay

Binding is initiated by the addition of 150 mg of P_2 membrane to test tubes containing [^{3}H]CP-55,940 (79 Ci/mmol), a cannabinoid analogue (for displacement studies), and a sufficient quantity of buffer C (50 mM Tris–HCl, 1 mM Tris-EDTA, 3 mM $MgCl_2$, 5 mg/ml BSA) to bring the total incubation volume to 1 ml. The concentration of [^{3}H]CP-55,940 in displacement studies is 400 pM, whereas that in saturation studies varies from 25 to 2,500 pM. Nonspecific binding is determined by the addition of 1 mM unlabeled CP-55,940. The standard CP-55,940 and other cannabinoid analogues are prepared in suspension buffer C from a 1 mg/ml ethanolic stock without evaporation of the alcohol.

After incubation at 30 °C for 1 h, binding is terminated by the addition of 2 ml ice-cold buffer D (50 mM Tris–HCl, 1 mg/ml BSA) and vacuum filtration through pretreated filters in a 12-well sampling manifold. Reaction vessels are washed once with 2 ml of ice-cold buffer D, and the filters washed twice with 4 ml of ice-cold buffer D. Filters are placed into 20 ml plastic scintillation vials with 1 ml of distilled water and 10 ml of Budget-Solve (RPI Corp., Mount Prospect, IL). After shaking for 1 h, the radioactivity present is determined by liquid scintillation photometry.

Evaluation

The B_{max} and K_D values obtained from Scatchard analysis are determined via a suitable computer program. Displacement IC_{50} values are determined by unweighted least-squares linear regression of log concentration-percent displacement data and then converted to K_i values.

Modifications of the Method

To further characterize neuronal cannabinoid receptors, Thomas et al. (1998) compared the ability of cannabinoid analogues to compete for receptor sites labeled either with [^{3}H]SR141716A or [^{3}H]CP-55940.

Herkenham et al. (1991) characterized and localized cannabinoid receptors in rat brain by a quantitative autoradiographic study.

Felder et al. (1995) compared the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors.

Ross et al. (1998) compared cannabinoid-binding sites in guinea pig forebrain and small intestine.

Rinaldi-Carmora et al. (1998) tested the affinity of an antagonist of the CB2 cannabinoid receptor for rat spleen and cloned human CB2 receptors.

Bilkei-Gorzo et al. (2005) described early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors.

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Vanilloid (Capsaicin) Activity

General Considerations on Vanilloids

Purpose and Rationale

Several authors reviewed the recent development of capsaicin and vanilloid receptors (Holzer 1991; Bíró et al. 1997; Sterner and Szallasi 1999; Szallasi and Blumberg 1999; Caterina and Julius 2001; Piomelli 2001; Gunthorpe et al. 2002). Capsaicin was isolated by Thresh (1846). The chemical structure was determined by Nelson (1919). The analgesic use of capsaicin was reviewed by Lembeck (1987). Capsaicin excites a subset of primary sensory neurons with somata in the dorsal root ganglion or trigeminal ganglion. As a general rule, these vanilloid-sensitive neurons are peptidergic, small-diameter (50 μm) neurons, giving rise to thin, unmyelinated C fibers. Among the sensory neuropeptides, the tachykinin substance P shows the best correlation with vanilloid sensitivity. Vanilloid-sensitive neurons transmit noxious information (usually perceived as itching or pain) to the CNS, whereas rare peripheral terminal sites release a variety of pro-inflammatory neuropeptides. Among the irritant compounds acting on primary sensory neurons, capsaicin and related vanilloids are unique in that the initial stimulation by vanilloids is followed by a long-lasting refractory state. Neurotoxicity has been observed when capsaicin is given to newborn rats (Janscó et al. 1977; Nagy and van der Kooy 1983).

Besides capsaicin, several natural vanilloid agonists were described (Jonassohn and Sterner 1997; Liu et al. 1997; Sterner and Szallasi 1999; Mendes et al. 2000). The irritant principle from *Euphorbia resinifera*, named resiniferatoxin, was isolated by Hergenhahn et al. (1975). In several assays, resiniferatoxin and its derivatives are several thousandfold more potent than capsaicin (Szolcsanyi et al. 1990; Ács et al. 1995), which is explained by specific receptor binding (Szallasi and Blumberg 1990; Ács et al. 1994). Lee et al. (2001) described simplified resiniferatoxin derivatives as potent vanilloid receptor agonists with potent analgesic activity and reduced pungency.

The high affinity of vanilloid receptors argues for the existence of endogenous vanilloids. Hwang et al. (2000) and Piomelli (2001) reported a direct activation of capsaicin receptors by products of lipogenases. Pain-inducing substances, such as bradykinin, may activate phospholipase-linked receptors in sensory neurons, mobilizing arachidonic acid from phospholipids and generating 12-HPETE. This lipid second messenger interacts in turn with a cytosolic domain of the VR1 receptor channel, increasing its opening probability and causing the sensory neuron to become depolarized.

The endogenous ligand of CB_1 cannabinoid receptors, anandamide, is also a full agonist at vanilloid VR1 receptors (Zygmunt et al. 1999; Maccarrone et al. 2000; Smart et al. 2000; DePetrocellis et al. 2001). Prekumar and Ahern (2000) showed that activation of protein kinase C activates VR1 channel activity.

The vanilloid receptor-1 (VR1) is a nonselective, ligand-gated, cation channel expressed predominantly by the sensory neurons. Vanilloid receptor-1 mainly responds to the noxious stimuli like capsaicin, which is the pungent component of chilli peppers, heat, and extracellular acidification. The vanilloid receptor-1 (VR1) is able to integrate simultaneous exposure to these kinds of stimuli and hence is considered important for pain sensation.

The first capsaicin or vanilloid receptor, termed VR1, was cloned by Caterina et al. (1997). Hayes et al. (2000) reported the cloning and functional expression of a human orthologue of rat vanilloid receptor-1. Pharmacological differences between the human and rat vanilloid receptor-1 were observed (McIntyre et al. 2001). VR1 functions as a molecular integrator of painful chemical and physical stimuli including capsaicin, noxious heat, and low pH (Tominaga et al. 1998; Michael and Priestley 1999; Davis et al. 2000; Welch et al. 2000). In mice lacking the capsaicin receptor, impaired nociception and pain sensation were observed (Caterina et al. 2000).

Vanilloid receptors are differently distributed in the central and peripheral nervous system (Szallasi 1995; Szallasi et al. 1995;

Mezey et al. 2000; Ichikawa and Sugimoto 2001). Bíró et al. (1998) reported characterization of functional vanilloid receptors expressed by mast cells. Biological and electrophysiological data indicate heterogeneity within the vanilloid receptors. Caterina et al. (1999) described a capsaicin-receptor homologue, named vanilloid receptor-like protein (VRL-1), with a high threshold for noxious heat. A novel human vanilloid receptor-like protein, named VRL-2, was identified and characterized by Delany et al. (2001).

Price et al. (2004) reported modulation of trigeminal sensory neuron activity by the dual cannabinoid–vanilloid agonists anandamide, *N*-arachidonoyl-dopamine, and arachidonyl-2-chloroethylamide.

Several vanilloid antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodo-resiniferatoxin (Wahl et al. 2001).

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Vanilloid Receptor Binding

Purpose and Rationale

Ács et al. (1994) described [^{3}H] resiniferatoxin binding by the human vanilloid (capsaicin) receptor. Receptor types and species differences of the vanilloid receptor were described by Szallasi et al. (1994, 1996). The rat vanilloid receptor (rVR1) was cloned and stably expressed in HEK293 cells by Jerman et al. (2000). A detailed pharmacological characterization was conducted using the Ca^{2+}-sensitive dye, Fluo3AM, in a fluorimetric imaging plate reader (FLIPR). Ross et al. (2001) studied structure–activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors in transfected CHO cells.

Procedure

Cell Culture

Rat vanilloid receptor (rVR1)-transfected CHO cells are maintained in MEM alpha minus media containing 2 mM L-glutamine supplemented with 10 % hyclone fetal bovine serum, 350 μg/ml G418 (Sigma-Aldrich), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells are maintained in 5 % CO_2 at 37 °C and passed twice a week using nonenzymatic cell dissociation solution. For the radioligand binding assay, cells are removed from flasks by scraping and then frozen as a pellet at −20 °C for up to 1 month.

Radioligand Binding Experiments

Assays are performed in DMEM containing HEPES (25 mM) and BSA (0.25 mg/ml). The

total assay volume is 500 µl containing 20 µg of cell membranes. Binding is initiated by addition of [^{3}H] resiniferatoxin ([^{3}H]-RTX). Assays are carried out at 37 °C for 1 h before termination by addition of ice-cold wash buffer (50 mM Tris buffer, 1 mg/ml BSA, pH 7.4) and vacuum filtration using a 12-well sampling manifold (Brandell cell harvester) and Whatman GF/B filters that have been soaked in wash buffer at 4 °C for at least 24 h. Each reaction is washed nine times with a 1.5 ml aliquot of wash buffer. The filters are oven-dried and then placed in a 5 ml scintillation fluid. Radioactivity is quantified by liquid scintillation spectrometry. Specific binding is determined in the presence of 1 µM unlabelled RTX. Protein assays are performed using a Bio-Rad De Kit. Unlabelled compounds are added in a volume of 50 µl after serial dilution using assay buffer from a 10 mM stock in ethanol or DMSO. [^{3}H]-RTX is also added in a 50 µl volume following dilution in assay buffer.

Evaluation

The K_D value and B_{max} for [^{3}H]-RTX and the concentration of competing ligands to produce 50 % displacement of the radioligand (IC_{50}) from specific binding sites are calculated using GraphPad Prism (GraphPad Software, San Diego). Dissociation constant (K_i) values are calculated using the Cheng and Prussoff equation.

Modifications of the Method

Wardle et al. (1997) used a 96-well plate assay system to characterize pharmacologically the vanilloid receptor in the dorsal spinal cord of the rat.

Hayes et al. (2000) described the cloning and functional expression of a human orthologue of rat vanilloid receptor-1.

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Evaluation of Vanilloid Receptor Antagonists

Purpose and Rationale

Several vanilloid receptor antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodoresiniferatoxin (Wahl et al. 2001). Kirschstein et al. (1999) described the inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists.

Procedure

Adult Sprague–Dawley rats of both sexes are deeply anesthetized with diethyl ether and rapidly decapitated. The spine is chilled at 4 °C in F12 Dulbecco's modified Eagle's medium saturated with carbogen gas and additionally containing 30 mM $NaHCO_3$, 100,000 units/l penicillin, and 100 mg/l streptomycin. Thoracic and lumbar

dorsal root ganglions are quickly dissected and freed from connective tissue. Neurons are dissociated in an incubation chamber enriched with carbogen gas at 37 °C using collagenase CLS II (5–10 mg/ml, 10–12 min) and trypsin (0.2–1 mg/ml, 10–12 min) dissolved in F12 medium. After trituration (four to six times with a Pasteur pipette), neurons are plated in 35 mm culture dishes, which also serve as recording chambers, and stored at 37 °C in a humidified 5 % CO_2 atmosphere before used for electrophysiological recordings.

Only round- or oval-shaped neurons without any processes are included in the study. The average of the major and the minor diameter is used to measure the size of oval-shaped neurons. Whole cell patch-clamp experiments are performed in carbogen gas saturated F12 medium (pH 7.4) at room temperature using an Axo-patch 200A amplifier (Axon Instruments) in voltage-clamp mode at a holding potential of −80 mV controlled by pCLAMP6 software. Data are also registered on a chart recorder. Patch pipettes are fabricated from borosilicate glass using a horizontal micropipette puller and filled with a solution containing (in mM) 160 KCl, 8.13 EGTA, and 10 HEPES (pH 7.2, $R_{Tip} = 5.3 \pm 0.2$ MΩ, mean ± SE). Cell diameter, cross-sectional area, and membrane capacitance are measured, and excitability is tested by depolarizing voltage steps for each neuron. Cells lacking a fast inward current with a reversal potential close to the equilibrium potential of sodium followed by a prolonged outward current are excluded from further investigation. Experiments in current-clamp mode are performed to measure the resting membrane potential of each neuron and to investigate single action potentials elicited by short (3 ms) depolarizing current pulses in neurons that are hyperpolarized by constant current injection resulting in membrane potentials between −70 and −80 mV. Inflections in the repolarizing phase are qualitatively detected as second negative peak in the first derivative (dV/dt) of each action potential; the duration of repolarization is quantitatively assessed by the 10–90 % decay time.

Applications of ∼50 µl of heated extracellular solution through a puffing system fixed on a micromanipulator are used to elicit heat-evoked currents. Control measurements with a fast temperature sensor (BAT-12, Physitemp; $\tau = 5$ ms) in place of the neurons are made revealing an effective temperature of ∼53 °C, a rise time of ∼250 ms, and a decay with a time constant of ∼20 s. Effects are compared with those of the application of the same amount of medium at room temperature. Heat stimuli with or without vanilloid receptor antagonists and control applications at room temperature are repeated 2–10 times, and the elicited currents are averaged. A neuron is considered as heat sensitive when the heat-evoked inward current is significantly greater than any fluctuations caused by superfusion of solution at room temperature. Heating the buffered solution may change its pH, and acid solution of pH 6.2 is known to activate nociceptive dorsal root ganglion neurons (Bevan and Yeats 1991). The pH of a HEPES-buffered solution decreases while heating (e.g., pH 7.1 at 50 °C). In contrast, higher temperatures increase the pH of a $NaHCO_3/CO_2$ buffer, because the solubility of CO_2 is reduced and thus reverses the HEPES effect. The pH of the F12 medium maximally changes in a range of 7.28–7.52 while heating to 50 °C and cooling down to room temperature. The membrane conductance is measured in voltage-clamp mode by hyperpolarizing pulses (5 mV, 10 ms, 50 s^{-1}), and conductance changes are determined at the maximum amplitude of heat-evoked currents.

Reversal potentials of heat- and capsaicin-induced currents are measured as described by Liu et al. (1997) using fast depolarizing ramps (−80 to +30 mV in 22 ms every 550 ms). Patch pipettes are filled with a potassium-free solution containing (in mM) 140 CsCl, 10 HEPES, 10 EGTA, and 4 $MgCl_2$ (adjusted to pH 7.2). Tetrodotoxin (100 µM) and nifedipine (1 µM) are added to the extracellular solution to block voltage-gated Na^+ and Ca^{2+} channels. Capsaicin is dissolved in ethanol, diluted to its final concentration with F12 medium, and applied through the puffing system. Capsazepine (dissolved in DMSO) and ruthenium red are prepared as concentrated stock solutions, diluted to final concentration in F12 medium, and applied either at room temperature or at ∼53 °C. Reversibility of

antagonist action is tested by reapplication of heated extracellular solution without any agents.

Evaluation

Off-line measurements and statistical analysis are done using pCLAMP6 (Axon Instruments) and EXCEL 5.0 (Microsoft). Data are presented as means ± SE. Treatment effects are statistically analyzed by Student's *t*-test for paired data and χ^2 test for analysis of incidences.

Modifications of the Method

Nagy et al. (1983) described dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat.

Lopshire and Nicol (1998) performed whole-cell and single-channel studies in rat sensory neurons and found a prostaglandin E_2-induced enhancement of the capsaicin-elicited current.

Jung et al. (1999) performed patch-clamp experiments in dorsal root ganglion neurons of neonatal rats and concluded that capsaicin binds to the intracellular domain of the capsaicin-activated ion channel.

Nagy and Humphrey (1999) compared the membrane responses of rat sensory neurons to noxious heat and capsaicin, using electrophysiological and ion flux measurements.

Baumann and Martenson (2000) found that extracellular protons both increase the activity and reduce the conductance of capsaicin-gated channels.

Liu et al. (2001) investigated mechanisms underlying capsaicin-mediated inhibition of action potentials and modulation of voltage-gated sodium channels in cultured trigeminal ganglion neurons.

Gunthorpe et al. (2004) identified and characterized a potent and selective vanilloid receptor antagonist isolated via high-throughput screening of a large chemical library in an FLPR-based C^{2+} assay.

For further information on the vanilloid receptor, see chapter "▶ Cardiovascular Analysis In Vivo".

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In Vivo Methods for Testing Central Analgesic Activity

General Considerations

Although the in vivo methods have been used more extensively in the past, they are still necessary in present research analgesic tests in animals before a compound can be given to man. Mostly, rodents, such as mice or rats, are used for analgesic tests, but in some instances, experiments in higher animals such as monkeys are necessary.

Several methods are available for testing central analgesic activity:

- Haffner's tail clip method in mice
- Tail-flick or other radiant heat methods
- Tail-immersion tests
- Hot plate methods in mice or rats
- Electrical stimulation (grid shock, stimulation of tooth pulp or tail)
- Monkey shock titration
- Formalin test in rats

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Haffner's Tail Clip Method

Purpose and Rationale

The method was described as early as 1929 by Haffner who observed the raised tail (Straub phenomenon) in mice treated with morphine or similar opioid drugs and found the tail after drug treatment to be less sensitive to noxious stimuli. He already described the high sensitivity of this method to morphine. Since then, the method has been used and modified by many authors.

Procedure

An artery clip is applied to the root of the tail of mice and the reaction time is noted. Male mice (Charles River strain or other strains) with a weight between 18 and 25 g are used. The control group consists of 10 mice. The test compounds are administered subcutaneously to fed mice or orally to fasted animals. The test groups and the control group consist of 7–10 mice. The drug is administered 15, 30, or 60 min prior testing. An artery clip is applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to this noxious stimulus by biting the clip or the tail near the location of the clip. The time between stimulation onset and response is measured by a stopwatch in 1/10 s increments.

Evaluation

A cutoff time is determined by taking the average reaction time plus three times the standard deviation of the combined latencies of the control mice at all time periods. Any reaction time of the test animals which is greater than the cutoff time is called a positive response indicative of analgesic activity. The length of time until response indicates the period of the greatest activity after dosing. An ED_{50} value is calculated at the peak time of drug activity. ED_{50} values found by this method were 1.5 mg/kg s.c. for morphine and 7.5 mg/kg for codeine s.c.

Critical Assessment of the Test

The test does not need any sophisticated equipment but a skilled, preferably "blind," observer. Peripheral analgesics of the salicylate type are not detected by this test.

Modifications of the Method

Bartoszyk and Wild (1989) described a modification of the original Haffner clip test using pressure on the tail of rats instead of mice. Additionally, hyperalgesia was induced by injection of carrageenan suspension into the tail. In this case, not only an effect of a nonsteroidal anti-inflammatory

agent but also a potentiation by B-vitamins could be shown.

Takagi et al. (1966) published a modification of HAFFNER's method for testing analgesics.

Ossipov et al. (1988) used the Haffner test to compare the antinociceptive effects of intrathecally administered opiates, α_2-adrenergic agonists, and local anesthetics.

Yanagisawa et al. (1984) described a tail-pinch method in vitro for testing antinociceptive drugs consisting of an isolated spinal cord, spinal nerve roots, and the functionally connected tail of a newborn rat. Changes of electric potential in the ventral root are induced by noxious pressure on the tail. In addition, responses after electric stimulation of the dorsal root were recorded. The authors recommend the method for studying actions of analgesic drugs.

Pinch of the toes of guinea pigs was recommended as a test for opioid analgesics by Collier (1965).

Tail-pinch feeding in rats after intracerebroventricular injection of various opioid antagonists has been used to differentiate opioid receptor subtypes (Koch and Bodnar 1993).

Person et al. (1985) used three different techniques of mechanical tail stimulation (reaction threshold determined with an analgesy meter at two different cutoff values and HAFFNER's tail clip) to study morphine–caffeine analgesic interaction in rats.

Arndt et al. (1984) studied pain responses (increase of heart rate and arterial pressure, respiratory effects) to tail clamping in trained unanesthetized spontaneously breathing dogs after administration of fentanyl.

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Radiant Heat Method

Purpose and Rationale

Originally, the method was developed by Schumacher et al. (1940) and Wolff et al. (1940) for quantitative measurements of pain threshold in man against thermal radiation and for evaluation of analgesic activity of opiates. Later on, the

procedure has been used by many authors to evaluate analgesic activity in animal experiments by measuring drug-induced changes in the sensitivity of mice or rats to heat stress applied to their tails. The test is very useful for discriminating between centrally acting morphine-like analgesics and non-opiate analgesics.

Mice are placed into cages leaving the tail exposed. A light beam is focused to the proximal third of the tail. Within a few seconds, the animal flicks the tail aside or tries to escape. The time until this reaction occurs is measured.

Procedure

The method was described by Ther et al. (1963) as a modification of earlier publications (D'Armour and Smith 1941). Groups of 10 mice (NMRI-strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard, the normal reaction time is determined. The animal is put into a small cage with an opening for the tail at the rear wall. The tail is held gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. For about 6 s, the reaction of the animal is observed by the investigator. The mouse tries to pull the tail away and turns the head. With a switch, the shutter is closed as soon as the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test. The escape reaction which is the endpoint of this test can be regarded as a complex phenomenon mediated by the brain. In contrast, the simple tail flick as an endpoint of this test may be mediated as a spinal reflex. Therefore, the observation of the escape reaction can be regarded as a true assessment of the influence of the drug on the brain.

The test compounds and the standard are administered either orally or subcutaneously. The animals are submitted to the same testing procedure after 30, 60, and eventually 120 min. For each individual animal, the reaction time is noted. Other time intervals can be used according to the question to be investigated.

Evaluation

There are two possibilities for evaluation:

- The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
- At each time interval, only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose, and ED_{50} values are calculated according to LITCHFIELD and WILCOXON.

As standards, codeine, pethidine, and morphine can be used. The ED_{50} values of these drugs are:

- Codeine 12 mg/kg s.c.
- Pethidine 12 mg/kg s.c.
- Morphine 2 mg/kg s.c.

Critical Assessment of the Test

The radiant heat test on the tail of mice is very effective to estimate the efficacy and potency of central acting analgesic drugs. With pyrazolones, ED_{50} values still can be calculated, but these are achieved only with relatively high doses. Compounds like acetylsalicylic acid and phenyl-acetic acids show only slight effects making it impossible to calculate ED_{50} values.

Modifications of the Method

Originally, the method has been described for testing analgesic properties in the rat (D'Armour and Smith 1941, Winter et al. 1954, Harris and Pierson 1964). Goldstein and Malseed (1979) adapted the procedure for utilization in **cats**. The effect of morphine could be antagonized by naloxone in this test. No response to sodium salicylate or pentobarbital was observed. Lutz et al. (1994) used a modification of the rat tail-withdrawal test to investigate the structure–activity profile of a series of opioid analgesics. One day before testing, polyethylene tubings were implanted in the femoral vein and externalized behind the neck for intravenous application of test substances.

Various instruments have been described for measuring tail-flick latencies by several authors, e.g., Davies et al. (1946), Owen et al. (1981), Isabel et al. (1981), Walker and Dixon (1983), Yoburn et al. (1984), and Harris et al. (1988).

Tail-flick analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Green and Young (1951) compared the heat and pressure analgesiometric methods in rats. Mohrland et al. (1983) described an ultrasound-induced tail-flick procedure.

Hargreaves et al. (1988), Costello and Hargreaves (1989), and Hylden et al. (1991) exposed the plantar surface of hindpaws of unrestrained rats to a beam of radiant heat applied through the glass floor of a testing chamber. Paw withdrawal latency was automatically recorded by a photocell.

This method was also used by Schuligoi et al. (1994).

Taylor et al. (1997) used this method to investigate the brief (phase 1) and persistent (phase 2) nociceptive responses of rats after injection of dilute formalin into the hindpaw.

Carmon and Frostig (1981) used brief laser-induced heat applied to the rat ear for pharmacological testing of analgesics.

Perkins et al. (1993) and Perkins and Kelly (1993) used ultraviolet-induced hyperalgesia in rat paw. Female Sprague–Dawley rats weighing about 100 g were exposed on the plantar surface of one hindpaw to UV light (intensity maximum 365 nm, 69 mW/cm^2) for 90 s, and this was repeated 18 h later. On the following days, each group of rats was placed in a transparent Perspex box, and the withdrawal threshold to a focused beam of radiant heat applied to the underside of each hindpaw was measured.

McCallister et al. (1986) directed radiant heat to the **ears of rabbits** and measured ear-withdrawal time.

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Hot Plate Method

Purpose and Rationale

The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws, and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenylacetic acid type do not generally affect these responses.

Procedure

The method originally described by Woolfe and MacDonald (1944) has been modified by several investigators. The following modification has been proven to be suitable:

Groups of 10 mice of either sex with an initial weight of 18–22 g are used for each dose. The hot plate, which is commercially available,

consists of an electrically heated surface. The temperature is controlled for 55–56 °C. This can be a copper plate or a heated glass surface. The animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stopwatch. The latency is recorded before and after 20, 60, and 90 min following oral or subcutaneous administration of the standard or the test compound.

Evaluation

The prolongation of the latency times comparing the values before and after administration of the test compounds or the values of the control with the experimental groups can be used for statistical comparison using the *t*-test. Alternatively, the values which exceed the value before administration for 50 % or 100 % can be regarded as positive, and ED_{50} values can be calculated.

Doses of 7.5 mg/kg s.c. morphine hydrochloride, 30 mg/kg s.c. codeine hydrochloride, 30 mg/kg s.c. pethidine hydrochloride, and 400 mg/kg s.c. phenazone were found to be effective, whereas aspirin showed no effect even at high doses.

Critical Assessment of the Test

The hot plate test has been used by many investigators and has been found to be suitable for evaluation of centrally but not of peripherally acting analgesics. Mice as well as rats have been used. The method has the drawback that sedatives and muscle relaxants (Woolfe and MacDonald 1944) or psychotomimetics (Knoll 1967) cause false positives, while mixed opiate agonists–antagonists provide unreliable results. The validity of the test has been shown even in the presence of substantial impairment of motor performance (Plummer et al. 1991). Mixed opiate agonists–antagonists can be evaluated if the temperature of the hot plate is lowered to 49.5 °C (O'Callaghan and Holtzman 1975; Zimer et al. 1986).

Modifications of the Method

O'Neill et al. (1983) described an automated, high-capacity method for measuring jump latencies on a hot plate. A hot plate test with increasing temperature was recommended by Tjølsen et al. (1991).

Hot plate analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

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Tail-Immersion Test

Purpose and Rationale

The method has been developed to be selective for morphine-like compounds. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 55 °C.

Procedure

Young female Wistar rats (170–210 g body weight) are used. They are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55 °C. Within a few seconds, the rat reacts by withdrawing the tail. The reaction time is recorded in 0.5 s units by a stopwatch. After each determination, the tail is carefully dried. The reaction time is determined before and periodically after either oral or subcutaneous administration of the test substance, e.g., after 0.5, 1, 2, 3, 4, and 6 h. The cutoff time of the immersion is 15 s. The withdrawal time of untreated animals is between 1 and 5.5 s. A withdrawal time of more than 6 s therefore is regarded as a positive response.

Evaluation

ED_{50} values can be calculated for each compound and time response curves (onset, peak, and duration of the effect) be measured. All the morphine-like analgesics have been shown to be active at doses which do not produce gross behavioral changes. For example, an ED_{50} of 3.5 mg/kg s.c. for morphine and an ED_{50} of 1.7 mg/kg s.c. methadone was found. Acetylsalicylic acid at a dose of 640 mg/kg p.o., phenylbutazone at a dose of 160 mg/kg s.c., as well as nalorphine at a dose of 40 mg/kg s.c. were inactive.

Critical Assessment of the Test

The test is useful to differentiate central opioid-like analgesics from peripheral analgesics.

Modifications of the Method

Ben-Bassat et al. (1959) described the receptacle method in mice. Each mouse was inserted in a conoid paper receptacle with its tail protruding, the cone being closed by a stapler. The protruding tail was entirely immersed in a water bath (58 °C) and the time until withdrawal of the tail was measured by a stopwatch.

Pizziketti et al. (1985) modified the tail-immersion test in rats in this way that they used a 1:1 mixture of ethylene-glycol and water **cooled** to a temperature of minus 10 °C as noxious stimulus. Linear dose–response curves were found with levo-methadone and morphine. Low ceiling effects or curvilinear dose–response curves were obtained with narcotic agonist–antagonist analgesics such as pentazocine. Diazepam and aspirin were inactive.

Tiseo et al. (1988) could show that the endogenous kappa agonist dynorphin A was inactive in the rat tail-immersion test at 55 °C but gave dose–response curves in the cold water version of the test.

Abbott and Melzack (1982) examined the effects of brainstem lesions on morphine analgesia using the formalin test which produced moderate pain that lasted about 2 h and the tail-flick hot water immersion test which measured brief threshold-level pain.

Abbott and Franklin (1986) used two forms of the rat tail-flick test: In the restrained form of the test, rats were placed in wire restraining tubes from 10 min before drug injection till the end of the test. In the unrestrained form of the test, rats

were left free in their home cages and handheld during each test for approximately 30 s. Responses to the thermal pain stimulus were assessed by the latency with which the rat removed its tail from 55 °C water. Two types of morphine analgesia have been postulated in animals: one type, exemplified in rats that are restrained during tail-flick testing, is sensitive to an interaction between morphine and brain 5-HT, the level of which is elevated by restrained stress (Kelly and Franklin 1984).

Luttinger (1985) determined the antinociceptive activity of drugs using different water temperatures in a tail-immersion test in mice. The results roughly paralleled the differences in the severity of pain for which various analgesics are effective.

Dykstra et al. (1986, 1987) described a tail-withdrawal procedure for assessing analgesic activity in **Rhesus monkeys** by immersion of the tail into water of 55 °C. This procedure was used by Rothman et al. (1989) to determine the pharmacological activities of optically pure enantiomers of the κ-opioid agonist, U50,488, and its cis diastereomer.

Using this method, Ko et al. (1999) found that activation of peripheral κ-opioid receptors inhibits capsaicin-induced nociception in Rhesus monkeys.

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Electrical Stimulation of the Tail

Purpose and Rationale

Since the tail of mice is known to be sensitive to any stimulus, a method of electrical stimulation has been described as early as 1950 by Burn et al. The stimulus can be varied either by the duration of the electric shock or by an increase in the electric current.

Procedure

As described by Kakunaga et al. (1966), male mice with a weight of 20 g are placed into special cages. A pair of alligator clips is attached to the tail whereby the positive electrode is placed at the proximal end of the tail. Rectangular wave pulses from a constant voltage stimulator at an intensity of 40–50 V are applied. The frequency of the stimulation is 1 shock/s and the pulse duration 2.5 ms. The normal response time range of the stimuli is 3–4 s. Following administration of the drug, the response time is registered at 15 min intervals until the reaction time returns to control levels.

Evaluation

The data for each animal are plotted with reaction times on the ordinate and time intervals following administration on the abscissa. The area under the time response curve is calculated. In control animals, the reaction time remains fairly constant and the area under the curve is approximately zero. Effects of morphine at 5 mg/kg s.c. and meperidine 30 mg/kg s.c. could easily be demonstrated.

Critical Assessment of the Test

The effect of central analgesics can be clearly demonstrated; however, also the activity of peripheral analgesics given at higher doses can be detected.

Modifications of the Method

Vidal et al. (1982) measured the thresholds of three nociceptive reactions (tail withdrawal, vocalization, vocalization afterdischarge) following electrical stimulation of the tail.

A variation of the test has been introduced by Yanaura et al. (1976), using ultrasonic stimulation instead of electric stimulation. The method is considered to be fast, simple, and precise. The stimulus can be applied repeatedly without causing injury to the tissue. A vocalization test in rats with electrical stimulation of the tail has been described by Hoffmeister (1968).

Ludbrook et al. (1995) described a method for frequent measurement of sedation and analgesia in sheep using the response to a ramped electrical stimulus. Sheep were placed in a canvas sling in their metabolic crates to allow their limbs to partially bear weight in order to minimize spontaneous limb movements. Two needles were placed subcutaneously 0.5 cm apart in the anterior aspect of the lower third of the sheep's hind and connected to the nerve stimulator. The current ramp rate was set at one mA per sec. As soon as limb withdrawal was observed, the stimulus was switched off and the highest current and ramp duration were recorded.

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Grid Shock Test

Purpose and Rationale

The electric grid shock test in mice has been described by Blake et al. (1963) as a modification of an earlier approach (Evans 1962) to measure the analgesic properties by the "flinch–jump" procedure in rats.

Procedure

Male mice with a weight between 18 and 20 g are individually placed into clear plastic chambers. The floor of the box is wired with tightly strung stainless steel wire, spaced about 1 mm apart. The stimulus is given in the form of square wave pulses, 30 cycles per second with a duration of 2 ms per pulse. The output of the stimulator has to be connected to alternate wires of the grid. A fixed resistance is placed in series with the grid and in parallel to an oscilloscope to allow calibration in milliamperes. With increasing shock intensities, the mice flinch, exhibit a startling reaction, increase locomotion, or attempt to jump. The behavior is accurately reflected on the oscilloscope by marked fluctuations of the displayed pulse and defined as pain threshold response. Pain thresholds are determined in each individual mouse twice before administration of the test drug and 15, 30, 60, 90, and 120 min after dosing. Groups of 10 animals are used for control and for the test drugs.

Evaluation

The current as measured in milliamperes is recorded for each animal before and after administration of the drug. The average values for each group at each time interval are calculated and statistically compared with the control values. Placebo-treated controls show a slight increase of threshold over time. Not only morphine sulfate in a dose of 10 mg/kg p.o. but also acetylsalicylic acid in a dose of 200 mg/kg p.o. definitely increases the threshold.

Critical Evaluation of the Method

The modification of the method as described by Blake et al. (1963) showed an effect not only of morphine but also of acetylsalicylic acid which is not easily picked up by other tests based on stimulation by physical means.

Modifications of the Test

Weiss and Laties (1961) in a "fractional escape" procedure trained animals to press a lever to reduce the intensity of shock delivered continuously through the floor grids of the experimental chamber. Each time the rat depresses the lever, it reduces the intensity of the shock. An external timer is programmed to increase the intensity of the shock every few seconds. If the animal fails to press the lever, the shock continues to increase in intensity until lever-pressing behavior drives it

down. Thus, the level of shock fluctuates depending on the rat's lever pressing. The action of an analgesic in altering the level of shock which the rat will "tolerate" can then be measured by comparing the average level at which the rat maintains the shock under control conditions with the average level at which the rat maintains the shock during treatment.

Painful stimulation of the paws of mice placed into cages equipped with metal bands for electrical stimulation was described by Charlier et al. (1961) as "pododolorimetry."

A modification of the jump–flinch technique for measuring pain sensitivity in rats based on four categories of responses was described by Bonnet and Peterson (1975).

Eschalier et al. (1988) described an automated method to analyze vocalization of unrestrained rats submitted to noxious stimuli.

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Tooth-Pulp Stimulation

Purpose and Rationale

The method has been first described by Koll and Reffert (1938) and by Ruckstuhl and Gordanoff (1939) for testing central analgesic activity in rabbits and has since applied by several authors to various animal species. Stimulation of the tooth pulp induces characteristic reactions, such as licking, biting, chewing, and head flick, which can be observed easily.

Procedure

Rabbits of either sex with a weight between 2 and 3 kg are anesthetized with 15 mg/kg thiopental or 0.2 mg/kg fentanyl citrate intravenously. Pulp chambers are exposed close to the gingival line in the lateral margins of the two front upper incisors with a high-speed dental drill. On the day of the experiment, clamping electrodes are placed into the drilled holes. After an accommodation period of 30 min, stimulation is started to determine the threshold value. The stimulus is applied

by rectangular current with a frequency of 50 Hz and a duration of the stimulus of 1 s. The electrical current is started with 0.2 mA and increased until the phenomenon of licking occurs. In some cases, the current has to be increased and then to be decreased again in order to find the appropriate threshold. For assessing the basic value, the threshold is determined three times in each animal. Each animal serves as its own control. For testing analgesic activity of a new drug and determination of an ED_{50}, 8–10 animals are used for each dose of the analgesic. The test substance is either injected intravenously or given orally by gavage. The threshold as the indicator of the antinociceptive effect is determined again after 15, 30, 60, and 120 min. The animals serve as their own controls. Threshold current is determined again 5, 15, 30, 45, and 60 min after intravenous application and 15, 30, 60, and 120 min after oral application.

Evaluation

For screening procedures, the increase of threshold, expressed in mV, is the indicator of intensity and duration of the analgesic effect. For determination of the ED_{50}, 8–10 rabbits are used for each dose, using three doses, which provided effects between 10 % and 90 %. An antinociceptive effect is defined as an increase of the threshold versus the initial control by a factor of 2 or more.

Critical Assessment of the Method

Central analgesics, especially opioid agonists, have been found to be very active in this test. Compared with other tests for central analgesic activity, like the hot plate test in mice, the tests result in lower ED_{50} values indicating a high sensitivity of the method. In addition, non-opiate analgesics like ketamine and peripheral analgesics like pyrazolone derivatives gave a positive response.

Modifications of the Method

The method has been performed primarily in rabbits (Hertle et al. 1957; Hoffmeister 1962, 1968; Piercey and Schroeder 1980), but also **dogs** (Koll and Fleischmann 1941; Skingle and Tyers 1979) and **cats** (Mitchell 1964) have been used.

Among several methods in different species, Fleisch and Dolivo (1953) found the electrical stimulation of the tooth pulp in the rabbit as the only satisfactory method to test the efficacy of different analgesic drugs.

The effects of tooth-pulp stimulation in the thalamus and hypothalamus of the **rat** have been investigated by Shigena et al. (1973).

The method has been adapted for freely moving rats (Steinfels and Cook 1986). Medium effective doses could be determined for ì and ê agonists. Nonsteroidal anti-inflammatory drugs were also effective in this test procedure, but the slopes of the dose–response curves for these compounds were lower than for the opioid analgesics. Microinfusion of bradykinin solution onto the tooth pulp of unrestrained rats was described by Foong et al. (1982) as a reliable method for evaluating analgesic potencies of drugs on trigeminal pain.

Kidder and Wynn (1983) described an automatic electronic apparatus for generating and recording a ramp stimulus for analgesia testing.

Thut et al. (1995) used the rabbit tooth-pulp assay to quantify efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

Shyu et al. (1984) studied the role of central serotoninergic neurons in the development of dental pain in the **monkey**.

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Monkey Shock Titration Test

Purpose and Rationale

Generally, analgesic tests in rats and mice result in correlation with the analgesic activity of a drug in man. To clarify the mode of action in more detail and to find a suitable dose for therapy in man, experiments in monkeys may be necessary.

Procedure

This test has been recommended by Weiss and Laties (1958) and later developed further by several authors. The monkeys are seated in restraining chairs. Electrical current is delivered by a Coulbourn Instrument Programmable Shocker through electrodes coupled to two test tube clamps which are attached to a shaved portion of the tail. The current ranges from 0 to 4 mA through 29 progressive steps. The monkey presses a bar to interrupt the shock. A stable baseline shock level is established for each monkey on the day prior to drug administration. After drug administration, shock titration activity is rated according to the change in maximum level of median shock intensity attained for drug as compared to control levels. Doses of 3.0 mg/kg i.m. morphine, 1.7 mg/kg i.m. methadone, and 10 mg/kg i.m. pentazocine were found to be effective.

Critical Assessment

The monkey shock titration test may be used for final evaluation of a new compound before administration to man. For screening activities, the procedure cannot be recommended since the test is too time-consuming and the apparatus too complicated. Furthermore, higher animals such as monkeys should only be used if absolutely necessary.

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Formalin Test in Rats

Purpose and Rationale

The formalin test in rats has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis (1977).

Procedure

Male Wistar rats weighing 180–300 g are administered 0.05 ml of 10 % formalin into the dorsal portion of the front paw. The test drug is administered simultaneously either s.c. or orally. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and 60 min and scored according to a pain scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated if both paws are resting on the floor with no obvious favoring of the injected paw.

Evaluation

Using various doses, ED_{50} values for protection can be calculated. Doses of 1.7 mg/kg morphine s.c. and 15 mg/kg s.c. pethidine were found to be effective.

Critical Assessment

The formalin test identifies mainly centrally active drugs, whereas peripherally acting analgesics are almost ineffective. Therefore, the formalin test may allow a dissociation between inflammatory

and noninflammatory pain, a rough classification of analgesics according to their site and their mechanism of action (Chau 1989). Cowan (1990) underlined the aspect that the formalin test is a model of chronic pain, whereas most other methods measure only the effect on acute pain.

Modifications of the Method

Murray et al. (1988) used **mice** instead of rats. They injected 0.020 ml of 5 % formalin solution into the subplantar region of the hindpaw. Morphine at a dose of 2.1 mg/kg s.c. and pentazocine at a dose of 23.8 mg/kg s.c. were active, whereas the cyclooxygenase inhibitor zomepirac was inactive even at a dose of 100 mg/kg s.c.

Hunskaar et al. (1986) and Hunskaar and Hole (1987) injected a small amount of formalin (20 µl of 1 % solution) under the skin of the dorsal surface of the right hindpaw of mice. A biphasic response with an early (0–5 min) and a late (20–30 min) phase with high licking activity was observed. Central acting analgesics were active in both phases, whereas nonsteroidal anti-inflammatory drugs and corticosteroids inhibited only the late phase. Acetylsalicylic acid and paracetamol were antinociceptive in both phases.

Shibata et al. (1989) again used lower concentrations of formalin (0.025 ml of 0.5 % formalin solution) and also mice instead of rats. They found a characteristic biphasic pain response. Centrally acting drugs such as morphine inhibited both phases, whereas according to their data, peripherally acting drugs such as acetylsalicylic acid, oxyphenylbutazone, and corticosteroids inhibited only the second phase.

Abbott et al. (1995) used the formalin test for scoring properties of the first and second phases of the pain response in rats.

Abbadie et al. (1997) determined the pattern of c-fos expression in the rat spinal cord to study the two phases of the formalin test.

Clavelou et al. (1989), Dallel et al. (1995), and Gilbert et al. (2001) used a modification of the formalin test for assessing pain and analgesia in the **orofacial region of the rat**. After injection into the upper lip, pain intensity was evaluated by the animal's behavior of rubbing of the injected area. A subcutaneous injection of 0.05 ml of 0.92 % formaldehyde solution was made into the upper lip, just lateral to the nose. Following injection, the rat was immediately brought back in a test box equipped with a video camera for a 45 min observation period. The recording time was divided into 15 blocks of 5 min, and a pain score was determined for each block, by measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral fore- or hindpaw. The animals were sacrificed after the end of the experiment to avoid unnecessary suffering.

Tjølsen et al. (1992) attributed the early phase to C-fiber activation, whereas the late phase appeared to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord.

Alreja et al. (1984) used the formalin test for assessing pain in **monkeys** after one of the authors volunteered to carry out the same procedure on himself.

Corrêa and Calixto (1993) studied the participation of B_1 and B_2 kinin receptors in the formalin-induced nociceptive response in the **mouse**. Pain response was increased after ACE-inhibition and decreased by bradykinin receptor antagonists.

Herman and Felinska (1979) proposed a rapid test for screening of narcotic analgesics in mice by evaluation of behavioral symptoms after subcutaneous injection of EDTA.

Legat et al. (1994) and Dumas et al. (1997) induced hyperalgesia in rats by subplantar injection of collagenase (100 µg in 100 µl saline) and rated the behavioral reactions after treatment with analgesics according to a modified formalin test.

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Neuropathic Pain

Neuropathic pain and neuropathy are manifestations of a large spectrum of different diseases that have deleterious effects on normal neural

functioning. Neuropathic pain is part of the larger syndrome of "neuropathy" which is a disorder/dysfunction of peripheral nerves. Neuropathic pain is a complex chronic "disease or disorder" involving the nerve itself as the source of the pain, where the nerve fibers themselves may be injured, damaged, or become dysfunctional, and consequentially these damaged nerve fibers send incorrect signals to other pain centers. The nerve fiber injury can result in the change of the nerve both at the site of injury and areas around the injury. Peripheral neuropathies can include sensory, motor, and autonomic fiber derangements that go into the central nervous system. All conditions classified as neuropathies do not always result in pain; they can be very debilitating in other ways such as sensory numbness/paresthesia, muscle weakness/paralysis, and gastric dysfunction.

Most neuropathic conditions can coexist with inflammatory, visceral, and/or nociceptive pain (or pain resulting from the stimulation of free nerve endings by a chemical, thermal, or mechanical event), as well as neuropathic dysfunction seen in diabetes resulting in autonomic nervous system involvement.

The major cause of neuropathic pain can include:

Infectious: Viral conditions are known to cause long-standing neuropathic pain. The postherpetic neuralgia is caused by reactivation of the varicella–zoster virus. Leprosy (mycobacterium), Lyme disease (spirochetes), Chagas disease (trypanosomes), HIV, and Guillain–Barré syndrome (postinfectious), all can cause neuropathic pain.

Metabolic: Neuropathic pain in diabetes is caused by metabolic dysfunction. Glycosylation end products inhibit axonal transport and Na^+/K^+ ATPase function resulting in axonal degeneration and nerve damage. Alcohol-induced polyneuropathy is often due to thiamine (B1) deficiency although it can produce its own small fiber pain pathology as opposed to a thiamine-deficient axonal sensorimotor burning neuropathy. It is defined by axonal degeneration in neurons of both the sensory and motor systems and initially occurs at the distal ends of the longest axons in the body. Deficiencies like Beriberi (vitamin B1) can also produce neuropathic pain.

Toxic: The most common toxic condition causing neuropathic pain is seen in the treatment of cancer as a result of chemotherapy and radiation. Thallium and isoniazid are also known to cause neuropathic pain conditions. Exposure to chemicals like lead and arsenic can also result in nerve damage. Toxic exposure generally results in abnormalities in genetic/protein processing.

Trauma: Phantom limb syndromes and/or complex regional pain syndromes (CRPS) can happen due to the consequence of trauma. Phantom limb pain refers to mild to extreme pain felt in the area where limb has been amputated. Phantom limb pain is thought to be a result of abrupt loss of sensory input from the peripheral limb to the brain. Although the limb is no longer there, the nerve endings at the site of the amputation continue to send pain signals to the brain that make the brain think the limb is still there. There is no known mechanism that causes CRPS, but many hypotheses have been suggested, including dysfunctional processing throughout the entire nervous system involving central peripheral, central, and autonomic neurons. Complex regional pain syndrome (CRPS) is a chronic pain condition most often affecting the arms, legs, hands, or feet, usually following an injury or trauma to that limb. CRPS is believed to be caused by involvement of the peripheral and central nervous systems. Complex regional pain syndrome occurs in two types, with different causes and with similar signs and symptoms. Illness or injuries that did not directly damage the nerves in the affected limb are classified as having CRPS-I (previously called reflex sympathetic dystrophy syndrome), and CRPS-II (previously called causalgia) follows a distinct nerve injury.

Autoimmune: This type of neuropathic pain can be quite diverse. They may have autoimmune antibodies involved in their pathophysiology and are usually amenable to immune therapy. Some examples of autoimmune neuropathic

pain include chronic inflammatory demyelinating polyneuropathy (CIDP) and vasculitic neuropathy.

Compressive: Carpal tunnel syndrome and compartment syndromes are common entrapment injuries. Both nerve entrapment and excessive external pressure on nerve axons can cause distortional (stretching) changes or ischemic changes. Prolonged injury results in Wallerian degeneration of the axon with resultant muscle atrophy.

Congenital/Hereditary: Charcot–Marie–Tooth disease (burning pain in extremities) and Fabry disease are good examples of peripheral neuropathic pain associated with congenital abnormalities. Other hereditary conditions like amyloidosis can also produce painful conditions.

General Considerations

Partial injury to somatosensory nerves sometimes causes causalgia in humans. Causalgia is characterized by spontaneous burning pain combined with hyperalgesia and allodynia and usually follows an incomplete peripheral nerve injury. Allodynia, a pain sensation due to normally innocuous stimulation, is a particularly troublesome symptom in patients. Neuropathic pains are classified according to either the etiological diagnosis of the neuropathy (e.g., painful diabetic neuropathy, post-herpetic neuralgia, posttraumatic neuralgia, etc.) or the anatomical lesion (e.g., central pain, peripheral neuralgia). See Hansson and Dickenson (2005). Various animal models are described to study neuropathic pain. See below.

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Chronic Nerve Constriction Injury

Purpose and Rationale

Bennet and Xie (1988) described a peripheral neuropathy due to nerve constriction in the rat that produces disorders of pain sensation like those seen in man. This method with slight modifications was used by Davar et al. (1991), Mao et al. (1992), Munger et al. (1992), Yamamoto and Yaksh (1992), and Tal and Bennet (1993) and reviewed by Bennett (1993).

Procedure

Anesthesia is induced in male Sprague–Dawley rats by inhalation with halothane 4 % and maintained at a concentration of 2–3 % as needed. After a local incision, the biceps femoris of each leg is bluntly dissected at mid-thigh to expose the sciatic nerve. Each nerve is then mobilized with care taken to avoid undue stretching. Four 4-0 chromic gut sutures are each tied loosely with a square knot around the right sciatic nerve. The left sciatic nerve is only mobilized. Both incisions are closed layer to layer with silk sutures and the rats allowed to recover. During the next days, the animals show a mild eversion of the affected paw and a mild-to-moderate degree of foot drop.

The thermal nociceptive threshold is measured according to the method of Hargreaves et al. (1988) (see section "Radiant Heat Method"). The rats are placed beneath a clear plastic cage ($10 \times 20 \times 24$ cm) upon an elevated floor of clear glass. A radiant heat source (halogen projector lamp) is placed beneath the glass floor on a movable holder and positioned such that it focuses at the plantar area of one hindpaw. The time interval between the application of the light beam and the brisk hindpaw withdrawal response is measured to the nearest 0.1 s.

The maximum hyperesthesia occurs between 7 and 14 days after nerve ligation. Before intrathecal injection of the drug or vehicle, the hindpaws are tested three times alternatively with 5 min intervals as the baseline data. The left and right test sequence is carried out at 5, 15, 30, 60, and 90 min after injection.

Evaluation

The mean $\pm$ SEM of the paw withdrawal latency (PWL) is plotted. To analyze the magnitude of hyperesthesia, the difference score (DS) is calculated by subtracting the maximum PWL of the control side (left side) from the maximum PWL of the affected side (right side). The maximum

PWL is defined as the PWL that was the maximum during the first 30 min after injection. To analyze the drug effects in hyperesthetic rats, the dose is plotted against the change in DS (postdrug difference score minus pre-drug difference score).

Modifications of the Method

The chronic constriction injury (CCI) model according to Bennett and Xie (1988) has been used by several authors: Sotgiu and Biella (1998), Toda et al. (1998), Blackburn-Munro and Jensen (2003), Keay et al. (2004), Bingham et al. (2005), Bomholt et al. (2005), Costa et al. (2005), and Howard et al. (2005).

Sotgiu et al. (1996) performed laminectomy from L1 to S2 in anesthetized rats with sciatic chronic constriction injury. For extracellular recording, two tungsten microelectrodes were positioned under a dissecting microscope on the surface of the spinal cord at L2 and L5–L6 levels ipsilaterally to the injured nerve and were advanced at steps of 2 μm. Neuronal activity was conventionally recorded and then digitized; frequency histograms were constructed by computer programs. The search stimulus for dorsal horn neurons at L2 and L5–L6 segments was the electrical stimulation of saphenous and sciatic nerve peripheral territories. Natural stimuli (brushing of the skin) and noxious stimuli (calibrated pinching) were defined. After the responses to saphenous stimuli in the neurons were recorded, a small pad of Gelfoam soaked with 0.5 ml lidocaine was placed around the intact epineurium proximally to the ligatures on the sciatic nerve. The saphenous stimulation was repeated during the block and after complete recovery of the preblock baseline activity. In this way, the effect of the local anesthetic on the spontaneous activity and on the response to a noxious stimulus could be evaluated.

The first animal model of painful neuropathy was reported by Wall et al. (1979a, b). The sciatic nerve of rats or mice was sectioned and either tied or implanted in a polyethylene tube sealed at its far end. Moreover, in one modification, also the saphenous nerve was cut, such that the hindpaw was completely denervated. This procedure, which is known as the neuroma model, is believed to replicate the human syndromes seen after amputation (phantom pain) or after nerve transection in an intact limb (anesthesia dolorosa). Within several days, the animals begin to self-mutilate the hindpaw on the side of the nerve transection: a behavior named "autotomy."

Seltzer et al. (1990) ligated only one-half of the sciatic nerve in rats unilaterally. The withdrawal thresholds to repetitive von Frey hair stimulation at the plantar side were decreased bilaterally as were the withdrawal thresholds to CO_2 laser heat pulses. The contralateral phenomena resemble the "mirror image" pains in humans with causalgia.

This "partial sciatic nerve injury model" has been used by several authors (Malmberg and Basbaum 1998; Lindenlaub and Sommer 2000; Bingham et al. 2005). Patel et al. (2001) studied the effects of $GABA_B$ agonists and gabapentin on mechanical hyperalgesia in models of neuropathic (partial sciatic ligation) and inflammatory (Freund's complete adjuvant) pain in the rat and the inhibitory action on spinal transmission in vitro.

Hofmann et al. (2003) described the tibial nerve injury model in rats as a surgically uncomplicated model of neuropathic pain based on unilateral transection (neurotomy) of the tibial branch of the sciatic nerve.

Walczak et al. (2005) characterized the saphenous nerve partial ligation in rats as a model of neuropathic pain.

Kim and Chung (1992) described an experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Either both the L_5 and L_6 spinal nerves or the L_5 spinal nerve alone on one side of the rat was tightly ligated. A modified version of this technique was used by LaBuda and Little (2005) and Bertorelli et al. (2005).

DeLeo et al. (1994) performed cryoneurolysis of the sciatic nerve in the rat using a CryoProbe cooled to −60 °C in a 30/5/30 s freeze–thaw–freeze sequence. Autotomy was observed after 4–14 days.

Coderre et al. (2004) produced a neuropathic-like pain syndrome in rats following prolonged hindpaw ischemia and reperfusion, creating an animal model of complex regional pain syndrome type I (CRPS-I; reflex sympathetic dystrophy),

called **chronic post-ischemia pain**. A tourniquet ring was placed on one hindlimb of an anesthetized rat just proximal to the ankle joint for 3 h, which was removed prior to termination of anesthesia to allow reperfusion. Rats exhibited hyperemia and edema/plasma extravasation of the ischemic hindpaw for a period of 2–4 h after reperfusion. Hyperalgesia to noxious mechanical stimulation (pin prick) and cold (acetone exposure) and mechanical allodynia to innocuous mechanical stimulation (von Frey hairs) are evident in the affected hindpaw as early as 8 h after reperfusion and extend for at least 4 weeks.

Mice that lack protein kinase C gamma (PKCγ) displayed normal response to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted (Malmberg et al. 1997).

Shimoyama et al. (2002) developed a **mouse model of neuropathic cancer pain** by inoculating Meth A sarcoma cells in the immediate proximity of the sciatic nerve in BALB/c mice. The tumor grows predictably with time and gradually compresses the nerve, thereby causing nerve injury. Time courses of thermal hypersensitivity and mechanical sensitivity to von Frey hairs were determined and signs of spontaneous pain were evaluated. The authors compared this model with the chronic constriction model.

Panesar et al. (1997) and Campbell et al. (1998) studied mechanical hyperalgesia associated with partial peripheral ligation in the **guinea pig**.

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Peripheral Nerve Injury Model

Purpose and Rationale

In addition to chronic ligation techniques, nerve dissection and nerve crush models have been used to study neuropathic pain. Nerve crush injury was described by Decosterd et al. (2002).

Procedure

Male Sprague–Dawley rats weighing 200–250 g were anesthetized with halothane (1.5–3 %). The left sciatic nerve was exposed at the mid-thigh level and crushed by a pair of hemostat forceps with smooth protective pads that were placed perpendicularly to the sciatic trunk for 30 s (Bester et al. 2000). Muscle and skin were closed in two layers.

Animals were habituated to the tester, the environment, and the handling procedures prior to commencement of the testing. A calibrated von Frey monofilament was applied five times until it bent on the lateral dorsal side of the hindpaw that is in crushed nerve territory or the intact sural nerve, ipsilateral to the nerve lesion. A series of monofilaments (1.0–200.0 g) were applied, and the test started with the lowest force filament. The mechanical withdrawal threshold corresponds to the minimum force required to elicit a reproducible flexor withdrawal movement after application of the von Frey hairs. Once baseline threshold was determined, eight light strokes were applied manually at 1 Hz to the dorsum of the paw at 5 min intervals, for 2 h. The mechanical withdrawal threshold was recorded immediately after the application of the light touch, at the same interval of 5 min during the whole test period.

Evaluation

Data are expressed as mean ± SEM of the recorded mechanical withdrawal threshold in grams or as a percentage of the baseline value. The differences between groups were analyzed with analysis of variance (ANOVA) two-way repeated measures.

Modifications of the Method

Vogelaar et al. (2004) described sciatic nerve regeneration in mice and rats. Under anesthesia, the sciatic nerve was carefully exposed. At a point immediately distal from the gluteus maximus muscle, the nerve was crushed for 30 s using a hemostatic forceps. The animals were followed for 70 (rats) and 32 (mice) days, and functional recovery of sciatic nerve function was monitored by the foot reflex withdrawal test, locomotor pattern, and mechanical withdrawal thresholds.

Rodrigues-Filho et al. (2003, 2004) published the technique of avulsion injury of the rat brachial plexus that triggers hyperalgesia and allodynia in the hindpaws. Male Wistar rats weighing 250–300 g were anesthetized by chloralose i.p. The brachial plexus was approached through a horizontal incision parallel to the clavicle, running from the sternum to the axillary region. The pectoralis major muscle was displaced, leaving the cephalic vein intact. The subclavian vessels were located and the lower trunk dissected and crushed three times for 5 s using microsurgical forceps. At the end of this procedure, the nerve was completely flattened and transparent. The tissue layers were then brought together and the skin closed with silk sutures.

For assessment of mechanical hyperalgesia, mechanical thresholds were measured in the hindpaws with an analgesy meter (Ugo Basile, Italy) according to the method of Randall and Selitto (1957). Furthermore, thermal hyperalgesia, mechanical allodynia, and cold allodynia were measured in the hindpaws, as well as grasping force in the forepaws.

Sweitzer et al. (2001) studied prevention of allodynia induced by transection of the L5 spinal nerve in rats.

Devor et al. (2005) studied heritability of symptoms in the neuroma model of neuropathic pain.

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Spared Nerve Injury Model

Purpose and Rationale

In addition to the above-described models of peripheral neuropathic pain [chronic constriction injury model according to Bennett and Xie (1988), partial ligation according to Seltzer et al. (1990), and segmental spinal nerve ligation according to Kim and Chung (1992)], Decosterd and Woolf (2000) described **spared nerve injury** as an animal model of persistent peripheral neuropathic pain.

Procedure

Adult male Sprague–Dawley rats were used. Under halothane (2 %) anesthesia, the skin on the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal, and tibial nerves. The procedure comprises an axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal and the tibial nerves were tightly ligated with 5.0 silk and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. Care was taken to avoid any contact with or stretching of the intact sural nerve. Muscle and skin were closed in two layers. Behavior testing was performed only after a period of at least 1 week.

For testing mechanical allodynia, animals were placed on an elevated wire grid and the plantar surface of the paw stimulated with a series of ascending force von Frey filaments. The threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli. The lateral and medial plantar surface of the paw as well as its dorsal surface was tested.

For testing mechanical hyperalgesia, a pin prick test was performed using a safety pin. The lateral part of the plantar surface of the paw was briefly stimulated at an intensity to indent but not penetrate the skin. The duration of paw withdrawal was recorded.

For testing cold allodynia, a drop of acetone solution was delicately dropped onto the lateral plantar surface of the paw, using a blunt needle connected to a syringe without touching the skin. The duration of the withdrawal response was recorded.

The lateral plantar surface was exposed to a beam of radiant heat through a transparent Perspex surface (Hargreaves et al. 1988). The withdrawal latency and duration were recorded. The heat stimulation was repeated three times at an interval of 5–10 min for each paw and the mean calculated.

Evaluation

Results were presented as mean ± SEM. The data were analyzed by one-way ANOVA and the nonparametric Wilcoxon matched-pairs signed-rank tests.

Modifications of the Method

The rat spared nerve injury model has been used by several authors: Blackburn-Munro and Jensen (2003), Rode et al. (2005), and Howard et al. (2005).

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Spinal Cord Injury

Purpose and Rationale

Spinal cord injuries result in a devastating loss of function. Chronic central pain syndromes frequently develop in the majority of affected patients. Several attempts have been made to find animal models of this situation. Xu et al. (1992), Hao and Xu (1996), Hao et al. (1998a, b, 2000), Wu et al. (2003), and Colpaert et al. (2004) performed studies in rats after ischemic spinal cord injury photochemically induced by laser irradiation.

Procedure

Female Sprague–Dawley rats were anesthetized with 300 mg/kg chloral hydrate i.p. and one jugular vein was cannulated. Vertebrae T11–L2 were exposed after a midline incision of the skin on the back. The animals were positioned beneath a tunable argon ion laser (Innova, Model 70, Coherent Laser Production Division) and irradiated with a knife edge beam, which was used to cover the single T13 vertebra with an average power of 0.16 W for 10 min. No laminectomy was performed. Immediately before the irradiation, erythrosine B (Red No. 3, Aldrich-Chemie) was injected intravenously in 0.9 % saline at a dose of 32.5 mg/kg. Since erythrosine B is rapidly metabolized, the injection at this dose was repeated at 5 min intervals during the irradiation in order to maintain an adequate blood concentration. Erythrosine B has an optimal absorption wavelength similar to that of the laser light. When it receives this light, a photochemical reaction occurs inside the spinal cord blood vessel where the laser is aimed. One of the reaction products accumulates in the vessel, injuring the endothelial layer and causing platelet release and coagulation and, thus, ischemia. After irradiation, the incision was

closed and the animals were kept warm for 2 h. The bladders were emptied manually two to three times a day until normal function was regained.

A set of calibrated von Frey hairs was used to test the vocalization threshold in response to graded mechanical pressure ranging from 0.021 to 410.0 g. During the test, the rats were gently restrained in a standing position by the experimenter, and the von Frey hair was pushed onto the skin until the filament became bent. The frequency of the stimulation was about every 3–4 s per stimulus and at each intensity. The stimuli were applied 5–10 times. The pressure which induced consistent vocalization (>75 % response rate) over a relatively large skin area was considered to be the pain threshold.

Evaluation

Rats were randomized for different treatment groups. The data are expressed as medians and variability as mean ± SEM. Data were analyzed with Kruskal–Wallis one-way ANOVA followed by Wilcoxon signed-rank test or by Mann–Whitney *U*-test.

Modifications of the Method

Christensen et al. (1996), Christensen and Hulsebosch (1997a, b), and Bennett et al. (2000a, b) used a rodent spinal hemisection model of spinal cord injury in which mechanical and thermal allodynia develop by 24 days after injury.

Male Sprague–Dawley rats (175–200 g) were deeply anesthetized by i.p. injection of 75 mg/kg ketamine and 15 mg/kg xylazine. The spinal cord was hemidissected at T13 on the left side by the following procedure: after palpation of the dorsal surface to locate the cranial borders of the sacrum and the dorsal spinous processes of the lower thoracic and lumbar vertebrae, the T11, T12, and T13 laminae were determined by locating the last rib, which attaches the cranial end of the T13 vertebrae. The surgical field was shaved and prepared with povidone–iodine, and a longitudinal incision was made exposing several segments. A laminectomy was performed at vertebral level T11, the lumbar spinal cord was identified with the accompanying dorsal vessel, and the spinal cord was hemisected at T13, cranial to the L1 dorsal root entry zone, with a scalpel blade without damage to the major dorsal vessel or vascular branches. The musculature and the fascia were then sutured and the skin was apposed by autoclips.

Siddall et al. (1995) and Drew et al. (2004) described mechanical allodynia following contusion injury of the rat spinal cord. Contusive spinal cord injury was produced in anesthetized Wistar rats weighing 200–300 g. Laminectomy was performed at the vertebral thoracic T10 level to expose a 3 mm window over the dorsal spinal cord, and adjacent rostral and caudal spinous processes were clamped to stabilize the spine. A brass guide tube 15 cm in length was positioned perpendicularly above the exposed cord, and a cylindrical 10 g steel weight (2 mm in diameter) with a rounded tip was suspended within the tube 2 cm above the cord surface. The weight was held within the tube by a metal pin. Removing the pin and allowing the weight to drop onto the exposed cord produced spinal cord injury at the T12–T13 segmental level. The wound was closed in layers and antibiotics were administered.

Abraham et al. (2001) and Caudle et al. (2003) described excitotoxic spinal cord injury induced by intraspinal injection of quisqualic acid.

Zochodne et al. (1994) induced a segmental chronic pain syndrome by lumbar intrathecal NMDA infusion.

Malmberg and Yaksh (1992) reported that hyperalgesia mediated by spinal glutamate or substance P receptor is blocked by spinal cyclooxygenase inhibition.

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Chemotherapy-Induced Pain

Purpose and Rationale

Within the numerous adverse effects associated with antineoplastic drugs, painful peripheral neuropathy is frequent. Chemotherapy-induced peripheral neuropathies are mainly sensory in nature and typically develop after a cumulative dose. Chemotherapy-induced peripheral neuropathies are an increasingly frequent problem. Chemotherapy-induced peripheral neuropathies are mainly most often characterized by the development of a subacute or chronic, length-dependent, distal, symmetrical polyneuropathy

with a predominant sensory involvement, with or without associated dysautonomia. Vincristine, an antineoplastic agent widely used in cancer therapy, was found to be neurotoxic for all treated patients and can induce peripheral neuropathy (Windebank 1999). Aley et al. (1996), Authier et al. (1999, 2003), and Marchand et al. (2003) developed an animal model of nociceptive neuropathy using repeated injections of vincristine.

Procedure

Male Sprague–Dawley rats weighing 180–200 g received five intravenous injections of 150 μg/kg vincristine every 2 days until a cumulative dose of 750 μg/kg was reached. Thresholds to paw pressure were determined before and 14 days after vincristine treatment. Test drug was then applied and the vocalization thresholds were determined 15, 30, 45, 60, 90, and 120 min after this injection.

The C-fiber-evoked flexor reflex elicited in the right hind limb (Falinower et al. 1994; Mestre et al. 1997) was recorded from halothane-anesthetized rats. Rectangular electric pulses of 6–7 mA strength and 2 ms duration were applied every 10 s to the sural nerve receptive field by means of two stainless steel needles inserted into the skin of toes 4 and 5. The C-fiber-evoked reflex response (electromyographic responses) was recorded from the ipsilateral biceps muscle by utilizing another pair of stainless steel needles. Once a stable threshold C reflex response was obtained, the stimulus strength was increased by a factor of threefold. Test drug was then injected in various doses and the mean C-fiber reflex (mean of the 12 C-fiber reflexes recorded during the 2 min period) was calculated every 2 min between 25 and 35 min.

Evaluation

The data analysis was performed by a two-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test for the time course of the effect of drug on mechanical nociceptive thresholds. For the C reflex, results were expressed as mean percentage inhibition of the integrated C reflex responses obtained between 25 and 35 min after drug injection and plotted against log dose, allowing ED_{50} calculation.

Modifications of the Method

Lynch et al. (2005) tested the effect of a nicotinic acetylcholine agonist against allodynia in rats in the vincristine-induced pain model.

Polomano et al. (2001) described painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel.

Dalziel et al. (2004) described allodynia in rats infected with varicella–zoster virus as an animal model for **post-herpetic neuralgia**.

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Trigeminal Neuropathic Pain Model

Purpose and Rationale

Trigeminal neuralgia is an extreme form of neuropathic pain. Trigeminal neuralgia is a chronic pain condition that affects the trigeminal nerve, which carries sensation from face to your brain. Even mild stimulation of the face can trigger an episode of excruciating pain. Trigeminal neuralgia is often considered one of the most painful conditions seen in clinical medicine. The pain usually is felt on one side of the jaw or cheek, but some people experience pain at different times on both sides.

Although the pathophysiology of the disorder is uncertain, vascular compression of the trigeminal root resulting in damage to primary afferent neurons is thought to play a major role in the generation of pain. Idänpään-Heikkilä and Guilbaud (1999) used a rat model of **trigeminal neuropathic pain**, developed by Gregg (1973), Jacquin and Zeigler (1983), Vos and Maciewicz (1991), Vos et al. (1994), and Vos and Strassman (1995), where the neuropathy is produced by a chronic constriction injury of the infraorbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain.

Procedure

Male Sprague–Dawley rats weighing 175–200 g were used. The head of the rat, which was anesthetized with sodium pentobarbital 50 mg/kg i.p. and treated with 0.4 mg/kg atropine i.p., was fixed in a stereotaxic frame. A midline scalp incision was made exposing skull and nasal bone. To expose the intra-orbital part of the left infraorbital nerve, the edge of the orbit, formed by the maxillary, frontal, lacrimal, and zygomatic bones, was dissected free. To give access to the infraorbital nerve, the orbital contents were gently deflected with a cotton-tipped wooden rod. The infraorbital nerve was dissected free at its most rostral extent of the orbital cavity, just caudal to the infraorbital foramen. Two chromic catgut (5–0) ligatures (2 mm apart) were loosely tied around the infraorbital nerve. The ligatures reduced the diameter of the nerve by just a noticeable amount and retarded, but did not interrupt, the epineural circulation. The scalp incision was closed with silk sutures.

Before the first actual stimulation session, the rats were allowed to adapt to the observation cage and to the testing environment. During this period, the experimenter reached slowly into the cage to touch the walls with a plastic rod, similar to the ones on which the von Frey filaments were mounted.

For mechanical stimulation, a graded series of 10 von Frey filaments with a bending force of between 0.217 and 12.5 g was used. The stimuli were applied within the infraorbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. The complete series of von Frey hair intensities was presented in an ascending series, and either a brisk withdrawal of the head or an attack/escape reaction was considered as the mechanical threshold. Local injection of a local anesthetic (articaine) into the rostral orbital cavity of the lesioned side, in close proximity to the ligated infraorbital nerve, increased the mechanical threshold to the upper level. The duration of the effect was dose dependent.

Evaluation

Data are expressed as means ± SEM. The nonparametric Kruskal–Wallis one-way ANOVA was used, and comparisons between the groups were performed using the Mann–Whitney *U*-test.

Modifications of the Method

Christensen et al. (2001) studied the effect of gabapentin and lamotrigine on mechanical allodynia-like behavior in the rat model of trigeminal neuropathic pain.

Deseure et al. (2002) studied the effects of acute i.p. injections of 5-HT_{1A} receptor agonists on mechanical allodynia in a rat model of trigeminal pain.

Cutrer and Moskowitz (1996) studied the actions of valproate and neurosteroids in a **guinea pig model** of trigeminal pain. Hartley guinea pigs were pretreated with valproate or allopregnanolone 30 min prior to activation of trigeminal afferent fibers via intracisternal injection of capsaicin. The effects were examined on c-fos expression within the trigeminal nucleus caudalis.

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Migraine Model in Cats

Purpose and Rationale

In order to simulate pain experienced by humans in migraine attacks, Storer and Goadsby (1997) developed a model of **craniovascular pain in cats** by stimulating the superior sagittal sinus and monitoring trigeminal neuronal activity using electrophysiological techniques.

Procedure

Adult cats were anesthetized with α-chloralose (60 mg/kg i.p.), paralyzed (gallamine 6 mg/kg i.v.), and ventilated. The superior sagittal sinus was accessed and isolated for electrical stimulation by a midline circular craniotomy. The region of the dorsal surface of C_2 spinal cord was exposed by a laminectomy and an electrode placed for recording evoked activity by sinus stimulation and spontaneous activity of the same cells. Signals were amplified and monitored online. Cells that were activated by stimulation of the sinus were recorded and were also spontaneously activated. Cells fired with latencies consistent with Aδ and C fibers, generally firing three or four times per stimulus (0.3 Hz, 250 μs duration, 100 V) delivered to the sinus. Both evoked and spontaneous firing could be inhibited by iontophoretic application of serotonin $(5\text{-HT})_{1B/1D}$ agonists.

Evaluation

The suppression or activation of cell firing was determined from both peri-stimulus and poststimulus histograms using the criteria of a shift of >30 % from baseline. Drug comparisons were made using the Kruskal–Wallis one-way analysis of variance assessing significance at the $P < 0.05$ level.

Modifications of the Method

Using the same method, Goadsby et al. (2002) studied the inhibition of trigeminovascular nociceptive transmission by adenosine A_1 receptor agonists.

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Peripheral Analgesic Activity

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General Considerations

The differentiation between central and peripheral analgesic drugs is nowadays of more or less historical value. Most of the so-called peripheral analgesics possess anti-inflammatory properties and in some cases also antipyretic activity besides analgesia. For many of them, the mode of action has been elucidated as an inhibition of cyclooxygenase in the prostaglandin pathway. Nevertheless, new peripheral analgesics have to be tested not only for their in vitro activity on cyclooxygenase but also for their in vivo activity.

The most commonly used methods for measuring peripheral analgesic activity are the writhing tests in mice (various modifications) and the Randall–Selitto test in rats.

Writhing Tests

Purpose and Rationale

Pain is induced by injection of irritants into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior which is called writhing. The test is suitable to detect analgesic activity although some psychoactive agents also show activity. An irritating agent such as phenylquinone or acetic acid is injected intraperitoneally to mice and the stretching reaction is evaluated. The reaction is not specific for the irritant.

Procedure

Mice of either sex with a weight between 20 and 25 g are used. Phenylquinone in a concentration of 0.02 % is suspended in a 1 % suspension of carboxymethyl cellulose. An aliquot of 0.25 ml of this suspension is injected intraperitoneally. Groups of six animals are used for controls and treated mice. Preferably, two groups of six mice are used as controls. Test animals are administered the drug or the standard at various pretreatment times prior to phenylquinone administration. The mice are placed individually into glass beakers and 5 min are allowed to elapse. The mice are then observed for a period of 10 min and the number of writhes is recorded for each animal. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing percent inhibition is average writhes in the control group minus writhes in the drug group divided by writhes in the control group times 100 %. The time period with the greatest percent of inhibition is considered the peak time. A dose range is reserved for interesting compounds or those which inhibit writhing more than 70 %. Compounds with less than 70 % inhibition are considered to have minimal activity.

Evaluation

A dose range is run in the same fashion as the time response except eight animals/group are tested at the peak time of drug activity. Four drug groups and a vehicle control group are employed. Animals are dosed and tested in a randomized manner. An estimated ED_{50} is calculated. Doses of 1.0 mg/kg p.o. indomethacin, 30 mg/kg p.o. acetylsalicylic acid, 40 mg/kg p.o. amidopyrine, and 80 mg/kg p.o. phenacetin have been found to be ED_{50} values.

Critical Assessment of the Test

In this test, both central and peripheral analgesics are detected. The test, therefore, has been used by many investigators and can be recommended as a simple screening method. However, it has to be mentioned that other drugs such as clonidine and haloperidol also show a pronounced activity in this test. Because of the lack of specificity, caution is required in interpreting the results, until other tests have been performed. Nevertheless, a good relationship exists between the potencies of analgesics in writhing assays and their clinical potencies.

Modifications of the Method

Instead of a phenylquinone suspension, 0.1 ml of a 0.6 % solution of acetic acid is injected

intraperitoneally to mice with a weight between 18 and 25 g (Koster et al. 1959; Taber et al. 1969). The response is similar to that after phenylquinone. Some authors have used this method together with the observation of changes in capillary permeability in order to distinguish between narcotic and nonnarcotic analgesics (Whittle 1964).

Eckhardt et al. (1958), Collier et al. (1968), and Loux et al. (1978) showed that several substances are able to elicit the writhing response. For example, Amanuma et al. (1984) as well as Nolan et al. (1990) used as irritant intraperitoneal injections of acetylcholine.

Emele and Shanaman (1963) and Burns et al. (1968) proposed bradykinin being an endogenous transmitter of pain as irritant.

Sancillo et al. (1987) induced abdominal constriction in mice by intraperitoneal injection of 31.6 µg/kg of prostaglandin E_1.

Bhalla and Bhargava (1980) described a method for assessing aspirin-like activity using aconitine to induce writhing.

Adachi (1994) described a device for automatic measurement of writhing in mice.

Analgesic effects of nonacidic nonsteroidal anti-inflammatory drugs in the acetic acid writhing test after intracisternal administration have been found by Nakamura et al. (1986).

The writhing phenomenon can also be observed in **rats** (Fukawa et al. 1980). The writhing responses were induced by intraperitoneal injection of 4 % sodium chloride solution. Narcotic and nonnarcotic analgesics, antipyretics, and nonsteroidal anti-inflammatory drugs were effectively evaluated at relatively low doses. Methamphetamine also showed an analgesic action. VonVoigtlander and Lewis (1982, 1983) induced writhing in rats by injection of 7 ml air or 6 % aqueous saline into the peritoneal cavity.

Ethacrynic acid-induced writhing response in rats was used by Björkman et al. (1992).

Schweizer et al. (1988) described a photoelectronic motility monitoring apparatus to measure automatically the writhing movements. A good correlation was found between ED_{50} values after oral administration in mice and the clinically effective oral doses in man.

Heapy et al. (1993) induced the abdominal constriction response in mice by intraperitoneally injecting 0.4 ml of either 0.25 % acetic acid, 7.5 mg/ml kaolin suspension, 2.4 mg/ml zymosan solution, or 25 µg/ml bradykinin solution.

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Pain in Inflamed Tissue (RANDALL-SELITTO-Test)

Purpose and Rationale

This method for measuring analgesic activity is based on the principle that inflammation increases the sensitivity to pain and that this sensitivity is susceptible to modification by analgesics. Inflammation decreases the pain reaction threshold and this low pain reaction threshold is readily elevated by nonnarcotic analgesics of the salicylate-amidopyrine type as well as by the narcotic analgesics. Brewer's yeast has been used as an inducer for inflammation which increases pain after pressure.

Procedure

Groups of male Wistar rats (130–175 g) are used. Only for oral testing, the animals are starved 18–24 h prior to administration. Otherwise, the route of administration can be intraperitoneal or subcutaneous. To induce inflammation, 0.1 ml of a 20 % suspension of brewer's yeast in distilled water is injected subcutaneously into the plantar surface of the left hind paw of the rat. Three hours later, pressure is applied through a tip to the plantar surface of the rat's foot at a constant rate by a special apparatus to the point at which the animal struggles, squeals, or attempts to bite. The apparatus being used has been modified by various authors such as the Analgesy-Meter (Ugo Basile, Apparatus for Biological Research, Milan, Italy). Each animal is tested for its control pain threshold. Any animal with a control pain threshold greater than 80 g is eliminated and replaced.

For a time response, groups of at least seven animals are used, four groups for the agent to be tested and one for the vehicle control. The tests are done at 15-min intervals after subcutaneous administration and at 30-min intervals after oral administration for any change in pain threshold. The interval of time which indicates the greatest increase in pain threshold is regarded as the peak time.

A dose range is obtained in the same manner as the time response. The drug to be tested is administered in a randomized manner. The pain threshold is recorded at time zero and again at the determined peak time.

Evaluation

The mean applied force is determined for each time interval tested. The percentage increase in pain threshold is calculated by subtracting the applied force of the vehicle control from the applied force of the drug group which is divided by the applied force of the vehicle control in order to give the percentage of increase in pain threshold of the drug group. Doses of 50 mg/kg s.c. Na salicylate, 50 mg/kg amidopyrine, 3 mg/kg s.c. morphine, and12.5 mg/kg s.c. codeine or pethidine have been found to be effective.

Critical Assessment of the Method

The method originally described by Randall and Selitto has been used by many investigators and has been proven to detect central analgesics as well as peripheral analgesics. Peripherally acting analgesics such as the nonsteroidal anti-inflammatory drugs increase only the threshold of the inflamed paw, whereas opiate analgesics increase also the threshold of the intact paw (Dubinsky et al. 1987). In most modifications, the assay has a shallow dose-response curve. Nevertheless, the ED_{50} values of nonsteroidal anti-inflammatory drugs in this test showed a good correlation with human doses (Romer 1980).

Modifications of the Method

The test has been modified by various authors. In some instances, the pressure on the inflamed paw has been omitted. Instead the animals were allowed to walk on a metal grid. The gait of the animals is assessed by an observer using a scoring system:

- 0 = three-legged gait
- 0.5 = marked limping
- 1 = normal gait

The scores are transformed into percent analgesia.

Other noxious stimuli were used to induce inflammation and hyperalgesia, such as carrageenan (Winter et al. 1962), Freund's adjuvant, or prostaglandin E_2 (Ferreira et al. 1978a).

Vinegar et al. (1990) injected 0.1 ml of 0.25 % solution of trypsin into the subplantar region and applied the load force 60 min later. They found a biphasic hyperalgesia and relatively low ED_{50} values for central and peripheral analgesics.

Technically, the method has been improved by several authors, such as Takesue et al. (1969).

Chipkin et al. (1983) modified the test by decreasing the rate of acceleration of the noxious stimulus (mechanical pressure) on the inflamed paw from 20 to 12.5 mmHg/s and an extension of the cutoff time from 15 to 60 s. This

modification is claimed to discriminate analgesics active against mild to severe clinical pain (narcotic-like) from those only useful against mild to moderate pain (nonnarcotic-like).

Randall–Selitto analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Central and peripheral analgesic action of aspirin-like drugs has been studied with a modification of the Randall–Selitto method applying constant pressure to the rat's paw by Ferreira et al. (1978b).

A modification of an analgesia meter for paw pressure antinociceptive testing in neonatal rats was described by Kitchen (1984).

Learning and retention has been tested in rats by Greindl and Preat (1976) inducing pain by a light quantifiable pressure applied to the normal hind paw.

Hargreaves et al. (1988) described a sensitive method for measuring thermal nociception in cutaneous hyperalgesia in rats. One paw was injected with 0.1 ml carrageenan solution and the other paw with saline. The rats were placed in chambers with glass floor and radiant heat was directed to the paws. A photoelectric cell detected the light reflected from the paw and turned off the radiant heat when paw movement interrupted the reflected light.

Perkins et al. (1993) described hyperalgesia after injection of 100 µl of Freund's adjuvant into the knee of anesthetized rats. After 64–70 h, the animal was placed with each hind paw on a pressure transducer, and a downward force was exerted until the uninjected leg was bearing 100 g. At this point, animals were less tolerant to a load on the injected leg, indicating a hyperalgesic response.

Davis et al. (1996) induced mechanical hyperalgesia by injection of substance P and capsaicin in the rat knee joint and measured the download force tolerated by the injected leg.

Ferreira et al. (1993a, b) induced hyperalgesia by intraplantar injection in the hind paw of rats of various agents, e.g., bradykinin, carrageenan, LPS, PGE_2, dopamine, TNFα, IL-1β, IL-6, and IL-8. A constant pressure of 20 mmHg was applied to the hind paws and discontinued when the rats presented a typical freezing reaction.

Subplantar injection of 0.1 µg of serotonin in the rat results in a brief period (up to 20 min) of increased pain sensitivity to an applied force (hyperalgesia) which precedes a longer period of decreased pain sensitivity (hypoalgesia). Vinegar et al. (1989) used this phenomenon for pharmacologic characterization of the algesic response.

Similarly, a biphasic algesic behavior after subplantar injection of 250 µg of trypsin was described by Vinegar et al. (1990).

Courteix et al. (1994) proposed the Randall–Selitto paw pressure test in rats with streptozotocin-induced diabetes as a model of chronic pain with signs of hyperalgesia and allodynia that may reflect signs observed in diabetic humans.

Amann et al. (1955, 1996) evaluated local edema and effects on thermal nociceptive threshold after intraplantar injection of nerve growth factor into the rat hind paw and studied the effect of a 5-lipoxygenase inhibitor in this test.

Zhou et al. (1996) tested the effects of peripheral administration of NMDA, AMPA, or KA on pain behavior in rats. A 28-gauge needle was inserted in the skin of rats proximal to the footpads and advanced about 1 cm so that the tip reached the base of the third toe. A bolus dose of 20 µl containing concentrations between 1 and 10,000 µM of KA, NMDA, or AMPA. For behavioral testing, each animal was placed in a Plexiglas box on a wire mesh screen. Mechanical stimuli were applied using four von Frey filaments with different bending forces. Each von Frey filament was applied 10 times to the skin on the base of the third toe. The paw withdrawal was rated as scores allowing dose-response curves for the hyperalgesic effects of excitatory amino acids. Furthermore, using the highest concentration of the stimulant, effects of antagonists were tested.

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Mechanical Visceral Pain Model in the Rat

Purpose and Rationale

Animal models designed to test the effectiveness of analgesic agents against visceral pain typically rely on noxious chemical irritation of the peritoneum, e.g., acetic acid- and phenylquinone-induced writhing tests based on acute inflammation. Ethical constraints prevent repeated assessments in a single animal, thereby compounding the difficulty of assessing development of tolerance to analgesic agents. To overcome these constraints, a model for mechanical visceral pain was developed based on repeatable and reversible distension of the duodenum in the rat (Colburn et al. 1989; deLeo et al. 1989).

Procedure

A one-piece balloon catheter is prepared composed of PE 50 tubing with a terminal latex rubber balloon which is 7.5 mm long and distensible to hold more than 1.5 ml fluid. Male Sprague Dawley rats weighing 175–200 g are anesthetized with N_2O and halothane. The abdomen is shaved and a 2.5-cm incision is made transversely just below the left costal margin. On the greater curvature of the stomach, an incision is made 10–20 mm above the pylorus and a purse string is accomplished with 4–0 silk prior to gastrostomy. Through a 2-mm gastrostomy, the catheter is introduced and advanced through the pylorus to the first portion of the duodenum (approximately 15–20 mm from the pylorus). The purse string is tied snugly closing the gastrostomy around the catheter. The catheter is tunneled to the base of the skull, externalized, and anchored to the dermis with a silicon sleeve and suture. The animals recover from anesthesia within 5 min. Following a 4–5-day recovery period, the duodenal distension volume is determined by the mean threshold that produces writhing (usually 0.5–0.7 ml). For the test, the animals are randomized and administered either saline, the standard (0.1, 0.25, 1, and 10 mg/kg indomethacin i.p.) or the test drug in various doses prior to challenging. The animals are placed in a polypropylene box and challenged by inflating the balloon with saline, using a 1-ml calibrated syringe, pulsed five times over 30 s and then distended for 1 min. Behavioral responses are scored:

- 0 = normal behavior defined as exploration, escape attempts, and resting
- 1 = slightly modified behavior defined as cessation of exploration, focusing, wet-dog shake, excessive facial grooming, teeth chattering, and deep breathing
- 2 = mildly to moderately modified behavior defined as retching-like activity, hunching, abdominal grooming or nipping, and immobility of the hind limbs (disappears with the removal of the stimulus)
- 3 = severely modified behavior defined as stretching of the hind limbs, arching, and dorsiflexion of the hind paws
- 4 = intensive visceromotor activity defined as repetitive stretching of the body, extension of the hind limbs and pelvis, and frequent rotating sideward, i.e., writhing

Evaluation

The average scores of the groups are plotted on semilog paper and ED_{50} values are determined by best line fit.

Critical Assessment of the Method

In the mechanical visceral pain model in the rat, morphine and indomethacin have been found to be active but not other agents involved in prostaglandin inhibition, like acetylsalicylic acid and mefenamic acid. Other mechanisms besides those involving the arachidonic acid cascade have to be investigated.

Modifications of the Method

Ness and Gebhart (1988) used colorectal distension as a noxious visceral stimulus in awake, unanesthetized, unrestrained rats. A 7–8-cm flexible latex balloon was inserted intra-anally under ether anesthesia and kept in position by taping to the base of the tail. Opening a solenoid gate to a constant pressure air reservoir initiated a 20-s, constant pressure stimulus in the descending colon and rectum. Femoral arterial and venous catheters were tunneled subcutaneously and exteriorized at the back of the neck. Teflon-coated stainless steel wire electrodes were stitched into the external oblique musculature immediately superior to the inguinal ligament for electromyographic recordings. Blood pressure and heart frequency increase were proportional to the degree of colorectal distension. These effects could be dose dependently antagonized by morphine and clonidine.

Renal pelvis distension with a pressure of 80 cm H_2O causes a decline in mean arterial blood pressure in pentobarbital-anesthetized rats. Brasch and Zetler (1982) used this blood pressure response, which disappears rapidly after cessation of the distension, to study the effects of analgesic drugs known to be effective in renal colic pain in man.

Moss and Sanger (1990) measured falls in diastolic blood pressure and intragastric pressure after distension of the duodenum by rapid application of intraluminal pressure (10–75 cm H_2O) in anesthetized rats. The distension-induced responses were blocked by pretreatment with morphine, an action reversible by injection of naloxone. Bilateral cervical vagotomy reduced the distension-evoked fall in intragastric pressure but had no effect on the corresponding fall in blood pressure.

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Antagonism Against Local Effects of Bradykinin

Purpose and Rationale

Guzman et al. (1962) and Lim et al. (1964) described the responses (vocalization, respiratory, and blood pressure changes) to intra-arterial injection of bradykinin and other algesic agents in cats and dogs. Deffenu et al. (1966) and Blane (1968) used the bradykinin-induced effects after intra-arterial injection in rats as an assay for analgesic drugs. Perivascular sensory nerves which accompany blood vessels throughout the body to end in unmyelinated free-branching terminals close to the capillaries and venules most likely carry the chemoreceptors of pain (Lim 1970). Due to rapid enzymatic degradation, bradykinin is ineffective as noxious stimulus after intravenous or oral administration.

Procedure

Male Wistar rats weighing 280–320 g are lightly anesthetized with ether. A polyethylene catheter

with an internal diameter of 0.5 mm is inserted centripetally into the right carotid artery. The catheter is passed through the subcutaneous tissues to protrude from the back of the animal. One hour after recovery from anesthesia, the first dose of bradykinin is injected into the catheter producing dextrorotation of the head, flexing of the forelimb, and occasionally squeaking. For each rat, the minimum dose of bradykinin is determined necessary to provoke these effects. The test compounds are applied subcutaneously or intraperitoneally 15 min prior to injection of the threshold dose of bradykinin. The bradykinin injections are repeated in 5-min intervals until the bradykinin effect reappears. Each rat receives one drug at one dose level.

Evaluation

The criterion for protection is the disappearance of the bradykinin effect after at least two consecutive doses of bradykinin. Using groups of 10 rats for various dose levels, ED_{50} values are calculated.

Critical Assessment of the Method

Not only narcotic analgesics but also pyrazolones and phenacetin or acetylsalicylic acid are active in this test. In some animals, the bradykinin-induced response can be diminished after repeated injections, classified as the noxious-adaptable group (Satoh et al. 1979).

Modifications of the Method

Haubrich et al. (1990) tested analgesic activity by the intracarotid bradykinin-induced head/forepaw flexion in the rat. Male Charles River rats (280–320 g) fasted overnight were prepared surgically under light ether anesthesia by insertion of a capped polyethylene cannula (PE-60) centripetally into the right carotid artery and then exteriorizing the cannula to a harness on the back, to permit repeated i.a. injections. The rats were allowed to recover at least 2 h from the surgery and then given single i.a. injections of bradykinin (triacetate salt in 0.2 ml 0.9 % NaCl per injection) at 10-min intervals to determine the threshold dose which produced marked dextrorotation of the head and flexion of the right forepaw of each rat. This response was elicited by threshold doses of bradykinin ranging from 0.1 to 0.5 μg/injection. After administration of the test drugs, the response of the threshold dose of bradykinin was determined at 10-min intervals for 1 h and then at 20-min intervals during the second hour. ED_{50} values were determined by probit analysis of the maximum percentage of rats that failed to respond to bradykinin at each dose of test drug any time within the 2-h test period.

Collier and Lee (1963) described nociceptive responses of **guinea pigs** to intradermal injections of bradykinin and kallidin-10.

Vargaftig (1966) measured the effect of nonnarcotic analgesics on the hypotension induced by intra-arterial injection of bradykinin in **rabbits**.

Adachi and Ishii (1979) used the response to injection of bradykinin into the femoral artery of guinea pigs for quantitative assessment of analgesic agents.

Griesbacher and Lembeck (1987) and Lembeck et al. (1991) used the reflex hypotensive response as an indicator of nociception after injection of bradykinin into the ear artery of anesthetized rabbits. Rabbits were anesthetized and the blood pressure was recorded from the carotid artery. The central artery of one ear was cannulated and the ear was separated from the head with the exception of the auricular nerve, which remained connected to the head. The ear was perfused with Tyrode solution to which acetylcholine and bradykinin were added. The reflex fall in blood pressure induced by bradykinin and acetylcholine was monitored. The effect could be inhibited by bradykinin antagonists.

Heapy et al. (1993) tested the effects of the bradykinin antagonist HOE 140 on the abdominal constriction response after intraperitoneal injection of bradykinin to **mice**.

Further Methods Used to Study the Role of Bradykinin and Bradykinin Antagonists in Inflammation and Algesia

Teixeira et al. (1993) investigated the mechanisms of inflammatory response induced by extracts of *Schistosoma mansoni* larvae in guinea pig skin. *Biomphalaria glabrata* snails with patent *Schistosoma mansoni* infections were induced to shed cercariae by exposure to light and water with a temperature of 31 °C. The cercariae were concentrated and homogenized and extracts prepared. Purified eosinophils or neutrophils obtained from peritoneal exudates were radiolabeled by incubation with ^{111}In chelated to 2-mercaptopyridine-*N*-oxide. Radiolabeled leukocyte infiltration and edema formation were measured simultaneously at injected skin sites. [^{125}I]Human serum albumin was added to the labeled leukocytes and these were injected i.v. into anesthetized guinea pigs. After 15 min, the extracts of cercariae were locally injected with or without the inhibitors. After 2 h, the animals were sacrificed and the injected sites were punched out with a 17-mm punch. Serum exudation and leukocyte infiltration were measured by counting the two isotopes. The bradykinin antagonist HOE 140 reduced substantially the extract-induced inflammation.

Ahluwalia et al. (1994) induced plasma protein extravasation in the rat urinary bladder by i.p. injection of cyclophosphamide mediated by capsaicin-sensitive primary afferent neurons which could be significantly inhibited by the bradykinin B_2 receptor antagonist HOE 140 and the tachykinin NK_1 receptor antagonist RP67,580.

Davis and Perkins (1994a, b) described a model of persistent inflammatory mechanical hyperalgesia using intra-articular injections of bradykinin or cytokines into the knee joint of rats.

Lecci et al. (1995) analyzed the local and reflex responses to bradykinin on rat urinary bladder motility in vivo.

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Effect of Analgesics on Spinal Neurons

Purpose and Rationale

The mode of action of peripheral analgesics is still a matter of debate. Besides inhibition of the arachidonic acid-derived pathway, activities on the spinal and central level have been discussed. Schaible and Schmidt (1983a, b, 1985, 1987, 1988) performed electrophysiological experiments in anesthetized cats and rats after local mechanical stimulation and after induction of acute arthritis of the knee joint. With this method, He et al. (1990a, b) and Neugebauer et al. (1994) found evidence for a spinal antinociceptive action of antipyretic analgesics, such as dipyrone.

Procedure

In cats weighing 2.0–4.0 kg, anesthesia is induced by i.m. injection of 15–30 mg/kg ketamine hydrochloride followed by i.v. injection of 60 mg/kg α-chloralose. After immobilization with i.v. pancuronium bromide, the cats are artificially ventilated. The skin of the right thigh is incised from rostral of the inguinal fossa to a point below the medial condyle of the tibia. The tendon of the sartorius muscle is cut close to its insertion at the capsule of the knee joint. The muscle is removed to expose the medial aspects of the joint and the medial articular nerve (MAN). The thigh is rigidly fixed to the mounting table by a threaded bolt fitted through the femur so that the lower leg can be flexed and extended in the horizontal plane. The saphenous nerve is cut in the inguinal fossa for recording. Bipolar electrodes are inserted at the MAN near the knee for en passent stimulation of articular afferents. **Extracellular recordings from single MAN units** in the saphenous nerve are performed using platinum wire electrodes. According to their conduction velocities, units are classed as group IV afferents (<2.5 m/s, unmyelinated axons) or group III afferents (2.5–20 m/s, thinly myelinated axons).

For **recordings from spinal cord neurons,** the spinal segments T12–L7 are exposed by laminectomy. The spinal cord is transected at the lower thoracic region after injection of 0.1 ml of 1 % procaine hydrochloride solution to prevent mechanical activation of axons in the long spinal tracts. The animals are fixed to a rigid frame with spinal and pelvic clamps. A pool is formed by skin flaps and filled with warm paraffin oil. The upper lumbar spinal cord is mounted on a pair of

platinum wire stimulating electrodes surrounding the whole cord. Ascending tract neurons are identified by electrical stimulation (Neugebauer and Schaible 1990). Single spinal neurons that can be excited by mechanical stimulation of the knee joint tissue are recorded extracellularly using glass-insulated carbon filament electrodes. The neurons are either nociceptive specific neurons responding only to noxious mechanical stimuli or wide dynamic range neurons responding to innocuous stimuli but showing strongest responses to stimuli of noxious intensity.

Acute arthritis in the right knee joint is induced several hours before recordings are started by injecting 0.3–0.5 ml of 4 % kaolin suspension and 15–20 min later 0.3 ml of 2 % carrageenan solution. Acute arthritis develops within 1–3 h.

Action potentials are displayed on a storage oscilloscope, amplified, filtered, fed to a window discriminator, and processed using an interface and a personal computer for construction of peristimulus time histograms. After a control period of at least 40 min during which a stable discharge rate of the afferent or spinal cord unit is obtained, the test substances are administered i.v. in various doses. Effects of the test substance on ongoing and mechanically evoked activity (by movements, pressure stimuli) are determined.

Evaluation

Ongoing activity is counted every minute. The means and standard deviations in 10-min periods are calculated before and after drug application. The values after drug injection are calculated as percentage of control values. To calculate the net effects of the different mechanical stimuli, the number of impulses in the preceding 30 s is subtracted from the total discharges during the stimulus. The responses to at least four stimuli before drug application are averaged and set to 100 %. The responses to the different mechanical stimuli after drug administration are expressed as a percentage of the controls. Statistical significance is evaluated using the *t*-test for unpaired samples.

Modifications of the Method

Using the model of kaolin-induced arthritis in the knee of rats, Han and Neugebauer (2005) and Han et al. (2005) developed a computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior.

Several other electrophysiological methods have been applied to elucidate the mode of action of non-opioid analgesic agents. Carlsson et al. (1986, 1988) and Jurna and Brune (1990) recorded the activity from single neurons in the dorsomedial part of the ventral nucleus of the thalamus in rats. Activity was elicited by supramaximal stimulation of nociceptive afferents in the sural nerve. In addition, activity was recorded in ascending axons of the spinal cord.

Chapman and Dickenson (1992) studied the spinal and peripheral roles of bradykinin and prostaglandins in nociceptive processing in the rat by recording C-fiber activity in the dorsal spinal horn after injection of formalin into the center of the respective field of the toe of the hind paw.

Dray et al. (1992) described a preparation of the neonatal rat spinal cord with functionally connected tail maintained in vitro. The preparation was placed in a chamber and the spinal cord and tail were separately superfused with a physiological salt solution. Peripheral nociceptive fibers were activated by superfusion of the tail with bradykinin and capsaicin or by a superfusate heated to 48–50 °C (noxious heat). The activation of peripheral fibers was assessed by measuring the depolarization produced in a spinal ventral root.

Malmberg and Yaksh (1992) described a direct analgesic action of NSAIDs through spinal cyclooxygenase inhibition by blocking the thermal hyperalgesia in rats induced after intrathecal administration of excitatory amino acids or substance P.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized mice was described by Hylden and Wilcox (1980).

Mestre et al. (1994) described a method for performing direct intrathecal injections in rats without introducing a spinal catheter.

Bahar et al. (1984) performed chronic implantations of nylon catheters into the subarachnoid

space of Wistar rats and marmosets and tested the effects of local anesthetics.

McQueen et al. (1991) investigated the effects of paracetamol and lysine acetylsalicylate on high-threshold mechano-nociceptors by recording neural activity from the inflamed ankle joint in anesthetized rats with mild adjuvant-induced monoarthritis.

Yamamoto and Yaksh (1992) studied the effects of excitatory amino acid antagonists administered through chronically implanted lumbar intrathecal catheters on the thermal hyperesthetic state induced by unilateral partial ligation of the sciatic nerve in rats.

Hashimoto and Fukuda (1990) described a spinal cord injury model produced by spinal cord compression in the rat.

Aanonsen and Wilcox (1987) tested effects of spinally administered opioids, phencyclidine, and sigma agonists on the action of intrathecally administered NMDA in the tail-flick, hot-plate, and biting and scratching nociceptive tests in **mice**.

Brambilla et al. (1996) demonstrated that intrathecal administration of AMPA produced a dose-dependent behavioral syndrome in mice characterized by caudally directed biting, which could be antagonized by peripheral administration of AMPA-receptor and NMDA-receptor antagonists.

Aanonsen et al. (1990) tested the effect of iontophoretically applied excitatory amino acid agonists, such as NMDA, AMPA, quisqualate, and kainate, on the firing rate of rat spinal neurons after peripheral noxious stimulation.

Cumberbatch et al. (1994) studied the roles of receptors for AMPA in spinal nociceptive and non-nociceptive transmission on dorsal horn wide dynamic range neurons in anesthetized spinalized rats. The effects of systemically administered competitive and noncompetitive AMPA antagonists were examined on responses to peripheral noxious heat and non-noxious tap stimuli as well as to iontophoretic AMPA and NMDA.

With this technique, Chizh et al. (1994) studied the effects of intravenous administration of AMPA antagonists to iontophoretically applied excitatory amino acids.

Watkins et al. (1994) induced hyperalgesia in rats by intraperitoneally administered lipopolysaccharides as measured by radiation heat tail flick in rats. Intrathecal catheters were implanted into the subdural space surrounding the spinal cord to test the involvement of excitatory amino acids, substance P, CCK, and opioids assessing the effects of antagonists.

Mjellem et al. (1993) produced a behavioral syndrome of caudally directed biting in mice by intrathecal injection of either NMDA or AMPA.

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Antagonism to Nerve Growth Factor

General Considerations on Nerve Growth Factor

Nerve growth factor (NGF) is a member of the neurotrophin family of structurally related secreted proteins that includes brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4). The nerve growth factor (NGF) promotes the survival and differentiation of sensory and sympathetic neurons. The NGF is important in the development and survival of nerve cells (neurons), especially those that transmit pain, temperature, and touch sensations (sensory neurons). The NGFβ protein functions by attaching (binding) to its receptors, which initiates signaling pathways inside the cell. The NGFβ protein can bind to two different receptors, the NTRK1 receptor or the p75NTR receptor. Both receptors are found on the surface of sensory neurons and other types of neurons. Mature neurotrophins are homodimers that are derived by proteolytic cleavage from precursor proteins encoded by separate genes. They bind to two types of receptor: a common receptor, $p75^{NTR}$, which binds all neurotrophins with a similar affinity, and members of the trk family of receptor tyrosine kinases, trkA, trkB, and trkC, which bind different neurotrophins.

NGF and BDNF have a crucial role in the generation of pain and hyperalgesia in several acute and chronic pain states (Woolf et al. 1994; McMahon 1996; Mannion et al. 1999; Thompson et al. 1999; Pezet et al. 2002; Allen and Dawbarn 2006). The expression of NGF is high in injured and inflamed tissues, and activation of the receptor tyrosine kinase trkA in nociceptive neurons triggers and potentiates pain signaling by multiple mechanisms (McMahon et al. 1995; Huang and Reichardt 2003; Hefti et al. 2006).

An effective pain therapeutic needs to prevent the activation of trkA by NGF. This may be achieved by agents that remove free NGF, by molecules that prevent binding to trkA, and by molecules that prevent activation of trkA.

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In Vitro Assays of Nerve Growth Factor

Purpose and Rationale

The nerve growth factor (NGF) is a protein molecule that is important for the growth, survival, maintenance, and differentiation of sensory and sympathetic neurons. It can also function as a signaling molecule. The neurotrophin nerve growth factor (NGF) binds to two receptor types: the tyrosine kinase receptor TrkA and the common neurotrophin receptor $p75^{NTR}$. Although many of the biological effects of NGF (such as neuronal growth and survival) are associated with TrkA activation, $p75^{NTR}$ also contributes to these activities by enhancing the action of TrkA when receptors are coexpressed (Verdi et al. 1994; Kaplan and Miller 1997). Colquhoun et al. (2004) studied the NGF antagonist PD90780 (Spiegel et al. 1995), which interacts with NGF, preventing its binding to $p75^{NTR}$. In this study, the actions of this compound were further explored, and it was found that PD90780 is not able to inhibit the binding of either brain-derived neurotrophic factor or neurotrophin-3 to $p75^{NTR}$, consistent with the direct interactions of the antagonist with NGF. In addition, it was demonstrated that the ability of PD90780 to inhibit NGF–$p75^{NTR}$ interactions is lower when receptors are coexpressed, compared with when $p75^{NTR}$ is the only neurotrophin receptor expressed.

Procedure

Radiolabeled Neurotrophin and Receptor Preparation

The iodination of NGF (mouse 2.5 s; Cedarlane Labs, Toronto, ON) and rhBDNF (Alomone Labs, Jerusalem, Israel) was performed as described by Sutter et al. (1979) with modification (Ross et al. 1997). The ability of PD90780 to block neurotrophin binding to the $p75^{NTR}$ receptor was evaluated under various receptor conditions. This was accomplished with the use of PC12 cells (TrkA and $p75^{NTR}$), $PC12^{nnr5}$ cells ($p75^{NTR}$ only), and truncated $p75^{NTR}$. The two cell types were cultured in RPMI 1640 medium with 10 % fetal calf serum. Recovery of the cells was permitted with the replacement of the medium with calcium/magnesium-free balanced salt solution followed by a 15-min incubation at 37 °C. Cells were centrifuged, and pellets were suspended in HKR buffer (10 mM HEPES, pH 7.35 containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 g/l glucose, and 1 g/l bovine serum albumin). In the case of truncated $p75^{NTR}$, the culture medium used to grow PC12 cells was removed and centrifuged to ensure it was free of cells. This medium contained $p75^{NTR}$ extracellular domains previously sloughed by the cells (molecular weight approximately 50 kDa) (DiStefano and Johnson 1988).

Chemical Cross-Linking of ^{125}I-NGF to TrkA and/or $p75^{NTR}$ in the Presence of Antagonists and Immunoprecipitation

^{125}I-NGF (0.1 nM) alone or in combination with NGF (100 nM), BDNF (10 nM), or PD90780 (100 μM) was incubated with PC12 cells at a concentration of 10^6 cells/ml in HKR buffer in a volume of 1 ml for 2 h at 4 °C with rocking. After binding, 20 μl of the cross-linker bis(sulfosuccinimidyl) suberate (BS^3) was added (final concentration of 0.4 mM) to each sample and incubated at room temperature for 30 min. The cells were washed three times with TBS, after which reducing SDS sample buffer was added to the pelleted cells to dissolve the proteins, or in the case of immunoprecipitations prepared as described below. Cell samples undergoing immunoprecipitations for TrkA or $p75^{NTR}$ were solubilized in lysis buffer (TBS containing 10 % glycerol, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) and incubated for 40 min at 4 °C. After centrifugation, the lysates were removed to a new

tube, and either rabbit polyclonal anti-Trk cytoplasmic domain antibody or rabbit polyclonal anti-$p75^{NTR}$ antibody (9992) (antisera against glutathione *S*-transferase fusion protein containing the cytoplasmic domain of $p75^{NTR}$) was added to the soluble proteins to isolate the respective receptors. The samples were left to incubate at 4 °C overnight. Antibody complexes were removed through application and incubation with 70 μl of a 50 % slurry of immobilized Protein G (Pierce Chemical, Rockford, Il., USA) for 2 h at 4 °C. The solid phase was washed with lysis buffer three times, with distilled water once, and then the proteins were dissolved in SDS sample buffer. Proteins from cross-linking and immunoprecipitation experiments were separated via 6 % SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical Cross-Linking of ^{125}I-NGF to $p75^{NTR}$ and Concentration Effect Assays

^{125}I-NGF was incubated at 4 °C for 2 h with or without PD90780. PC12 or $PC12^{nnr5}$ cells were added at 10^6 cells/ml, and samples were incubated at 4 °C for 2 h with rocking. Bound ^{125}I-NGF and $p75^{NTR}$ proteins were cross-linked with final concentrations of 5 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2 mM sulfo-*N*-hydroxysulfosuccinimide (SNHS) (20 μl of each) and incubated with rocking at room temperature for 30 min. Samples were washed with TBS [10 mM tris (hydroxymethyl)aminomethane, pH 8.0, and 150 mM NaCl] three times before the addition of reducing SDS sample buffer to dissolve the proteins. The proteins were then separated on a 6 % SDS-PAGE gel. In the experiments involving truncated $p75^{NTR}$, ^{125}I-NGF or ^{125}I-BDNF was exposed to the same concentrations of PD90780 and incubated with medium containing truncated $p75^{NTR}$ before cross-linking with EDC/SNHS. The reaction was quenched by adding 15 μl of 1 M glycine followed by 10 min of mixing. The samples were then immunoprecipitated using 192 IgG, which recognizes the extracellular domain of $p75^{NTR}$ (Calbiochem, San Diego, Calif., USA).

Neurotrophin Receptor Binding

Neurotrophins NGF, BDNF, and NT-3 were iodinated, and PC12 and $PC12^{nnr5}$ cells were cultivated and recovered as previously described. Tubes were set up containing single data points that held iodinated neurotrophin (0.5 nM), PD90780 (10 μM), a final concentration of 10^6 cells/ml, and NGF (at 50 nM for nonspecific binding) as required and were then incubated at 4 °C for 2 h. Aliquots (100 μl) were layered on top of 200 μl of 10 % glycerol in HKR buffer in 0.4-ml tubes. Samples were then centrifuged at 5,000 rpm for 2 min, after which the tip containing the cell pellet was cut off and radioactivity present was determined.

TrkA Phosphorylation Assay

Modification of the methods described permitted determination of TrkA phosphorylation (Ross et al. 1998). NGF (40 pM) was incubated with varying concentrations of PD90780 (3, 30, or 300 μM) for 2 h in HKR buffer. PC12 cells used at 10^6 cells/ml were incubated with NGF and PD90780 solutions for 15 min at 37 °C. Samples were washed once with cold PBS and then lysed with solutions containing 500 μM orthovanadate and immunoprecipitated with anti-Trk antibody as previously described. An SDS-PAGE run on 6 % gel followed by Western blot analysis performed with antiphosphotyrosine antibody (4G10; UBI, Lake Placid, NY, USA) and visualized with ECL (Amersham) permitted the resolution of isolated phosphoproteins. The resulting bands were quantified via densitometry analysis.

NGF Protomer Cross-Linking

^{125}I-NGF (0.1 nM) was incubated for 2 h at 4 °C with $ZnCl_2$ (100 μM), PD90780 (30 μM), or $ZnCl_2$ and PD90780 (100 and 30 μM, respectively), along with HKR buffer, for a total volume of 0.1 ml. After incubation, BS^3 was added (final concentration of 0.4 mM) in 5 μl volume and set at room temperature for 30 min. Proteins were dissolved with the addition of 50 μl of SDS sample buffer and heating to 95 °C for 10 min. Separation of ^{125}I-NGF dimers and ^{125}I-NGF monomers was completed using a 15 % acrylamide gel SDS-PAGE.

Evaluation

Following SDS-PAGE, gels were fixed and dried, and the radioiodinated ligands cross-linked to receptors were detected via autoradiography. Receptor ligand bands within SDS-PAGE gels were excised, and radioactivity within each band was detected with a Beckman gamma counter. The concentration effect curves, SEM, IC_{50} values, and 95 % confidence intervals (CIs) described in the concentration effect studies were determined by nonlinear regression analyses and carried out by the program GraphPad Prism, version 3.00 (GraphPad Software, San Diego, Calif., USA).

Modifications of the Method

Debeir et al. (1999) described a nerve growth factor mimetic TrkA antagonist that causes withdrawal of cortical cholinergic boutons in the adult rat. A small peptide, C(92–96), which blocks NGF–TrkA interactions, was delivered stereotactically into the rat cortex over a 2-week period, and its effect and potency were compared with those of an anti-NGF monoclonal antibody (mAb NGF30). Two presynaptic antigenic sites were studied by immunoreactivity, and the number of presynaptic sites was counted by using an image analysis system. Synaptophysin was used as a marker for overall cortical synapses, and the vesicular acetylcholine transporter was used as a marker for cortical cholinergic presynaptic sites.

Owolabi et al. (1999) characterized the antiallodynic actions of ALE-0540, a novel nerve growth factor receptor antagonist that inhibits the binding of NGF to tyrosine kinase (Trk) A or both p75 and TrkA (IC_{50} 5.88 ± 1.87 μM, 3.72 ± 1.3 μM, respectively), as well as signal transduction and the biological responses mediated by TrkA receptors.

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In Vivo Assays of Nerve Growth Factor Antagonism

Purpose and Rationale

Many studies indicate the role of NGF in pain perception.

Herzberg et al. (1997) reported NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve.

Ma and Woolf (1997) reported that the progressive tactile hyperalgesia induced by peripheral inflammation is nerve growth factor dependent. An i.p. injection of anti-NGF antiserum (5 μl/g) 1 h before induction of inflammation by intraplantar complete Freund's adjuvant (CFA) injection and 24 h after reduced the basal inflammatory hypersensitivity and significantly attenuated the progressive increase of spontaneous activity and touch-, pinch-, and Aβ-afferent-evoked responses, as well as the progressive reduction of the mechanical threshold of biceps femoris/semitendinosus alpha motoneurons normally evoked by repeated (every 5 min) tactile stimulation of the inflamed hind paw, in decerebrate spinal rats.

Ro et al. (1999) described the effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve.

Theodosiou et al. (1999) studied the role of nerve growth factor in hyperalgesia due to nerve damage.

Gwak et al. (2003) found attenuation of mechanical hyperalgesia following spinal cord injury by administration of antibodies to nerve growth factor in the rat.

Procedure

Adult Sprague Dawley rats (200–250 g) were spinally hemisected at T13 (Christensen et al. 1996). Under enflurane anesthesia (induction 3 % and maintenance 2 %), the T11–T12 laminae were determined by counting the dorsal spinous processes from the sacrum. The surgical field was then shaved, a longitudinal incision made exposing several segments, and a laminectomy performed at two vertebral segments, T11–T12. The spinal cord was hemisected just cranial to the L1 dorsal root entry zone with a microdissecting knife without damaging the major dorsal vessel or vascular branches. An insulin syringe with a 28-gauge needle was placed dorsal-ventrally at the midline of the cord and pulled laterally to ensure the completeness of the hemisection. The incised skin was sutured, and postoperative care was done. After the hemisection, animals were either treated once a day for 10 days with anti-NGF (anti-nerve growth factor-2.5S, Sigma, i.p., 2 μg, 0.2 ml), or with saline (0.2 ml), or were untreated.

In the behavioral experiments, rats were housed in clear plastic boxes (8 × 8 × 24 cm) above a metal mesh (0.5 × 0.5 cm) and acclimatized for 30 min to avoid the stress associated with environmental change. Mechanical paw withdrawal threshold to the application of a von Frey filament was measured by using the up-down testing paradigm (Ro et al. 1999). An ascending series of von Frey filaments of incremental force (0.35, 0.53, 0.78, 2.5, 3.7, 5.2, 6.0, and 12.5 g) was applied for 3 s to the middle of the plantar surface of the hind paw, starting with the 2.5-g stimulus (Chaplan et al. 1994).

For electrophysiology experiments, rats were anesthetized with sodium pentobarbital (40–50 mg/kg), with supplementary pancuronium bromide (2–4 mg/kg per h), and artificially ventilated. Extracellular recordings of neuronal activity were made from the dorsal horn neurons in the lumbar spinal cord (L4–L5), using a recording glass microelectrode with a carbon filament (3–5 MΩ) while mechanical stimuli were applied onto the receptive fields. The single-unit responses of the

dorsal horn neurons, characterized as wide dynamic range (WDR) neurons by their graded responses to increased intensities of mechanical stimuli, were amplified, filtered, and displayed on an oscilloscope. The output signals were also fed into a data acquisition system (CED 1401 plus) via a window discriminator for the construction of real-time recordings of peristimulus time histograms, which were displayed as the number of spikes per second.

The mechanical stimuli were applied for 10 s and included (1) brushing the skin with a camel hairbrush in a stereotypic manner (brush), (2) sustained application of a large clamp that produced a sense of firm pressure when placed on human skin (pressure), and (3) sustained application of a small clamp that produced a distinctly painful sensation (pinch).

Evaluation

Statistical analysis was performed using Mann–Whitney's unmatched pairs rank-sum test to evaluate the differences between the scores in two groups. All data are displayed as means ± standard error.

Modifications of the Method

Spinal cord injury often leads to central pain syndrome including hyperalgesia to mechanical stimulation. Several authors studied the influence of nerve growth factor in **models of neuropathic pain** (Ramer and Bisby 1999; Li et al. 2002, 2003; Cahill et al. 2003; Ruiz et al. 2004).

Nerve Ligation Injury

Nerve ligation injury was performed according to the method described previously (Kim and Chung 1992). This technique produces signs of tactile allodynia and thermal hyperalgesia. Rats were anesthetized with halothane and the L5 and L6 spinal nerves were exposed, carefully isolated, and tightly ligated with 4–0 silk suture distal to the dorsal root ganglion (DRG). After ensuring homeostatic stability, the wounds were sutured, and the animals were allowed to recover in individual cages. Sham-operated rats were prepared in an identical fashion except that the L5 and L6 spinal nerves were not ligated.

Intrathecal Catheter Placement

Two routes of administration, a systemic i.p. and a spinal intrathecal (i.th.) route, were used explore the activity of compounds. For the spinal route, test compounds were injected through indwelling i.th. catheters in the manner described by Yaksh and Rudy (1976). While under anesthesia, polyethylene-10 tubing (8 cm) was inserted through an incision made in the atlantooccipital membrane to the level of the lumbar enlargement of the rat and secured. Drug injections were made in a volume of 5 µl followed by a 9-µl saline flush.

Thermal Sensitization

Rats were lightly anesthetized with ether. The left hind paw was placed in a water bath maintained at 50 °C for 1 min. Inflammation suggested by rubor of the paw developed immediately. The rats were allowed to recover from anesthesia and tactile testing was begun 2 h after thermal sensitization. This procedure has produced signs of thermal hyperalgesia and tactile allodynia that persisted for over 12 h.

Evaluation of Tactile Allodynia

Mechanical allodynia was determined in the manner described previously (Chaplan et al. 1994). The paw withdrawal threshold was determined in response to probing with calibrated von Frey filaments. The rats were kept in suspended cages with wire mesh floors and the von Frey filaments were applied perpendicularly to the planar surface of the paw of the rat until it bent slightly, and was held for 3–6 s, or until the paw was withdrawn. A positive response was indicated by a sharp withdrawal of the paw. The 50 % paw withdrawal threshold was determined by the nonparametric method (Dixon 1980).

Shelton et al. (2005) found that nerve growth factor mediates **hyperalgesia and cachexia in**

autoimmune arthritis. Function-blocking antibodies to NGF completely reverse established pain in rats with fully developed arthritis despite continuing joint destruction and inflammation. Likewise, these antibodies reverse weight loss while not having any effect on levels of the pro-cachectic agent tumor necrosis factor (TNF).

Banik et al. (2005) reported that increased nerve growth factor after rat plantar incision contributes to **guarding behavior and heat hyperalgesia**. The therapeutic effect of a monoclonal antibody against endogenous NGF was evaluated by intraperitoneal administration of a single preoperative dose of anti-NGF.

Adult male, 225–275 g, Sprague Dawley rats were used in a plantar incision animal model. The animals were anesthetized with 1.5–2 % halothane and the surgical field was prepared in a sterile manner. A 1-cm longitudinal incision was made in the plantar aspect of the hind paw beginning 0.5 cm from the end of heel; the skin, fascia, and muscle were incised and the skin was closed with 5–0 nylon suture. Topical antibiotics were administered. On the second postoperative day, sutures were removed under brief anesthesia.

For measuring guarding behaviors, unrestrained rats were placed on a small plastic mesh floor (grid 8 × 8 mm). Using an angled magnifying mirror, the incised and non-incised paws were viewed. Both paws of each animal were closely observed over a 1-min period repeated every 5 min for 1 h. Depending on the position in which each paw was found during the majority of the 1-min scoring period, a 0, 1, or 2 was given. A score of 0 was given for full weight bearing with the area of the wound blanched or distorted by the mesh, 1 for the wound area just touching the mesh without blanching or distortion, and 2 for the wound area completely off the mesh. The sum of the 12 scores (0–24) obtained during 1-h session for each paw was obtained.

For measuring heat sensitivity, rats were placed individually on a glass floor covered with a clear plastic cage and allowed to acclimate. Withdrawal latencies to radiant heat were assessed by applying a focused radiant heat source underneath a glass floor on the middle of the incision. The latency time to evoke a withdrawal was determined with a cutoff value of 30 s. The intensity of the heat was adjusted to produce a withdrawal latency in normal rats of 25–30 s. Each rat was tested at least three times, at an interval of 10 min. The average of at least three trials was used to obtain paw withdrawal latency.

For measuring mechanosensitivity, rats were placed individually on a plastic mesh floor covered with a clear plastic cage and allowed to acclimate. The withdrawal response to punctate mechanical stimulation was determined using calibrated von Frey hairs applied underneath the cage to an area adjacent to the incision. Each filament was applied once starting with 10 mN and continuing until a withdrawal response occurred or 250 mN was reached. If a rat did not respond to the 250-mN filaments (522 mN), the next filament was recorded. This was repeated a total of three times with at least a 5- to 10-min test-free period between withdrawal responses. The lowest force from the three tests producing a response was considered the withdrawal threshold.

Zahn et al. (2004) described the effect of blockade of nerve growth factor and tumor necrosis factor on pain behaviors after plantar incision.

Obata et al. (2002) described the expression of neurotrophic factors in the dorsal root ganglion in a **rat model of lumbar disk herniation**. The left L4/L5 nerve roots were exposed after hemilaminectomies, and autologous intervertebral disks, which were obtained from coccygeal intervertebral disks, were implanted on each of the exposed nerve roots without mechanical compression.

Lamb et al. (2003) studied nerve growth factor (NGF) and **gastric hyperalgesia** in the rat. Male Sprague Dawley rats (300–400 g) were anesthetized and the stomach exposed and placed in a circular clamp. Acetic acid (60 %) or saline was injected into this area and aspirated 45 s later, resulting in kissing ulcers. A balloon was surgically placed into the stomach and electromyographic responses to gastric distension were recorded from the acromiotrapezius muscle. Animals received a daily injection of neutralizing NGF antibody or control serum for 5 days; NGF in the stomach was measured with an ELISA. The severity of gastric injury was assessed

microscopically and by determination of myeloperoxidase activity.

Winston et al. (2003) investigated molecular and behavioral changes in nociception in a novel **rat model of chronic pancreatitis** induced by pancreatic infusion of trinitrobenzene sulfonic acid as a model of painful pancreatitis. Nociception was assessed by measuring mechanical sensitivity of the abdomen and by recording the number of nocifensive behaviors in response to electrical stimulation of the pancreas. Expression of neuropeptides, calcitonin gene-related peptide (CGRP) and substance P (SP), in the thoracic dorsal root ganglia receiving input from the pancreas and nerve growth factor in the pancreas was measured.

Guerios et al. (2006) reported that nerve growth factor (NGF) mediates peripheral mechanical hypersensitivity that accompanies **experimental cystitis in mice**. Cystitis was induced by intraperitoneal injection of cyclophosphamide (CYP) in female mice. Sensitivity of hind paws to mechanical stimuli was determined prior to and 4, 9, and 24 h after CYP, and the sensitivity of the tail to thermal stimuli was determined prior to and 4 and 24 h after CYP treatment. To investigate the role of NGF in these processes, other groups of mice received NGF antiserum or normal serum intravenously 30 min after CYP administration. CYP induced bladder inflammation that was not ablated by treatment with NGF antiserum. Sensitivity to mechanical stimuli was increased 4 and 9 h after CYP administration. This was reversed by NGF antiserum but not by normal serum.

Cyclophosphamide-induced cystitis was also used as a model for visceral pain by Lanteri-Minet et al. (1995), Boucher et al. (2000), and Bon et al. (2003).

Jaggar et al. (1999) studied hyperalgesia to thermal stimulation of the hind limb of rats after inflammation of the urinary bladder by instillation of 0.5 ml of 50 % turpentine in olive oil.

Dmitrieva and McMahon (1996) and Dmitrieva et al. (1997) reported sensitization of visceral afferents by NGF in the adult rat.

Delafoy et al. (2003) studied the role of NGF in **trinitrobenzene sulfonic acid-induced colonic hypersensitivity**. The function of NGF as a mediator of persistent pain states was tested in a model of colonic hypersensitivity measured by isobaric distension in conscious rats. The effects of exogenous NGF on colonic pain threshold, the involvement of NGF in trinitrobenzene sulfonic acid-induced colonic hypersensitivity, and the involvement of sensory nerves in the effects of NGF and trinitrobenzene sulfonic acid using rats treated neonatally with capsaicin were studied.

Sevcik et al. (2005) found that anti-NGF therapy profoundly reduced **bone cancer pain** and the accompanying increase of markers of peripheral and central sensitization. Osteolytic murine sarcoma cells were injected into the intramedullary space of the mouse femur. Administrations of an NGF-sequestering antibody produced a profound reduction in cancer pain-related behavior that was greater than that achieved with the administration of morphine.

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Anti-Inflammatory Activity

Vino Daniel

Contents

General Considerations

Inflammation was characterized 2,000 years ago by Celsus by the four Latin words: rubor, calor, tumor, and dolor. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues, and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation, and chronic repair processes. In some cases, the screening is directed to test compounds for local application. Predominantly, however, these studies are aimed to find new drugs against polyarthritis and other rheumatic diseases. Since the etiology of polyarthritis is considered to be largely immunological, special tests have been developed to investigate various immunological and allergic factors (see Part IX, "Antiarthrotic and Immunomodulatory Activity").

In Vitro Methods for Anti-inflammatory Activity

General Considerations

An array of physiological substances, sometimes called autacoids, are involved in the process of inflammation and repair. These include histamine, serotonin, bradykinin, substance P, and the group

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of eicosanoids (prostaglandins, thromboxanes, and leukotrienes), the platelet-activating factor (PAF), as well as cytokines and lymphokines. Their discovery makes the use of in vitro studies possible. The influence of nonsteroidal anti-inflammatory agents on the eicosanoid pathway gave rise to numerous studies.

^{3}H-Bradykinin Receptor Binding

Purpose and Rationale

Tissue injury or trauma initiates a cascade of reactions which results in the proteolytic generation of bradykinin and kallidin from high molecular weight precursors, kininogens, found in the blood and tissue. The rapid enzymatic cleavage of kininogens is accomplished by the kallikreins, a group of proteolytic enzymes which are present in most tissues and body fluids. Bradykinin produces pain by stimulating A and C fibers in the peripheral nerves, participates in the inflammatory reaction, and lowers blood pressure by vasodilatation. Since its breakdown occurs via the same enzyme responsible for converting angiotensin I into angiotensin II, some of the effects of converting enzyme inhibitors may be due to the presence of bradykinin. The ^{3}H-bradykinin receptor binding is used to detect compounds that inhibit binding of ^{3}H-bradykinin in membrane preparations obtained from guinea pig ileum. Two types of bradykinin receptors (B_1 and B_2 receptors) are known (Feres et al. 1992; Bascands et al. 1993; Tropea et al. 1994; Marceau et al. 1998; Calixto et al. 2004; Leeb-Lundberg et al. 2005). The existence of a pulmonary BK_3 receptor has been proposed by Farmer et al. (1989) and Meini et al. (2004). Evidence was obtained for the existence of three subtypes of B_2 receptors, B_{2a}, B_{2b}, and B_{2c} (Seguin et al. 1992; Seguin and Widdowson 1993).

Procedure

The ileum from guinea pigs is cleaned from its content and cut into pieces of 2 cm length. They are homogenized for 30 s in ice-cold TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, in a Potter homogenizer. The homogenates are filtered through three layers of gauze and centrifuged twice at 50,000 g for 10 min with an intermediate rehomogenization in buffer.

For routine studies the final pellets are resuspended in 40 vol of incubation buffer (25 mM TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, 0.1 % bovine serum albumin, 140 µg/ml bacitracin, 1 mM dithiothreitol, 0.1 µM captopril). In the competition experiment, 50 µl ^{3}H-bradykinin (one constant concentration of $0.5-2 \times 10^{-9}$ M), 50 µl test compound (six concentrations, $10^{-5}-10^{-10}$ M), and 150 µl membrane suspension from guinea pig ileum (approx. 6.6 mg wet weight/ml) per sample are incubated in a shaker bath at 25 °C for 90 min.

Saturation experiments are performed with 12 concentrations of ^{3}H-bradykinin ($14.2-0.007 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer, and nonspecific binding is determined in the presence of non-labeled bradykinin (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after the addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

Evaluation

The following parameters are calculated:

- Total binding of ^{3}H-bradykinin
- Nonspecific binding in the presence of 10 µM bradykinin
- Specific binding = total binding–nonspecific binding
- % inhibition: 100–specific binding as percentage of control value

Compounds are first tested at a single high concentration (10,000 nM) in triplicate. For those showing more than 50 % inhibition, a displacement curve is constructed using seven different concentrations of test compound. Binding potency of compounds is

expressed either as a relative binding affinity (*RBA*) with respect to the standard compound (bradykinin) which is tested in parallel or as an IC_{50}.

$$RBA = \frac{IC_{50}\,\text{standard compound}}{IC_{50}\,\text{compound}} \times 100\ \%$$

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-bradykinin versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

Tests for Bradykinin Receptor Types and Subtypes

Prado et al. (2002) described mechanisms regulating the expression, self-maintenance, and signaling function of the bradykinin B_2 and B_1 receptors.

Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor was reported by Hess et al. (1992).

Menke et al. (1994) reported the expression cloning of a human bradykinin B_1 receptor.

Bradykinin B_1 receptors have been studied in the isolated rabbit aorta (Bouthillier et al. 1987), in the isolated rabbit carotid artery (Pruneau and Bélichard 1993), and in the rabbit urinary bladder (Butt et al. 1995).

A potent bradykinin B_1 receptor antagonist has been described by Wirth et al. (1991).

Heterogeneity of B_1 receptors has been suggested by Wirth et al. (1992).

Heitsch (2002) reviewed non-peptide antagonists and agonists of the bradykinin B_2 receptor.

Bradykinin receptor ligands were described by Marceau and Regoli (2004) and Fortin and Marceau (2006).

Drummond and Cocks (1995) used rings of bovine left anterior descending coronary artery to study endothelium-dependent relaxations mediated by inducible B_1 and constitutive B_2 kinin receptors.

The production of cyclic GMP via activation of B_1 and B_2 kinin receptors in cultured bovine aortic endothelial cells was described by Wiemer and Wirth (1992).

Pharmacological characterization of bradykinin receptors in canine cultured tracheal smooth muscle cells has been reported by Yang et al. (1995).

Bradykinin B_2 receptors and their antagonists have been studied in human fibroblasts by Alla et al. (1993); with the high-affinity radioligand [^{125}I]PIP HOE 140 by Brenner et al. (1993); in guinea pig gall bladder by Falcone et al. (1993); in the smooth muscle of guinea pig taenia caeci by Field et al. (1994); in guinea pig ileum membranes by Graneß and Liebmann (1994) and Liebmann et al. (1994a); in isolated blood vessels from different species by Félétou et al. (1994); and in endothelial cells by Wirth et al. (1994).

Hallé et al. (2000) described the in vitro and in vivo effects of kinin B_1 and B_2 receptor agonists and antagonists in inbred control and cardiomyopathic hamsters.

The role of B_1 and B_2 receptors and of nitric oxide in bradykinin-induced relaxation and contraction of isolated rat duodenum was studied by Rhaleb and Carretero (1994).

Campos et al. (1996) investigated the effect of pretreatment with bacterial endotoxin on the bradykinin B_1 and B_2 receptor-induced edema in the rat paw and the interaction of B_1-mediated responses with other inflammatory mediators.

Characterization of kinin receptors by bioassays was described by Gobeil and Regoli (1994). Molecular cloning, functional expression, and pharmacological characterization of a human bradykinin B_2 receptor gene were performed by Eggerickx et al. (1992).

Simpson et al. (2000) characterized bradykinin analogues on recombinant human bradykinin B_1 and B_2 receptors using a high-throughput functional assay which measures intracellular Ca^{2+} responses.

Bradykinin B_2 receptor subtypes were discussed by Liebmann et al. (1994b) and Regoli et al. (1994).

Evidence for a pulmonary B_3 bradykinin receptor has been given by Farmer et al. (1989) and Meini et al. (2004).

Bradykinin B_3 receptors have been described by Field et al. (1992) in the smooth muscle of the guinea pig *taenia caeci* and trachea.

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Substance P and the Tachykinin Family

General Considerations

Substance P (SP) is a neuropeptide which can function as a neurotransmitter and as a neuromodulator. Substance P is an important element in pain perception. The sensory function of substance P is thought to be related to the transmission of pain information into the central nervous system. Substance P belongs to the tachykinin family of peptides that share a common carboxy-terminal sequence (Phe-X-Gly-Leu-Met-NH_2). It was first described by von Euler and Gaddum (1931) as a brain and gut extract that stimulates smooth muscle contraction. Bioassay extracts from spinal dorsal roots implicated substance P as a pain neurotransmitter (Lembeck 1953; Lembeck and Holzer 1979). After determination of the amino acid sequence (Chang et al. 1971), the distribution of substance P in the CNS could be studied (Hökfelt et al. 1975). Neurokinins belong like substance P to a group of neuropeptides named tachykinins. Following the discovery of neurokinin A and neurokinin B, three distinct G protein-coupled receptors, NK_1, NK_2, and NK_3, were described (Maggi et al. 1993; Mussap et al. 1993; Patacchini and Maggi 1995). Neurokinin A and substance P are preferred agonists of the tachykinin NK_1 and NK_2 receptors, whereas neurokinin B preferentially interacts with the tachykinin NK_3 receptor. The receptor sensitivity of these peptides is relatively poor, and it is possible that their actions could be mediated by interactions with their less preferred receptors.

Nomenclature of tachykinins and tachykinin receptors has been discussed repeatedly (Henry 1987; Maggi 2000).

Tachykinin NK_1 antagonists are potent antiemetics; however, other possible therapeutic uses, including rheumatoid arthritis, asthma, migraine, pain, and psychiatric disorders, were suggested (Longmore et al. 1995). The P-preferring NK_1 receptor has attracted most interest as a CNS target because it is the predominant tachykinin receptor expressed in the human brain, while NK_2 and NK_3 receptor expression is in extremely low abundance or absent. Several NK_1 receptor agonist antagonists were synthesized and evaluated (Snider et al. 1991; Emonds-Alt et al. 1993; Cascieri et al. 1992; Sakurada et al. 1993; Bristow and Young 1994; Jung et al. 1994; Rupniak and Williams 1994; Smith et al. 1994; Vassout et al. 1994; Patacchini and Maggi 1995; Bonnet et al. 1996; Chapman et al. 1996; Herbert and Bernat 1996; Palframan et al. 1996; Ren et al. 1996). Moreover, agonists and antagonists at the NK_2 receptor (Hagan et al. 1991, 1993; Beresford et al. 1995; Robineau et al. 1995; Kudlacz et al. 1997; Lecci et al. 1997) and at the NK_3 receptor (Guard et al. 1990; Edmonds-Alt et al. 1995; Patacchini et al. 1995; Nguyen-Le et al. 1996; Beaujouan et al. 1997; Sarau et al. 1997) were reported (Longmore et al. 1995; Rupniak 1999).

Understanding the role of substance P in the brain has been complicated by marked species differences in the distribution of the tachykinin receptor types. There appears to be a relative increase in NK_1 receptor density during evolution, such that NK_3 receptors are abundant in lower vertebrates and mammals but, like NK_2 receptors, are apparently absent in the human brain. Preclinical studies with substance P receptor antagonists have been hindered not only by phylogenetic differences in tachykinin receptor expression, but also by pharmacological heterogeneity of the NK_1 receptor and the NK_2 receptor. Another confounding feature of neurokinin receptor antagonist is the blockade of Na^+ and Ca^{2+} channels at high doses which produces effects in various assays that are independent of receptor antagonism (Patacchini and Maggi 1995; Rupniak 1999). Most developments were guided by the effects of substance P as a pain neurotransmitter. Surprisingly, most clinical studies of analgesic activity of NK_1 receptor antagonists were negative (Rupniak and Kramer 1999). However, clinical findings indicated that substance P receptor antagonists are able to alleviate depression and anxiety in patients suffering from major depressive disorder (Kramer et al. 1998).

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^{3}H-Substance P Receptor Binding

Purpose and Rationale

Substance P is an undecapeptide which is widely distributed in the central and peripheral nervous systems and functions as a neurotransmitter/neuromodulator in a variety of physiological processes. Substance P is a key mediator of inflammation due to immune complex formation. Substance P is secreted by nerves and inflammatory cells including macrophages, eosinophils, lymphocytes, and dendritic cells. Substance P acts by binding to the neurokinin-1 receptor. Substance P initiates inflammation by local deposition of immune complexes in vessel walls or tissues, and this initiates vascular permeability changes, leading to edema and leukocyte extravasation, the

two key components of an inflammatory response. The main leukocytes involved are neutrophils; in addition, there are the stimulation of macrophages and activation of the complement via the classical pathway and production of reactive oxygen intermediates. This type of immune response is an underlying mechanism in many medical conditions including systemic lupus erythematosus, rheumatoid arthritis, vasculitis, etc., where there is a correlation between the disease severity and the titer of the immune complexes. For many diseases the identification of substance P as a key player in the inflammation mechanism may have significant impact on therapeutic management for many diseases.

Substance P is released from neurons in the midbrain in response to stress where it facilitates dopaminergic neurotransmission and from sensory neurons in the spinal cord in response to noxious stimuli, where it excites dorsal neurons. In the periphery, release of substance P from sensory neurons causes vasodilatation and plasma extravasation, suggesting a role in neurogenic inflammation. Selective antagonists to substance P found in receptor binding studies may elucidate the physiological role of substance P and may be candidates for anti-inflammatory and analgesic drugs.

Procedure

Fresh porcine brains are obtained from the slaughterhouse. Striata are dissected and homogenized (Ultra-Turrax) in 50 mM ice-cold Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 150 mM KCl, 12 mM EDTA, 200 μM phenylmethylsulfonyl fluoride, 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 2 μg/ml chymostatin. These homogenates are then incubated for 30 min at 4 °C before being centrifuged at 30,000 g for 20 min at 4 °C and washed twice with 50 mM Tris–HCl (pH 7.4) buffer. Pellets are resuspended in 0.32 M sucrose containing 200 μM phenylmethylsulfonyl fluoride and 40 μg/ml bacitracin before storage at −80 °C until use.

Sixty-minute incubations is carried out at room temperature in 50 mM Tris–HCl buffer, pH 7.4, containing various concentrations of [^{3}H]substance P ([^{3}H]SP) (0.05–20 nM), 5 mM $MgSO_4$, 40 mg/ml bacitracin, 4 mg/ml leupeptin, and 2 mg/ml chymostatin in the presence of 0.8–1 mg of membrane protein in a final volume of 1 ml. Total binding and nonspecific binding are determined in triplicate in the absence or presence of 1 mM unlabeled substance P. Incubations are terminated by adding 4 ml of ice-cold Tris–HCl buffer (pH 7.4), and membranes are filtered on Whatman glass fiber filters that are presoaked in 0.5 % polyethylenimine for a minimum of 3 h to reduce absorption. Filters are then washed three times (5 ml each) using ice-cold Tris–HCl buffer (pH 7.4). Bound radioactivities are determined using a liquid scintillation counter.

Evaluation

Saturation and competition data are analyzed using a computer program as described by McPherson (1985).

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Neurokinin Receptor Binding

Purpose and Rationale

Tachykinins are a family of neuropeptides found in the central and peripheral nervous system. The actions of tachykinins are mediated through three subtypes of neurokinin receptors belonging to the G protein-linked receptor family, namely, NK_1, NK_2, and NK_3. Substance P displays the highest affinity to NK_1 receptors, whereas neurokinin A and neurokinin B preferably bind to NK_2 and NK_3 receptors, respectively. NK_1 receptors are expressed in a wide variety of peripheral tissues and in the CNS. NK_2 receptors are expressed primarily in the periphery, while NK_3 receptors are primarily expressed in the CNS.

Procedure

Tachykinin NK_1 receptor binding assay is performed in intact Chinese hamster ovary (CHO) cells expressing the human tachykinin NK_1 receptor (Cascieri et al. 1992). The receptor is expressed at a level of 3×10^5 receptors per cell. Cells are grown in a monolayer culture, detached from the plate with enzyme-free cell dissociation solution (Specialty Media), and washed prior to use in the assay. ^{125}I[Tyr^8]substance P (0.1 nM, 2,000 Ci/mmol; New England Nuclear) is incubated in the presence or absence of test compounds (dissolved on 5 µl DMSO) with 5×10^4 CHO cells. Ligand binding is performed in 0.25 ml of 50 mM Tris–HCl, pH 7.5, containing 5 mM $MnCl_2$, 150 mM NaCl, 0.02 % bovine serum albumin, 40 µg/ml bacitracin, 0.01 mM phosphoramidon, and 4 µg/ml leupeptin. The incubation proceeds at room temperature until equilibrium is achieved (>40 min), and the receptor ligand complex is harvested by filtration over GF/C filters presoaked in 0.1 % polyethylenimine using a Tomtec 96-well harvester. Nonspecific binding is determined using excess substance P (1 µM) representing <10 % of total binding.

For **NK_2 receptor binding assays**, membranes of CHO cells transfected with human ileum NK_2 receptor are used (Hagan et al. 1993; Beresford et al. 1995). The membrane suspensions (5 µg protein) in assay buffer (Tris base (50 mM), $MnCl_2$ (3 mM), bovine serum albumin (0.05 %), chymostatin (2 µg/ml), and leupeptin 4 µg/ml, pH 7.4) are incubated for 90 min at room temperature with wash buffer (Tris base (50 mM), $MnCl_2$ (3 mM), lauryl sulfate (0.01 %), pH 7.4) or test compound and [^{3}H]-GR100679 (0.5 nM final concentration). Nonspecific binding is defined by the use of GR159897 (1 µM).

For **NK_3 receptor binding assays**, guinea pig cortical membranes (Guard et al. 1990) are incubated at room temperature for 60 min with HEPES wash buffer or test compound and [^{3}H]-senktide (final concentration 0.8–1.0 nM). Nonspecific binding is defined by addition of eledoisin (10 µM).

Evaluation

Inhibition curves are analyzed and pIC_{50} values calculated by the use of a curve fitting program. pIC_{50} values are converted to inhibition constants (pK_i values) using the Cheng–Prusoff equation

$$K_i = IC_{50}/(1 + L/K_D)$$

where L is the ligand concentration and K_D is the dissociation constant. The K_D and B_{max} (maximum number of binding site per mg of tissue) are determined from saturation curves and analyzed by a curve fitting program. Values are expressed as means ± SEM.

Modifications of the Method

Watson et al. (1995) performed substance P binding assays in **ferret brain membranes** and assessed neurokinin NK_1 receptor binding using human lymphoblasts (IM9 cells).

Rupniak et al. (1997) studied displacement of ^{125}I-[Tyr^8]-substance P binding to cloned human tachykinin NK_1 receptors and to ferret brain membranes in vitro.

Beattie et al. (1995) used U373 MG cell membranes and cerebral cortical membranes from rat, ferret, and gerbil and [^{3}H]substance P for NK_1 receptor binding assays.

Bonnet et al. (1996) and McLean et al. (1996) used the IM9 lymphoblastoma cell line expressing the human NK_1 receptor.

Emonds-Alt et al. (1995) studied the binding of [^{125}I] Bolton–Hunter-labeled substance P to **NK_1**

receptors of rat brain cortex, human lymphoblast cells (IM9), and human astrocytoma cells (U373MG, STTG1); binding of [^{125}I] iodohistidyl-NKA (or [^{125}I]neuropeptide γ) to **NK_2 receptors** of rat or hamster urinary bladder or guinea pig ileum; and binding of [^{125}I] iodohistidyl-[Me-Phe^7]NKB (or [^{125}I]Eledoisin) to tachykinin **NK_3 receptors** of rat, guinea pig, and gerbil brain cortex and of [^{125}I]iodohistidyl-[Me-Phe^7]NKB to the human NK_3 receptor, cloned and expressed in CHO cells (Buell et al. 1992).

Cascieri et al. (1992) described the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor.

A radioligand of the tachykinin NK_2 receptor was described by Renzetti et al. (1998).

Jordan et al. (1998) evaluated the Cytosensor microphysiometer, a system that measures the extracellular acidification rate as an index of the integrated functional response to receptor activation, as a method to study the NK_3 receptor pharmacology and used this system to assess the functional activity of novel compounds at this receptor.

Appell et al. (1998) reported biological characterization of neurokinin antagonists discovered through screening of a combinatorial library. Using stably transfected CHO-K1 cell lines expressing human NK_1, NK_2, and NK_3 receptor subtypes and europium time-resolved fluorescence, primary receptor binding assays were designed to define active compounds. In addition, a secondary, functional assay measuring intracellular calcium flux with the calcium-sensitive fluorophore, fluo-3, in CHO cells transfected with the human NK_1 or NK_2 receptor was used to determine agonist or antagonist activities.

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Characterization of Neurokinin Agonists and Antagonists by Biological Assays

Purpose and Rationale

Several biological assays have been used to characterize neurokinin agonists and antagonists on their receptors (Review by Regoli et al. 1994).

The following functional assays are recommended for the evaluation of antagonists:

For NK_1 Receptors

- Inhibition of [Sar^9,$Met(O_2)^{11}$]substance P-induced endothelium-dependent **relaxation of rabbit pulmonary artery**, previously contracted with 0.1 μM noradrenaline (D'Orléans-Juste et al. 1986; Rubino et al. 1992; Emonds-Alt et al. 1993)
- Inhibition of [Sar^9,$Met(O_2)^{11}$]substance P or [Sar^9]substance P sulfone-induced contractions of **guinea pig ileum** in the presence of

3 μM atropine and 3 μM mepyramine and indomethacin (Dion et al. 1987; Emonds-Alt et al. 1993; Patacchini et al. 1995; Hosoki et al. 1998; Walpole et al. 1998)
- **Rabbit vena cava** stimulated by substance P or [Sar^9,$Met(O_2)^{11}$]substance P (Nantel et al. 1991; Regoli et al. 1994; Gitter et al. 1995; Robineau et al. 1995; Bonnet et al. 1996; Nguyen-Le et al. 1996)
- Inhibition of substance P-induced **relaxation of the isolated dog carotid artery** previously contracted with norepinephrine (Snider et al. 1991)
- **Rat urinary bladder**, stimulated by the selective agonist [Sar^9,$Met(O_2)^{11}$]substance P and treated with SR 48986 (1.7×10^{-7} mol/l) to eliminate NK_2 functional sites (Rouissi et al. 1993; Nguyen-Le et al. 1996)
- Ca^{2+} mobilization in **rat vas deferens** (Nagata et al. 1991)
- Inhibition of **substance P-induced plasma extravasation in the bladder and bronchi** of the guinea pig (Bonnet et al. 1996)
- Inhibition of **substance P-induced vasodilatation in the nasal mucosa of pigs** using an acoustic rhinometer (Rinder and Lundberg 1996)
- Inhibition of **[Sar^9,$Met(O2)^{11}$]substance P-induced plasma extravasation in guinea pig bronchi** (Cirillo et al. 1998)
- Inhibition of **methacholine-induced contractions of isolated rat tracheal strips** (Tian et al. 1997)
- Inhibition of **cyclophosphamide- and radiation-induced damage in the rat and ferret organs** (Alfieri and Gardner 1997, 1998)
- Inhibition of **edema formation induced by substance P and antigen in rat skin** (Herbert and Bernat 1996)
- Inhibition of **reciprocal hind limb scratching after intracerebroventricular injection of substance P, [Sar^9, $Met(O_2)^{11}$]substance P, or septide in mice** (Jung et al. 1994) **or gerbils** (Smith et al. 1994)
- Inhibition of **turning behavior after intracerebroventricular injection of substance P, [Sar^9,$Met(O_2)^{11}$]substance P, or septide in mice** (Jung et al. 1994)
- Inhibition of **hind paw tapping and chromodacryorrhea after intracerebroventricular injection of tachykinin agonists in gerbils** (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Rupniak et al. 1995, 1997; Vassout et al. 1994)
- Inhibition of **cisplatin-induced emesis in ferrets** (Rupniak et al. 1997; Singh et al. 1997; Minami et al. 1998)

For NK_2 Receptors
- Inhibition of neurokinin A-induced contraction of **isolated rabbit aorta** (Snider et al. 1991)
- Inhibition of neurokinin A-induced contraction of **isolated endothelium-deprived rabbit pulmonary artery or hamster trachea** (D'Orléans-Juste et al. 1986; Emonds-Alt et al. 1993; Patacchini et al. 1995)
- The **hamster urinary bladder** (Dion et al. 1987; Maggi et al. 1990; Regoli et al. 1994; Emonds-Alt et al. 1997; Tramontana et al. 1998)
- Inhibition of **motor responses induced by intravesical administration of capsaicin** in rats in vivo (Lecci et al. 1997)
- **Rat esophageal tunica muscularis** (Croci et al. 1995)
- Inhibition of **turning behavior induced by intrastriatal injection of Nle^{10}-neurokinin A in mice** (Emonds-Alt et al. 1997)

For NK_3 Receptors
- Inhibition of **senktide- or neurokinin B-induced contractions of the rat portal vein** (Mastrangelo et al. 1987; Snider et al. 1991; Emonds-Alt et al. 1993; Patacchini et al. 1995)
- Antagonism against **senktide-induced contractions in the isolated rabbit iris sphincter muscle** (Medhurst et al. 1997; Sarau et al. 1997)
- Inhibition of **colonic propulsion in rats** (Broccardo et al. 1999)
- Inhibition of **turning behavior induced by intrastriatal injection of senktide in gerbils** (Emonds-Alt et al. 1995)
- Inhibition of **citric acid-induced cough in guinea pigs** (Daoui et al. 1998)
- The failure of NK_1 receptor antagonists in most clinical tests for analgesia in spite of

clear preclinical data is a matter of discussion (Hill 2000a, b; Urban and Fox 2000).

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Assay of Polymorphonuclear Leukocyte Chemotaxis In Vitro

Purpose and Rationale

Leukocyte accumulation is an important aspect of host defense mechanisms. A crucial step in an innate immune response is the movement of leukocytes to the sites of infection. Increased concentrations of microbial toxins and proinflammatory mediators induce upregulation of endothelial adhesion molecules in inflamed tissue leading to the targeted accumulation of leukocytes at the site of infection. Selectin, local chemokines, and leukocyte integrins such as lymphocyte function antigen-1 (LFA-1, CD11a/CD18) and macrophage antigen-1 (MAC-1, CD11b/CD18) play a role in leukocyte adhesion. However intravascular shear forces prevent relevant leukocyte adhesion in a healthy individual. Chemotactic factors attract leukocytes to an infected or inflamed site. The method of Boyden (1962) has been widely employed to measure the chemotactic effects on polymorphonuclear leukocytes. Watanabe et al. (1989) described a rapid assay of polymorphonuclear leukocyte chemotaxis in vitro.

Procedure

Two 96-well tissue culture plates are utilized as one set of multiple Boyden chambers. One plate

as multiple lower compartments and the other plate upside down as multiple upper compartments can be tightly sandwiched with the aid of 12 sets of bolts. The holes, into which bolts are set and polymorphonuclear leukocytes (PMN) suspensions are introduced, are opened by a heated stick. Eight sets of 6 holes are made on the bottom of the upper plate to serve as multiple upper compartments for introducing PMN suspensions. Eight sets of polycarbonate filter (approximately 3.2 × 2.2 cm), cut from a round filter (Nuclepore Co.) with pores of 2 μm in diameter, are sandwiched between the upper and lower plates. One sheet of the filter can separate six sets of the upper compartments from the lower ones. Silicon grease is spread on all the plate surfaces that attach to the filters. The lower compartments are filled with a chemoattractant (400 μl/well) diluted in RPMI-1640 medium. The eight sheets of filter paper are carefully placed on each set of the lower compartments to avoid air bubbles. The upper plate is positioned over the lower plate and fastened with bolts. The upper compartments are filled with 0.3 ml of PMN suspension (at 10^7 cells/ml). The assembly is incubated at 37 °C for 60 min in a humidified atmosphere containing 5 % CO_2. Then the fluid in the upper compartment is decanted, and the upper compartments are completely washed with a jet of water. The lower plate is centrifuged at 2,400 rpm for 5 min at room temperature, and the supernatant in the well is decanted. The pellet of PMNs is dispersed in 200 μl of phosphate-buffered saline containing 0.1 % EDTA. Absorbance at 660 nm of each well containing PMN suspension is determined with a 96-well microplate reader. The number of PMNs in the lower compartments is further determined by a Coulter counter.

Evaluation

The migration rate is calculated as percentage from the number of PMNs in the lower compartment/number of PMNs applied in the upper compartment. The migration rate is dependent on the concentration of the chemoattractant (e.g., zymosan-activated serum). Moreover, a dose-dependent decrease of migration rate is achieved by chemotaxis inhibitors.

Modifications of the Method

Nelson et al. (1975) described chemotaxis under agarose as a simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes.

Migration of PMNs in agarose gel was measured after fixation with glutaraldehyde and staining with Giemsa by Shalaby et al. (1987).

Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood were described by Ferrante and Thong (1980).

PMN chemotaxis was measured in multiwell microchemotaxis chambers separated by 5-μm pore size polyvinylpyrrolidone-free polycarbonate membranes by Harvath et al. (1980) and Figari et al. (1987).

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Polymorphonuclear Leukocyte Aggregation Induced by FMLP

Purpose and Rationale

Aggregation of polymorphonuclear leukocytes (PMNs) can be induced by FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine). The aggregation can be inhibited by xanthine derivatives.

Procedure

PMN cell suspensions are prepared from peritoneal exudates obtained 17 h after intraperitoneal injection of 10 ml 6 % sodium caseinate into Sprague–Dawley rats. The cells are washed twice in Gey's balanced salt solution (Gibco GBSS) and resuspended to a final concentration of 15×10^6 cells/ml. The test compounds and the standard (pentoxifylline) are dissolved in GBSS. FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine) is dissolved in DMSO. The further dilutions are made up to a final concentration of 10^{-7} mol FMLP in GBSS. Before addition of FMLP, the cell suspensions are preincubated for 10 min with the drugs. PMN aggregation is carried out in a Born aggregometer.

Evaluation

The results are expressed as change in transmittance, measured in mm on the recorder. The mean peak of the untreated cells is set 100 %.

Modifications of the Method

Moqbel et al. (1986) measured the activation of human leukocytes after FMLP in a rosette assay by the change in the expression of complement (C3b) and IgG (Fc) receptors and in a cytotoxic assay by the in vitro capacity to adhere to and kill the complement-coated larvae (schistosomula) of *Schistosoma mansoni*.

Bradford and Rubin (1986) determined the effect of various drugs on IP_3 accumulation evoked by FMLP in neutrophils from New Zealand white rabbits.

Bourgoin et al. (1991) studied the influence of granulocyte–macrophage colony-stimulating factor (GM-CSF) on phosphatidylcholine breakdown by phospholipase D in human neutrophils.

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Constitutive and Inducible Cellular Arachidonic Acid Metabolism In Vitro

Purpose and Rationale

The various metabolites of arachidonic acid are involved in many inflammatory processes. Arachidonic acid is released from the cellular phospholipid fraction by the action of phospholipase A_2 and subsequently metabolized via two major routes: the cyclooxygenase pathway yielding the primary prostaglandins and thromboxane and the 5-lipoxygenase pathway yielding the leukotrienes. Thromboxanes, prostaglandins, and leukotrienes play a pathophysiological role in many diseases. Receptor assays for these autacoids were developed (Halushka et al. 1989). Murata et al. (1997) produced mice lacking the prostaglandin receptor which showed increased susceptibility to thrombosis and altered pain reception and inflammatory response.

Nonsteroidal anti-inflammatory drug and their analgesic effects have been explained on the basis of their inhibition of the enzymes that synthesize prostaglandins. NSAIDs exert their analgesic effect through a variety of peripheral and central mechanisms. Cyclooxygenase (COX) is the central enzyme in the biosynthetic pathway to prostaglandins from arachidonic acid. The two structurally different forms of the cyclooxygenase enzymes are the COX-1 and COX-2 forms. Cox-1 is seen in many tissues and predominantly in the kidneys and gastric mucosa. Cox-2 is generally seen in cells during inflammation when prostaglandins are regulated.

The therapeutic mode of action of the classical nonsteroidal anti-inflammatory drugs (NSAID), such as aspirin or indomethacin, is primarily explained by their inhibitory effect on cyclooxygenase, the key enzyme of the prostaglandin pathway. Inhibitors of the 5-lipoxygenase pathway have attracted considerable attention as potential anti-inflammatories with high potency. Appropriate assay systems for the determination of the different eicosanoids allow to study the influence of drugs toward the specific pathways of the arachidonic acid cascade in various cellular systems (Samuelsson 1986; Vane and Botting 1987).

According to recent discoveries there are two forms of cyclooxygenase (Xie et al. 1992; Lee et al. 1992; Gierse et al. 1996). Cyclooxygenase-1 (COX-1) is found as a constitutive enzyme in most tissues including blood platelets. Prostaglandins generated by constitutive pathways may exert cytoprotective effects and are involved in maintaining vital functions in vascular hemostasis, gastric mucosa, and kidney.

Chandrasekharan et al. (2002) described the cloning, structure, and expression COX-3, a cyclooxygenase-1 variant, which is selectively inhibited by analgesic/antipyretic drugs, such as acetaminophen, phenacetin, antipyrine, and dipyrone.

The inhibition of these prostaglandins by the classical cyclooxygenase inhibitors is now generally accepted as an explanation of their adverse side effects.

COX-2 which shares about 62 % amino acid homology with COX-1 is only expressed after cell activation, especially by mitogenic or inflammatory stimuli (Herrmann et al. 1990; Funk et al. 1991; Crofford 1997). Thus, specific suppression of the COX-2-pathway may represent a superior target for the evaluation of new anti-inflammatory drugs. Drugs which have a high potency on COX-2 and a favorable COX-2/COX-1 ratio have potent anti-inflammatory activity with fewer side effects (Riendeau et al. 1997; Vane 1998; Hawkey 1999; Chan et al. 1999). Shigeta et al. (1998) described the role of

cyclooxygenase-2 (COX-2) in the healing of gastric ulcers in rats. Hull et al. (2005) investigated the expression of cyclooxygenase-1 and cyclooxygenase-2 by human gastric endothelial cells.

The cardiovascular value of selective COX-2 inhibitors has been questioned because they selectively reduce prostacyclin production, thus disrupting the hormonal balance and promoting a prothrombotic state (Hankey and Eikelboom 2003).

These theoretical concerns were supported by the results of clinical trials demonstrating an increased risk of myocardial infarction with COX-2 inhibitors compared with conventional nonsteroidal anti-inflammatory drugs (NSAIDs) (Bombardier et al. 2000). The debate on benefit–risk assessment of COX-2 inhibitors is ongoing (Bing 2003; Schmidt et al. 2004).

Formation of Leukotriene B_4 in Human White Blood Cells In Vitro

Procedure

Human white blood cells are prepared according to the standard procedure published by Salari et al. (1984): 40 ml freshly drawn citrated blood is admixed with 8 ml of PM16 buffer, containing 6 % (v/v) dextran (MW = 480,000), and incubated at room temperature for 1 h. The supernatant containing the white blood cell fraction is removed, diluted 1:1 (v/v) with PM16, and centrifuged for 15 min at 300 g. The precipitate is resuspended in PM16 and adjusted to 10^{10} cells/l (Counter HT, Coulter Electronics, Krefeld, FRG).

The metabolism of endogenously bound arachidonic acid to LTB_4 is measured in a total volume of 0.3 ml of the cell suspension at 37 °C. The reaction tube contains 2 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, and the investigational drug. After 15 min preincubation the reaction is started by addition of 12.5 µg of the Ca ionophore A 23187 and 2 µg glutathione. After 5 min the reaction is stopped with 30 µl 0.1 M HCl at 0 °C. After centrifugation for 2 min at 0 °C, aliquots of the supernatant are subjected to HPLC, similarly as described by Veenstra et al. (1988), using a C-18 Nucleosil column (5 µm, 100 × 3 mm, Chrompack GmbH, Frankfurt, FRG) and, at a flow rate of 0.7 ml/min, a solvent mixture consisting of 725 ml methanol, 275 ml water, and 0.1 ml acetic acid. Authentic standard drugs are used to identify cis-, trans-, and epi-LTB_4. The separation of the three isomers can be followed photometrically at the UV maximum of 278 nm.

Evaluation

The peak areas of cis-, trans-, and epi-LTB_4 are measured as a function of drug concentration and related to a control experiment without drug.

Critical Assessment

The tested pathway involves the enzymatic steps of phospholipase A_2 and the 5-lipoxygenase. Thus inhibitors of these enzymes are to decrease formation of the three isomers of LTB_4. The step of phospholipase A_2 can be circumvented by the addition of exogenous arachidonic acid (1 µmol/l).

Inhibitors of LTA_4 hydrolase, which catalyzes the intermediary conversion of LTA_4 to the biologically active cis-LTB_4, can also be identified by this assay. Such drugs are to exhibit an increased formation of the nonenzymatic hydrolysis products trans- and epi-LTB_4 at the expense of decreased cis-LTB_4.

Modifications of the Method

Winkler et al. (1988) used differentiated U-937 cells expressing LTB_4 receptors to study Ca^{2+} mobilization in response to LTB_4.

Jones et al. (1995) tested inhibition of [^{3}H] leukotriene D_4-specific binding in guinea pig lung, sheep lung, and dimethylsulfoxide-differentiated U837 cell plasma membrane preparations.

Formation of Lipoxygenase Products from ^{14}C-Arachidonic Acid in Human Polymorphonuclear Neutrophils (PMN) In Vitro

Procedure

PMN are prepared by the standard procedure of Böyum (1976). The first steps are carried out at room temperature: 50 ml citrated human blood is

centrifuged at 130 g for 15 min. The pellet is resuspended in 20 ml Dulbecco's minimal essential medium and subsequently underlayered with 15 ml Lymphoprep. After centrifugation at 400 g for 25 min, the pellet is resuspended in 28 ml Dulbecco's HBSS containing 3 % dextran. After incubation at 0 °C for 120 min, the decanted supernatant is centrifuged at 400 g for 15 min. The pellet is resuspended in 1 ml Dulbecco's PBS, containing 11 µmol/l glucose, to a leukocyte count of 2×10^{10}/l.

The method of HPLC determination of cellular metabolites of exogenous 1-C-14-arachidonic acid, as published by Borgeat and Samuelsson (1979), is modified as briefly described:

0.1 ml of the leukocyte suspension is incubated in Dulbecco's phosphate-buffered saline (DPBS) at 37 °C with the test drugs for 15 min. The incubation is then interrupted by cooling in an ice bath. Calcium ionophore A 23187 (final concentration 7×10^{-5} mol/l) and 1-C-14-arachidonic acid (final concentration 8.4×10^{-5} mol/l, 0.5 µCi) are added and, after a second incubation period of 15 min at 37 °C, terminated by the addition of 0.4 ml methanol. The assay mixture is then extracted with chloroform. The chloroform is evaporated and, after redissolving the residue in a minor amount of methanol/water, analyzed by HPLC and radiomonitoring for the C-14-eicosanoids.

HPLC conditions are as follows: Column – Nucleosil C-18, 5 µm; organic phase, 700 ml methanol, 300 ml water, 0.1 ml acetic acid; after 35 min change to pure methanol. Flow, 1 ml/min, 2,000 psi. Radiomonitor, LB 507 (Berthold, Wildbad, FRG).

A viability assay (trypan blue exclusion) ascertains that cells remain intact during incubation periods.

Evaluation

The radioactivity of the separated 5-HETE and LTB_4 is measured as a function of drug concentration and related to a control experiment without drug. Two further lipoxygenase products, 12-HETE and 15-HETE, which are additionally generated under the test conditions, can be quantified in a similar way.

Critical Assessment

The measured reaction sequence starts with arachidonic acid and involves its transformation to 5-HETE by 5-lipoxygenase, as well as the subsequent enzymatic hydrolysis to LTB_4. Inhibitors of 5-lipoxygenase exhibit a decreased formation of 5-HETE and LTB_4. Effects of drugs on the side products 12-HETE and 15-HETE can also be studied in this test system.

Formation of Eicosanoids from ^{14}C-Arachidonic Acid in Human Platelets In Vitro

Procedure

Blood is drawn from the vena brachialis of healthy volunteers and collected into plastic tubes containing sodium citrate (0.38 % final concentration (w/v)). After centrifugation at 100 g for 15 min, the platelet-rich plasma (PRP) containing about $2{,}5 \times 10^{11}$ platelets/l (Counter HT, Coulter Electronics, Krefeld, FRG) is saved and kept at 20 °C no longer than 1 h until the experiment is started. Metabolism of ^{14}C-arachidonic acid is followed by the HPLC procedures published by Weithmann et al. (1994), modifying the method of Powell (1987). PRP is mixed with the same volume of a citrate/D-glucose solution (27.35 g trisodium citrate · $2H_2O$, 1.47 g citric acid, and 27.74 g D(+)-glucose · H_2O ad 1 l water) and centrifuged at 1,000 g for 20 min (4 °C). The saved precipitate is resuspended in Dulbecco's solution (DPBS, Serva, Heidelberg, FRG) in the original volume. 0.495 ml of this platelet suspension is incubated at 37 °C for 15 min with the test compound. Subsequently eicosanoid formation is started by the addition of 5 µl of 1-^{14}C-arachidonic acid solution (50 Ci/mol, 9×10^{-4} Ci/l, NEN, Dreieich, FRG). After 5 min (37 °C) the reaction is stopped by adding 0.5 ml of chilled acetone/0.1 ml 1n HCl, cooled to 0 °C, and extracted two times with 3 ml ethylacetate. The combined organic extracts are evaporated and the residue redissolved in 0.2 ml methanol. Aliquots are separated by HPLC at a flow rate of 1.5 ml/min, using a C-18 Nucleosil column (5 µm, 125 × 4.6 mm, Bischoff, Leongang, FRG) connected with

a pre-column C-18 Nucleosil (5 μm, 20 × 4.6 mm) of the same type. The formed ^{14}C-eicosanoids are analyzed using a liquid scintillation flow detector LB 507 (Berthold, Wildbad, FRG). The radiochromatogram is analyzed by comparison with tritiated authentic eicosanoids (NEN, Dreieich, FRG). The elution system consists of the following solvent mixtures (elution time in parenthesis): I 725 ml water/275 ml acetonitrile/1 ml acetic acid (40 min), II 700 ml methanol/300 ml H_2O/1 ml acetic acid (40 min), and III pure methanol (20 min).

Evaluation

The radioactivity of the separated TXB_2 and PGE_2 is measured as a function of drug concentration and related to a control experiment without drug addition.

A further lipoxygenase product, 12-HETE, which is additionally generated under the test conditions, can be quantified in a similar way.

Lasché and Larson determined PGI_2 by a bioassay based on its generation by aortic rings and assay by its ability to inhibit platelet aggregation.

Critical Assessment

The measured reaction sequence starts with arachidonic acid and involves its transformation to prostaglandin endoperoxides, which is catalyzed by cyclooxygenase. The unstable and short-living endoperoxides transform immediately into thromboxane A_2 by the action of thromboxane isomerase. TXA_2 is unstable, too, and yields the stable nonenzymatic hydrolysis product TXB_2.

Inhibitors of cyclooxygenase exhibit a decreased formation of TXB_2. Specific inhibition of the thromboxane isomerase step results in an accumulation of the mentioned endoperoxide intermediates, which due to their chemical instability are nonenzymatically transformed to the primary PGE_2. Thus, inhibitors of the TXA_2 isomerase lead to a significant increase of the PGE_2 peak at the expense of the TXB_2 peak. Effects of drugs on the side product 12-HETE can also be studied in this test system.

Stimulation of Inducible Prostaglandin Pathway in Human PMNL

Procedure

The procedure of Herrmann et al. (1990) with the modification of Weithmann et al. (1994) is used to stimulate cyclohexamide-inhibitable generation of PGE_2 in human PMNL by LPS.

H.2.5 × 10^9 PMNL/l culture medium (RPMI 1640, completed with 1 mmol/l sodium pyruvate, 5 % FCS (w/v), 2 mmol/l glutamine, and each 100 U/ml penicillin/streptomycin) are incubated with 100 μmol/l acetylsalicylic acid for 60 min (37 °C, 5 % CO_2), and the latter subsequently removed by four times washing with medium. 0.25 ml aliquots (containing 2.5 × 10^9 PMNL/l medium) are incubated with 0.1 mg/ml LPS (lipopolysaccharide from *Salmonella abortusequi*, Sigma GmbH, Deisenhofen, FRG) along with the test compound for 18 h (96-well plates, 37 °C, 5 % CO_2). The generation of eicosanoids is induced by the administration of 7 × 10^{-5} mol/l of the calcium ionophore A 23187. After incubation for 30 min at 37 °C, the plates are centrifuged at 400 g for 15 min. PGE_2 is determined in the supernatant, using an ELISA kit commercially available from several distributors. Alternatively the test compound is added along with the calcium ionophore. At least 90 % of the cells remain intact during incubation times (trypan blue exclusion assay).

Evaluation

The PGE_2 concentration in the sample is determined from appropriate calibration curves. The PGE_2 concentration is measured as a function of drug concentration and related to a control experiment without drug.

Critical Assessment

Long-term activation with LPS or other inflammatory effectors leads in human polymorphonuclear neutrophils to the stimulation of a prostaglandin-synthesizing capacity, which under normal conditions is not present in this system. The described assay system detects compounds which interfere with this activation process.

Incubation of drugs with already activated cells allows to search for drugs that directly influence the enzyme activity.

COX-1 and COX-2 Inhibition

Purpose and Rationale

Several assays were described to characterize COX-1 and COX-2-inhibitors, such as in vitro COX enzyme assay (Seibert et al. 1994), COX-2 protein extraction and analysis (Anderson et al. 1996), and a human whole blood assay using LPS-induced PGE_2 production as an index for cellular COX-2 activity (Riendeau et al. 1997) or whole-cell assays with transfected Chinese hamster ovary cells expressing COX-1 and COX-2 or COX-2 specific (osteosarcoma cells) and COX-1 specific (U937 cells) making use of PGE_2 production after arachidonic challenge as an index of cellular potency and selectivity of cyclooxygenase inhibitors (Chan et al. 1999; FitzGerald and Loll 2001; Rao et al. 2003).

Procedure

In Vitro Cyclooxygenase Inhibition

The ability of test compounds to inhibit COX-1 and COX-2 (IC_{50} values, μM) is determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Mich., USA, no. 560101) (Uddin et al. 2004). This COX (ovine) inhibitor screening assay directly measures the amount of the prostaglandin $PGF_{2\alpha}$ produced in the cyclooxygenase reaction. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds. Thus, this COX assay is more accurate and reliable than an assay based on peroxidase inhibition. The COX (ovine) inhibitor screening assay includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors. This assay is an excellent tool, which can be used for general inhibitor screening or to eliminate false-positive leads generated by less specific methods.

Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid to PGH_2. $PGF_{2\alpha}$ produced from PGH_2 by reduction with stannous chloride is measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody–PG complex binds to a mouse anti-rabbit monoclonal antibody that was previously attached to the well. The plate is washed to remove any unbound reagents, and then Ellman's reagent, which contains the substrate for acetylcholinesterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation. Percent inhibition is calculated by the comparison of compound treated to various control incubations. The concentration of the test compound causing 50 % inhibition (IC_{50}, μM) is calculated from the concentration–inhibition response curve.

Inhibition Studies with Recombinant Human COX-1 and COX-2

Microsomal preparations of recombinant human COX-1 and COX-2 are prepared from a vaccine virus-COS-7 cell expression system (O'Neill et al. 1994). Recombinant human COX-1 and COX-2 are expressed in baculovirus-Sf9 cells, and enzymes are purified (Ouellet and Percival 1995; Cromlish and Kennedy 1996). Enzymatic activity is monitored continuously by either a fluorescence assay measuring the appearance of the oxidized form of the reducing agent cosubstrate homovanillic acid or by oxygen consumption (Ouellet and Percival 1995).

Classical nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors are time-dependent, irreversible inhibitors of hCOX-2, which is consistent with a two-step process involving an initial rapid equilibrium binding of enzyme and inhibitor, followed by the slow formation of a tightly bound enzyme–inhibitor

complex. COX-2 inhibitors show a time-independent inhibition of hCOX-1, consistent with the formation of a reversible enzyme–inhibitor complex (Ouellet and Percival 1995; Riendeau et al. 2001).

HPLC Assay for Oxygenation of Radiolabeled Arachidonic Acid by COX-1

Purified recombinant human COX-1 (50 µl of 1 µg/ml in 100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 µM hematin) is preincubated with 2 µl of the inhibitor solution (50-fold concentrated stock in DMSO, 0–2.5 mM) for 15 min. The reaction is then initiated by the addition of 5 µl of 1 µM [^{14}C]-arachidonic acid (0.005 µCi) to obtain a final concentration of 0.1 µM. After 7 min incubation at room temperature, the reaction is stopped by the addition of 5 µl 1 M HCl and 50 µl acetonitrile. Aliquots of 50 µl of each reaction mixture are analyzed for substrate conversion by reverse phase HPLC onto a C-18 Nova-Pak column (3.9 × 150 mm) which is developed with acetonitrile/water/acetic acid (85:15:0.1) at 2 ml/min. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1 min and 2.2–2.5 min, respectively, are quantitated by a Packard radiochromatography detector. Percentages of inhibition are calculated from the difference in conversion of arachidonic acid to prostaglandin metabolites between inhibitor-treated samples and controls exposed to DMSO vehicle.

Determination of the Stoichiometry of Inhibitor Binding

Aliquots of purified COX-2 (0.25 mg/ml, concentration of subunit of 3.4 mM) are incubated in buffer (100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM phenol) in the presence of various inhibitors (0–8 µM) for 15 or 30 min. An aliquot (20 µl) is then removed for determination of the cyclooxygenase activity which is monitored continuously by oxygen consumption by a Clark-type polarographic oxygen probe. The oxygen chamber is filled with 0.6 ml of reaction buffer (100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 µM hematin, 1 mM phenol, 100 µM arachidonic acid at 30 °C or 37 °C), and the reaction is initiated by the addition of 20 µl of a solution of 4 mM hydrogen peroxide and 0.5 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) in assay buffer. Enzyme concentration is determined by amino acid concentration following acid hydrolysis.

Determination of the Dissociation Rate Constant of the Enzyme–Inhibitor Complex

Purified COX-2 (2.0 nmol, 2 ml) in 20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 2 µM hematin, and 0.1 % *β*-octylglucoside is treated with 2.0 nmol [^{14}C]-DFU (18 Ci/mol) and incubated at 20 °C for 3 h. A control (0.7 ml) is removed, and 13 nmol unlabeled DFU is added to the remaining 1.3 ml of the mixture containing COX-2 and [^{14}C]-DFU. At timed intervals, 0.1 ml (in duplicate) is transferred to a Microcon-30 micro concentration device (Amicon), and the free inhibitor is separated from the enzyme-bound inhibitor by centrifugation at 14,000 g for 6 min at 4 °C. Buffer (0.1 ml) is added to the retentate and the centrifugation repeated. The filtrate and retentate are then removed and mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter.

An aliquot of purified COX-2 (1.0 nmol) is treated with 1.25 mol equivalents of inhibitor or with DMSO vehicle control and incubated at 20 °C for 1 h. The enzyme–inhibitor mixture is then transferred to a Pierce Microdialyzer 100 apparatus and dialyzed continuously against 2 l of buffer (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM phenol, 0.1 % octylglucoside) for 5 h at 22 °C during which aliquots are moved and frozen at −70 °C until assayed for cyclooxygenase activity by oxygen uptake as described above.

Recovery of Inhibitor from the COX-2-Inhibitor Complex

Purified COX-2 (0.79 nmol) is treated with 1.0 mol equivalent of inhibitor, and the mixture is incubated for 60 min at room temperature. The remaining activity at this time is 4 % that of a vehicle-treated control. The sample is then divided in two and the protein denaturated by treatment with four volumes of ethyl acetate/methanol/1 M citric acid (30:4:1). After extraction and centrifugation (10,000 g for 5 min), the organic layer is removed and the extraction

repeated. The two organic layers are combined and dried under N_2. The extract is dissolved in 10 µl of HPLC solvent mixture consisting of water/acetonitrile/acetic acid (50:41:0.1), and 50 µl is injected onto a Nova-Pak C-18 column (3.9 × 150 mm) and developed at 1 ml/min. The inhibitor is detected by absorption at 260 nm and eluted with a retention time of 6.6 min in this system. Control experiments for inhibitor recovery are performed with incubation of the inhibitor in the absence of enzyme and processing of the samples in an identical fashion before quantitation by HPLC.

Spectrophotometric Assay of Recombinant Human COX-2

Enzymatic activity of the purified COX-2 is measured using a chromogenic assay based on the oxidation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 (Copeland et al. 1994). The assay mixture (180 µl) contains 100 mM sodium phosphate, pH 6.5, 1 µM hematin, 1 mg/ml gelatin, 2–5 µg/ml of purified COX-2, and 4 µl of the test compound in DMSO. The assay is also performed in the presence of the detergent Genapol X-100 at a final concentration of 2 mM. The mixture is preincubated at room temperature (22 °C) for 15 min before the initiation of the enzymatic reaction by the addition of 20 µl of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hematin). For assays in the presence of Genapol, the arachidonic acid and TMPD solution is prepared in 50 % aqueous ethanol. The enzyme activity is measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed from the increase in absorbancy at 610 nm. A low rate of nonenzymatic oxidation is observed in the absence of COX-2 and is subtracted before the calculation of the percentage of inhibition.

Whole-Cell Assays with Transfected Chinese Hamster (CHO) Cells Expressing COX-1 and COX-2

Stably transfected CHO cells expressing human COX-1 and COX-2 are cultured and assayed for the production of PGE_2 after stimulation with arachidonic acid (Kargman et al. 1996). Cells (0.3×10^6 cells in 200 µl) are preincubated in HBSS containing 15 mM HEPES, pH 7.4, with 3 µl of the test drug or DMSO vehicle for 15 min at 37 °C before challenge with arachidonic acid. Cells are challenged for 15 min with an arachidonic acid solution (10 % ethanol in HBSS) to yield final concentrations of 10 µM arachidonic acid in the CHO[COX-2] assay and 0.5 µM arachidonic acid in the CHO[COX-1] assay. In the absence of addition of exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] are <30 pg $PGE_2/10^6$ cells. In the presence of 0.5 µM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] cells increase to 260–1,500 pg $PGE_2/10^6$ cells. After stimulation with 10 µM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-2] cells increase from <120 to 700–1,600 pg $PGE_2/10^6$ cells. Compounds are tested in eight concentrations in duplicate using threefold serial dilutions in DMSO. COX activity in the absence of test compounds is determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus PGE_2 levels in cells mock-challenged with ethanol vehicle.

Arachidonic acid-dependent production of PGE_2 is measured in both cell lines after addition of test drugs. Indomethacin shows similar IC_{50} values in both CHO[COX-1] and CHO[COX-2] cells, whereas specific COX-2 inhibitors show a 1,000-fold specificity.

Assays with Murine Macrophages

Mitchell et al. (1994), Hu et al. (2003), and Joo et al. (2004) used mouse peritoneal macrophages for evaluation of COX-2 inhibitors.

Adherent peritoneal macrophages were harvested from the peritoneal cells of male C5BL-6 J mice after intraperitoneal injection of brewer thioglycollate medium (5 ml/100 g body weight) for 3 days. The peritoneal cells obtained from three to four mice were mixed and seeded in 48-well culture cluster at a cell density of 1 × 109 cell/l in RPMI-1640 supplemented with 5 % newborn calf serum, penicillin, and streptomycin. After settlement for 2–3 h, nonadherent cells were washed by D-Hanks' balanced salt solution.

Then macrophages were cultured in RPMI-1640 without serum. Almost all of the adherent cells were macrophages as assessed by Giemsa staining. Cell viability was examined by trypan blue dye exclusion. All incubation procedures were performed with 5 % CO_2 in humidified air at 37 °C.

COX-1 Assay

Macrophages were incubated with test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with calcimycin 1 μmol/l for 1 h. The amount of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) in supernatants was measured by RIA according to the manufacturer's guide. The inhibitory ratio was calculated as

$$IR = \frac{(C_s - C_t)}{(C_s - C_c)}$$

C_s, C_t, and C_c refer to 6-keto-PGFα concentration in supernatants of calcimycin, test compound, and control groups, respectively.

COX-2 Assay

Macrophages were incubated with the test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with lipopolysaccharide (LPS) 1 mg/l for 9 h. The amount of PGE_2 in supernatants was measured by RIA. The inhibitory ratio was calculated using the same formula as in COX-1 assay section. C_s, C_t, and C_c refer to PGE_2 concentration in supernatants of LPS, test compound, and control groups, respectively.

Statistical analysis data were expressed as the mean ± SD of more than three independent experiments. Dose–inhibitory effect curves were fit through "uphill dose–response curves, variable slope" using Prism, GraphPad version 3.00:

$$Y = \frac{1}{1 + 10^{[(\log IC_{50} - X) \times \text{Hillslope}]}}.$$

Whole-Cell Assays with Osteosarcoma Cells (COX-2) and U937 Cells (COX-1)

The human osteosarcoma cell line has been shown to selectively express COX-2 by reverse transcription polymerase chain reaction and immunoblot analysis, whereas undifferentiated human lymphoma U937 cells selectively express COX-1. The production of PGE_2 by these cells after arachidonic acid challenge is used as an index of cellular COX-2 and COX-1 activity, respectively. Test substances are preincubated for 5–15 min with the cells under serum-free conditions (HBSS) before a 10-min stimulation with 10 μM arachidonic acid and measurement of PGE_2 production (Wong et al. 1997). COX activity in each cell line is defined as the difference in PGE_2 concentrations in samples incubated in the presence or absence of arachidonic acid.

Human Whole Blood Assay

For the COX-2 assay, fresh heparinized human whole blood is incubated with lipopolysaccharide from *E. coli* at 100 μg/ml and with 2 μl of vehicle or a test compound for 24 h at 37 °C (Brideau et al. 1996). PGE_2 levels in the plasma are measured using radioimmunoassay after deproteination. For the COX-1 assay, an aliquot of fresh blood is mixed with either DMSO or test compound and is allowed to clot for 1 h at 37 °C. TBX_2 levels in the serum are measured using an enzyme immunoassay after deproteination.

Modifications of the Method

Young et al. (1996) and Khanapure et al. (2003) used a similar assay to determine COX-1 and COX-2 enzyme activity in human whole blood. Human blood from non-fasted donors, who had not taken any aspirin or NSAIDs for 14 days, was collected in sodium heparin and distributed in 1 ml aliquots per well in a 24-well tissue culture plate. The plate was placed on a gently rotating platform shaker in a 5 % CO_2 incubator at 37 °C for 15 min. Test compounds were dissolved and diluted in dimethylsulfoxide (DMSO), and 1 μl of each dilution of test compound was added per well in duplicate wells. To induce COX-2, lipopolysaccharide (LPS) from *E. coli* was added at 10 μg/ml to appropriate wells 15 min after addition of the test compounds. For the stimulation of COX-1, the calcium ionophore A23187 was added to a final concentration of 25 μM to separate wells 4.5 h after the addition of the test compounds. At 30 min after addition of A23187 or

5 h after LPS addition, all incubations were terminated by cooling on ice and adding EGTA to a final concentration of 2 mM. The blood samples were then transferred to 15 ml polypropylene centrifuge tubes and centrifuged at 1,200 g for 10 min at 4 °C. Then, 100 µl of plasma was removed from each blood sample and added to 1 ml of methanol in a 15 ml polypropylene centrifuge tube, mixed vigorously, and stored overnight at −20 °C. The next day, the samples were centrifuged at 2,000 g for 10 min at 4 °C, and the supernatants were transferred to glass tubes and evaporated to dryness. After reconstitution with EIA buffer and appropriate dilution (2,000-fold for COX-1 and 500-fold for COX-2), the samples were assayed for thromboxane B2 using EIA kits (Cayman, Ann Arbor, Mich., USA) in duplicate wells.

Kalajdzic et al. (2002) showed that a preferential COX-2 inhibitor suppresses peroxisome proliferator-activated receptor induction of COX-2 gene expression in human synovial fibroblasts.

Berg et al. (1997) developed a cell assay system using the human erythroleukemic cell line HEL as a source for COX-1 and the human monocytic cell line Mono Mac 6 as a source for COX-2.

Kalgutkar et al. (2000) exploited biochemical differences between the COX isoforms to improve upon the selectivity of carboxylate-containing NSAIDs as COX-2 inhibitors.

Faust et al. (2003) recommended human peritoneal macrophages in culture as a model for studying inflammatory disorders in vitro.

Krause et al. (2003) reported that the NSAIDs indomethacin and diclofenac and a selective COX-2 inhibitor uncouple mitochondria in intact cells. To analyze the effects on energy metabolism of rat thymocytes, top-down elasticity analysis (Brand 1996, 1998; Ainscow and Brand 1999) was applied. Energy metabolism was conceptually divided into three blocks of reactions that generated and consumed the central intermediate mitochondrial membrane potential (ψ_m). The substrate oxidation subsystem encompassed all cellular catabolic reactions that provide the respiratory chain with its substrates NADH and succinate (e.g., glucose, fatty acid, and amino acid metabolism) and the electron transport chain itself, which together generate ψ_m. The ψ_m-consuming reactions were further divided into the subsystems proton leak and ATP turnover. The ATP turnover subsystem encompassed ATP synthesis by ATP synthase and subsequent ATP consumption by cellular pathways (e.g., ion pumps or protein synthesis). Manipulation of the biochemical properties of one block of reactions prompted a change of the intermediate ψ_m. The whole system evolved into a new steady state, which was determined by the kinetic responses of the other blocks to changes of ψ_m. Successive inhibition of one block of reactions permitted determination of these kinetic responses of the other blocks to changes of ψ_m. A comparison of the kinetic responses in the presence and absence of effectors (e.g., drugs) allowed for the identification of sites of actions.

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Influence of Cytokines

Induced Release of Cytokines (Interleukin-1α, IL-1β, IL-6, IL-8, and TNF-α) from Human White Blood Cells In Vitro

Purpose and Rationale

Cytokines represent a class of different, biologically highly potent peptides that are endogenously synthesized upon stimulation. They are involved in numerous cellular processes, such as inflammation, immunological responses, and many others. Cytokines are small protein released by cells and are important in cell signaling. Cytokines have a specific effect on communications between the cells or on the interactions between cells. Cytokines are produced by a broad variety of cells including immune cells like T lymphocytes, B lymphocytes, mast cells, macrophages, fibroblasts, endothelial cells, and various stromal cells. They play an important role in host response to inflammation, infection, sepsis, cancer, immune responses, etc.

Their broad pleiotropic biological activities are best characterized by their former classification as:

- B-cell activating and differentiating factor, endogenous pyrogen, osteoclast-activating factor, thymocyte proliferation factor, monocyte cell factor, and leukocyte endogenous factor (IL-1)
- Hepatocyte stimulatory factor, hybridoma growth factor, and hematopoietic cell stimulatory factor (IL-6)
- Neutrophil chemotactic factor and adhesion inhibitor (IL-8)
- Tumor necrosis factor (TNFalpha)

There are presently 18 cytokines with the name interleukin (IL) (Dinarello 2000). The concept of proinflammatory cytokines and anti-inflammatory cytokines (IL-4, IL-10, IL-13) is fundamental to cytokine biology and novel drug discovery strategies.

Blocking IL-1 or TNF has been highly successful in patients with rheumatoid arthritis, inflammatory bowel disease, or graft-versus-host disease.

The following procedure is used to detect compounds that interact with the lipopolysaccharide-induced cytokine release from human mononuclear blood cells. The cytokines measured are interleukins 1alpha, 1beta, 6 and 8, as well as TNFalpha.

Procedure

According to Böyum (1976) 10 ml of freshly prepared human citrated blood is diluted 1:1 with PM 16-buffer (Serva, Heidelberg, FRG), and underlayered with 15 ml Lymphoprep (Molter GmbH, Heidelberg, FRG), and subsequently

centrifuged at 20 °C with 400 g (Minifuge, Heraeus, Hanau, FRG) for 30 min. The cell fraction appearing as a white ring between the two phases is carefully removed by means of a syringe, diluted 1:1 (v/v) with PM 16 and again centrifuged for 15 min. The pellet is washed with 10 ml of RPMI 1640 (Gibco, Berlin, FRG), containing in addition 300 mg/l L-glutamine. The washed cell fraction is taken up in 1 ml RPMI 1640, containing in addition 300 mg/l L-glutamine, 25 mmol/l HEPES, 5 % FCS, and 100 IU/ml penicillin/streptomycin (Gibco). Using a cell counter (type IT, Coulter Diagnostics, Krefeld, FRG), the cell suspension which consists of about 90 % lymphocytes and 10 % monocytes is adjusted to approx. 5×10^9 cells/ml.

Synthesis and release of cytokines according to Tiku et al. (1986) is performed in 96-well microtiter plates. To 0.23 ml of the cell fraction, 500 ng LPS (lipopolysaccharide from *Salmonella abortusequi*, Sigma GmbH, Deisenhofen, FRG), dissolved in 0.01 ml dimethylsulfoxide/water (1:10, v/v), and the investigational drug, dissolved in 0.01 ml, are added. The cell suspension is now kept at 37 °C/5 % CO_2 in a common incubator. Incubation time is usually 20 h (for Il-6 and Il-8 only 4 or 1 h, respectively). The reaction is stopped by placing the microtiter plate into an ice bath. The plate is then centrifuged at 2,000 rpm for 2 min. The cytokine levels are determined in various aliquots of the supernatant using the appropriate ELISA kit, which is commercially available from several distributors.

Evaluation

The cytokine concentration in the sample is determined from appropriate calibration curves. The cytokine concentration is measured as a function of drug concentration and related to a control experiment without drug. In general, Hostacortin (10 to 0.1 µmol/l) is used as the standard compound.

Critical Assessment

Drugs interfering with LPS activation, biosynthesis, and cellular release of cytokines are to exhibit activity in these assay systems. Usually cell viability is not altered, as assessed by the lactate dehydrogenase test.

Modifications of the Method

Van der Pouw-Kraan et al. (1992) examined the regulation of interleukin (IL)-4 production by human peripheral blood T cells. Production of IL-4 as measured by ELISA was shown to be regulated differently from IL-2 and INF-γ (also measured by ELISA).

A proinflammatory role for interleukin-18 (IL-18) in rheumatoid arthritis was attributed by Gracie et al. (1999).

The role of the interleukin-6 family of cytokines in inflammatory arthritis and bone turnover was reviewed by Wong et al. (2003).

Kim et al. (2005) reported an inhibitory effect of luteolin, a flavonoid from *Lonicera japonica*, on tumor necrosis factor-α-induced IL-8 production in human colon epithelial cells.

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Flow Cytometric Analysis of Intracellular Cytokines

Purpose and Rationale

Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including size and granularity, as well as the expression of surface and intracellular markers defined by fluorescent antibodies. Fluorescent anti-cytokine and anti-chemokine monoclonal antibodies are very useful for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within mixed populations. Multicolor immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high-resolution method to identify the nature and frequency of cells which express particular cytokines (Sander et al. 1991, 1993; Jung et al. 1993).

Procedure

Cells and Cell Culture

Peripheral blood is obtained from healthy human volunteers. Mononuclear cells are isolated by Ficoll gradient centrifugation. For purification of T cells or further cell sorting, mononuclear cells are incubated with neuraminidase-treated sheep red blood cells and centrifuged over Ficoll. Erythrocytes are eliminated by ammonium chloride lysis. For isolation of memory cells or naive cells, T cells are incubated with a cocktail of antibodies containing anti-CD16, anti-CD56, anti-CD20, anti-CD14 (anti-CD8), and anti-CD45RA or anti-CD45R0. Cell sorting is done with the magnetic cell sorter (MACS) according to Abt et al. (1989) and Miltenyi et al. (1990) using rat anti-mouse IgG1 or Ig2a antibodies labeled with superparamagnetic beads (Miltenyi, Bergisch Gladbach, Germany). Depleted cells are highly enriched with $CD4^+(CD3^+)CD45R0^+$ or $CD4^+(CD3^+)CD45R0^-CD45RA^+$ cells (>95 %) and are referred to as memory cells and naive cells

($CD4^+CD45R0^-CD45RA^+$), respectively. Only depleted cells are used for experiments.

Cells (2×10^5/100 ml) are cultured in 96-well flat bottom plates for various periods of time at 37 °C and 8 % CO_2 in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} M mercaptoethanol, and 10 % AB serum. Cells are stimulated with phorbol 12-myristate 13-acetate 1–10 ng/ml + 1 µM ionomycin, phytohemagglutinin 2.4 µg/ml, or phytohemagglutinin 2.4 µg/ml + phorbol 12-myristate 13-acetate 1 ng/ml in the presence or absence of 3 µM monensin (Sigma).

Staining

Cultured cells are washed twice in Hanks' balanced solution (HBSS) and then fixed in ice-cold HBSS containing 4 % paraformaldehyde for 10 min. After two further washes in HBSS, the cells are resuspended to 2×10^5 in 300 µl HBSS containing 0.1 % saponin, 10 % AB serum, 100 µg/ml goat IgG, and 0.01 M HEPES buffer. After 10 min, the cells are spun down, and cytokine-specific antibodies diluted in HBSS with 0.1 % saponin and 0.1 M HEPES buffer (saponin buffer) are added at a concentration of 1 µg/ml for 30 min at room temperature. Cells are washed twice in saponin buffer and subsequently incubated with isotype-specific second-step antibodies in a concentration ranging from 0.5 to 5 µg/ml for 20 min in the dark. Cells are washed in saponin buffer and stained with streptavidin conjugates or in the case of surface staining incubated with 200 µg/ml mouse IgG diluted in saponin buffer for 15 min. After subsequent washing in saponin buffer, cells are washed twice in HBSS and stained for 20 min with different antibodies in order to determine their surface phenotype. As the last step, cells are washed in HBSS.

Flow Cytometry

A FACScan flow cytometer (Becton Dickinson, Mountain View, USA) equipped with a 15 mW argon ion laser and filter settings for fluorescein-isothiocyanate (530 nm), phycoerythrin (585 nm), and TRI-Color (Medac, Hamburg, Germany) or PerCP (Becton Dickinson, USA) emitting in the deep red (>650 nm) is used.

Evaluation

Five thousand to ten thousand cells are computed in list mode and analyzed using the FACScan research software (Becton Dickinson).

Modifications of the Method

Slauson et al. (1999) combined the analytical power of flow cytometry with mitogen-driven, whole blood lymphocyte activation and proliferation assays to investigate the in vitro mechanism of action of malononitrilamides.

Protocols for immunofluorescent staining of intracellular cytokines for flow cytometric analysis are provided by BD Pharmingen, Life Science Research Europe, Heidelberg, Germany.

Ashcroft and Lopez (2000) highlighted the opportunities in high-throughput flow cytometry (HTFC) which are opened by commercial high-speed machines. The specifications of these machines are cell analysis rates over 100,000 cells/s and cell sorting rates of 55,000 cells/s with high purity.

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Screening for Interleukin-1 Antagonists

Purpose and Rationale

Interleukin-1α and -1β are potent regulators of inflammatory processes. The naturally occurring IL-1 receptor antagonist (IL-1ra) is effective in vitro and in vivo in modulating biological responses to IL-1 (Carter et al. 1990; Hannum et al. 1990; Schreuder et al. 1995). Using a combination of anion exchange, gel filtration, and reverse-phase HPLC, three species of native IL-1ra were identified. An unglycosylated, intracellular isoform is designated as icIL-1ra (Lennard 1995; Arend 1991; Arend et al. 1998).

A cell-free, non-isotopic assay has been developed to discover molecules that compete with the natural ligands for binding to the active sites of the type-I IL-1 receptor. The key reagents are the IL-1 receptor antagonist, a recombinant soluble form of the receptor (sIL-1R), and a specific anti-sIL-1R non-neutralizing monoclonal antibody (Sarrubi et al. 1996).

Procedure

Proteins

The extracellular portion of the type-I IL-1 receptor (sIL-1R) is expressed on the membrane of Chinese hamster ovary cells using a phosphatidylinositol glycan linkage (PIG-tail). Its expression, cleavage with phosphoinositol-specific phospholipase C, and purification are performed according to Whitehorn et al. (1995).

The three IL-1 ligands are expressed in *Escherichia coli* using synthetic genes (Dower et al. 1989; Yanosfky and Zurawski 1990). The purification of IL-1ra and IL-β is accomplished according to Schreuder et al. (1995) and Yem et al. (1988).

IL-1α is purified as follows: *E. coli* cell sonicates are precipitated with 2 M ammonium sulfate, and the pellet is resuspended in TE (25 mM Tris/HCl, pH 8.9, 1 mM EDTA), dialyzed against the same buffer, and loaded on a DEAE-Sepharose column equilibrated with TE. The protein is eluted with a linear NaCl gradient to 300 mM. Ammonium sulfate to 0.8 M is added to the IL-1α-containing fractions which are loaded onto a phenyl-Sepharose column equilibrated with TE containing 0.8 M ammonium sulfate. The elution is performed with a linear gradient to TE with no salt. IL-1α-containing fractions are concentrated and chromatographed on a Sephacryl S-200 column in PBS (phosphate-buffered saline: 20 mM sodium phosphate, pH 7.3, 150 mM sodium chloride).

Fluorescein-labeled IL-1α is obtained by incubating 1 mg/ml IL-1α with 1 mg/ml fluorescein isothiocyanate in PBS for 2 h at room temperature in the dark. The reaction solution is passed directly over a G-25 column (Pharmacia) equilibrated with PBS to remove unreacted fluorescein isothiocyanate.

The monoclonal antibody Mab79 is used as direct dilutions ($1:10^5$–10^6) of ascitic fluid in PBSA (PBS containing 0.3 % bovine serum albumin). Horseradish peroxidase-linked anti-mouse IgG polyclonal antibody is used.

Protein concentrations are determined using the Bio-Rad protein assay kit, based on the dye-binding procedure according to Bradford (1976). BSA is used as reference protein.

Immobilized-Ligand IL-1 Receptor Binding Assay

Essentially the same procedure can be used for both manual and automated versions of the assay, with all steps and incubations performed at room temperature. In the automated assay a Beckman Biomek 1,000 workstation was used for all steps, from coating to spectrophotometric measurements.

Ligand immobilization is obtained by incubation of 3.6 µg/ml IL-1ra in 50 µl PBS in flat-bottomed culture-treated microplate wells, equivalent to 10 pmol/well of IL-1ra. After overnight

incubation microplates are emptied, and 250 µl/well of 3 % BSA in PBS is added to block unreacted sites. After 2 h of incubation and three washes with an excess of PBS, the ligand-coated microplates are ready for the receptor binding reaction.

In separate microplates with U-shaped wells, 12 µl of samples (containing up to 50 % DMSO or DMF) or controls (the same solution without compound) is mixed with 48 µl of 150 pM sIL-1R in PBSA. Then 50 µl of these mixtures is transferred to the IL-1ra-coated plates (equivalent to 6 fmol/well of sIL-1R) and incubated for 2 h. Microplates are then washed twice with PBS, and 50 µl of 1:500,000 dilution of Mab79 ascitic fluid in PBSA is added to each well. After 1 h of incubation, 25 µl of 1:100 dilution of HRP-labeled anti-mouse IgG in PBSA is added and the incubation prolonged for an additional hour. Plates are finally washed four times with PBS, and bound peroxidase activity is measured spectrophotometrically, using either *o*-phenylenediamine (OPD) or tetramethylbenzidine (TMB) as substrate. In the first case, 150 µl of 1 mg/ml OPD in 0.1 M citric acid, pH 5.0, containing 0.03 % of a 35 % solution of hydroperoxide is added, and, after color development, the reaction is stopped with 50 µl of 4.5 M sulfuric acid. Alternatively, 100 µl of 0.1 mg/ml TMB in 25 mM citric acid and 50 mM sodium phosphate, containing 0.02 % hydrogen peroxide (35 % solution), is added, and the reaction is stopped with 50 ml of 2.5 M sulfuric acid.

Evaluation

Absorbance (at 492 nm for OPD and 450 nm for TMB) is measured using either a Titertek microplate reader (for the manual procedure) or directly by the Biomek 1,000 workstation (in the automated version). IC_{50} values can be calculated from dose–response curves.

Critical Assessment of the Method

Since no cells or cell membranes are used, the assay is very robust, with no interference from membrane-perturbing agents, and has high resistance to the organic solvent normally used to resuspend compounds of chemical libraries.

Modifications of the Method

High-affinity type I interleukin-1 receptor antagonists were discovered by screening recombinant peptide libraries (Yanosfky et al. 1996).

Akeson et al. (1996a) developed an ex vivo method for studying inflammation in cynomolgus monkeys using whole blood for analysis of IL-1 antagonists administered in vivo. Animals were given an i.v. infusion of IL-1ra, and blood samples were taken pre-infusion and during the infusion. The samples were incubated with or without IL-1β and the subsequent ex vivo induction of IL-6 determined. This allows the analysis of the in vivo efficacy of antagonists without exposing the animals to IL-1.

A novel low-molecular-weight antagonist, selectively binding the type I IL-1 receptor and blocking the in vivo responses to IL-1, was described by Akeson et al. (1996b).

Evaluation of the IL-1 receptor antagonist IL-1ra in a rodent abscess model of host resistance was published by Colagiovanni and Shopp (1996).

Blocking monoclonal antibodies (mAbs) specific to mouse IL-1 receptor antagonist (IL-1ra) were prepared by immunizing Armenian hamsters with recombinant mouse IL-1ra by Fujioka et al. (1995). A sensitive and specific ELISA against mouse IL-1ra was established.

Miesel et al. (1995) tested the antiarthritic reactivity of the IL-1 receptor antagonist IL-1ra in male DBA/1xB10A(4R) mice with arthritis induced by intraplantar injection of potassium peroxochromate. Then 3 µmol/kg K_3CrO_8 was administered topically into the left hind paws and 1 h after the induction of arthritis, and 2 mg/kg IL-1ra was administered intraperitoneally, which was repeated on day 2. An arthritis index was determined daily.

Nakae et al. (2003) found that IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist.

Redlich et al. (2003) reviewed rheumatoid arthritis therapy after tumor necrosis factor and interleukin-1 blockade.

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Inhibition of Interleukin-1β-Converting Enzyme (ICE)

Purpose and Rationale

Programmed cell death (apoptosis) is effected through a cascade of intracellular proteases known as caspases (Alnemri et al. 1996). The interleukin-1β-converting enzyme (ICE), alternatively known as caspase-1, was the first such protein identified on the basis of its sequence homology to the proapoptotic *Caenorhabditis elegans* gene product, ced-3 (Yuan et al. 1993). The caspase family includes 10 reported human homologues of ICE. By sequence homology, comparisons between three caspase subfamilies have been identified. The ICE subfamily includes three caspases: ICE, TX (caspase-4), and TY (caspase-5). The CPP32 subfamily includes CPP32 (caspase-3), CMH-1 (caspase-7), and MCH-2 (caspase-6). The third caspase subfamily includes ICH-1 (caspase-2), FLICE (caspase-8), and caspase-9 and caspase-10.

ICE processes pro-IL-1β to yield active IL-1β, which plays a pivotal role in inflammatory cell activation (Dinarello 1996) and is known to inhibit the expression of apoptosis (Tatsuda et al. 1996). Inhibition of IL-1β formation is an approach for the treatment of inflammatory disorders such as rheumatoid arthritis. Livingstone (1997) presented a review on in vitro and in vivo studies of peptidyl ICE inhibitors.

Procedure

Neutrophil Isolation

Neutrophils are isolated from healthy volunteers by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient (Lee et al. 1993). Isolated neutrophils are resuspended in polypropylene tubes at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10 % FCS, 1 % glutamine, and 1 % penicillin/streptomycin solution. Neutrophil purity is assessed by size and granularity on flow cytometry.

Quantification of Apoptosis

Neutrophil apoptosis is quantified by flow cytometry as the percentage of cells with hypodiploid DNA (Nicoletti et al. 1991). Cells are centrifuged at 200 g for 10 min, gently resuspended in 500 µl of hypotonic fluorochrome solution (50 µg/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, and 0.1 % Triton X-100), and stored in the dark at 4 °C for 3–4 h before analysis using a Coulter Epics XL-MCL cytofluorometer. A minimum of 5,000 events are collected and analyzed. Apoptotic nuclei are distinguished from normal neutrophil nuclei by their hypodiploid DNA; neutrophil debris is excluded from analysis by raising the forward threshold. Apoptotic nuclei appear as a broad hypodiploid DNA peak which is easily discernible from the narrow peak of cells with normal diploid DNA content. Apoptosis is assessed at 24 h after treatment.

Assay of Caspase-1 Activity

Cell lysates are prepared from the membrane fraction of 20×10^6 neutrophils following experimental manipulation. Aliquots of the lysates (10 µl) are diluted in an assay buffer 100 mM HEPES (pH 7.4), 10 % sucrose, and 0.1% 3-([[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate) containing 20 µM Ac-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin
(Calbiochem) and then incubated for 45 min at room temperature. The release of 7-amino-4-methylcoumarin is detected by continuous

measurement using a Perkin-Elmer LS50 luminescence spectrometer with an excitation of 380 nm and an emission slit at 460 nm. Specific ICE (caspase-1) activity is measured as pmol/s per milligram of protein.

Evaluation

Individual experiments are repeated a minimum of four times; results are expressed as the mean ± SD. Analysis is performed using the Student's *t*-test or ANOVA with Scheffè's correction.

Modifications of the Method

Norman et al. (1997) found that the severity and mortality of experimental pancreatitis are dependent on interleukin-1-converting enzyme.

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Nuclear Factor-κB

General Considerations

Nuclear factor kappa B (NF-κB) is an inducible transcription factor of the Rel family, sequestered in the cytoplasm by the IκB family of proteins. NF-κB exists in several dimeric forms, but the p50/p65 heterodimer is the predominant one. Activation of NF-κB by a range of physical, chemical, and biological stimuli leads to phosphorylation and proteasome-dependent degradation of IκB, leading to the release of free NF-κB. This free NF-κB then binds to its target site (κB sites in the DNA) to initiate transcription. This transcription is involved in a number of diseases including cancer, AIDS, autoimmune diseases, and inflammatory disorders. The nuclear factor-κB is essential for the transcriptional regulation of the proinflammatory cytokines IL-1, IL-6, and IL-8 and tumor necrosis factor-α. NF-κB is also the target for glucocorticoid-mediated IL-8 repression. Reduction–oxidation (redox) regulation is implicated in the activation of NF-κB (Mukaida et al. 1994; Aupperle et al. 1999; Christman et al. 2000a, b; Nichols et al. 2001; D'Acquisto et al. 2002; Nishi et al. 2002; Palanki 2002; Tian et al. 2002; Heynink et al. 2003; Aggarwal et al. 2004; Pande and Ramos 2003, 2005; Kaltschmidt et al. 2005). The IKK complex, as a critical activator of NF-κB function, consists of a core of three subunits, two of which, namely, IKKα and IKKβ, contain functional kinase domains and are capable of phosphorylating IκB at specific N-terminal residues to initiate its ubiquitination. In contrast, the third core subunit of the IKK complex, called NEMO (also known as IKKγ or IKKAP), is a non-catalytic component that functions as a key regulator of IKK activity (Gosh and Karin 2002; Katin et al. 2004).

Studying joint erosion in rheumatoid arthritis, O'Gradaigh et al. (2003) found that interactions between tumor necrosis factor-α, interleukin, and receptor activator of nuclear factor-κB ligand (RANKL) regulate osteoclasts.

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Inhibition of Nuclear Factor-κB

Purpose and Rationale

Several authors studied the inhibition of NF-κB by compounds.

Staal et al. (1990) found that intracellular thiols regulate activation of nuclear factor-κB and transcription of human immunodeficiency virus. Schrenk et al. (1992) reported dithiocarbamates as potent inhibitors of nuclear factor-κB activation in intact cells. Natarajan et al. (1996) described caffeic acid phenyl ester as a potent and specific inhibitor of activation of nuclear transcription factor NF-κB. Geng et al. (1997) reported that S-allyl cysteine inhibits the activation of nuclear factor-κB in human T cells. Hiramoto et al. (1998) described nuclear-targeted suppression of NF-κB by a quinone derivative. Ichiyama et al. (1999) found the

inhibition of peripheral NF-κB activation by a central action of α-melanocyte-stimulating hormone. Castrillo et al. (2001) described the inhibition of the nuclear factor κB pathway by tetracyclic kaurene diterpenes in macrophages with specific effects on NF-κB-inducing kinase activity and on the coordinated activation of ERK and p38 MAPK. Kang et al. (2001) reported that genistein prevents nuclear factor kappa B activation and acute lung injury induced by intratracheal treatment of rats with lipopolysaccharide. Lee et al. (2002) found that kamebakaurin, a kaurane diterpene, inhibits NF-κB by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF-κB target genes. Palanki et al. (2002) reported the structure–activity relationship studies of ethyl 2-[3-methyl-2,5-dioxo (3-pyrrolinyl) amino]-4-(trifluoromethyl)pyrimidine-5-carboxylate, an inhibitor or AP-1- and NF-κB-mediated gene expression. Kim et al. (2004) found that triptolide, a natural compound extracted from the Chinese herb *Tripterygium wilfordii*, inhibits murine-inducible nitric synthase expression by downregulating lipopolysaccharide-induced activity of nuclear factor-κB and c-Jun NH_2-terminal kinase. Jancso et al. (2005) studied the effect of acetylsalicylic acid on nuclear factor-κB activation and on late preconditioning against infarction of the myocardium. Kunsch et al. (2005) described redox-sensitive inflammatory gene expression of AGIX-4207, an antioxidant and anti-inflammatory compound.

Tse et al. (2005) found that honokiol, a small molecular weight lignan isolated from *Magnolia officinalis*, inhibits tumor necrosis factor-α-stimulated NF-κB activation and NF-κB-regulated gene expression through suppression of inhibitor κB kinase (IKK) activation.

Procedure

Honokiol was dissolved in DMSO as a 100 mM stock solution and stored at −20 °C.

Cell Culture

The cell lines used in this experiment were obtained from American Type Culture Collection (Manassas, Va., USA). U937 and HL-60 cells were grown in RPMI-1640 medium containing 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, NY, USA) at 37 °C in humidified 5 % CO_2 atmosphere. MCF-7 and HeLa cells were cultured in Eagles' minimum essential medium containing 10 % fetal bovine serum under the same condition.

Electrophoretic Mobility Shift Assay (EMSA)

For the electrophoretic mobility shift assay according to Chaturvedi et al. (2000), equal quantities of nuclear protein (5 μg) from each sample were incubated with radiolabeled gel shift oligonucleotides for 15 min at 37 °C and then resolved on a non-denaturing 5 % (w/v) polyacrylamide gel. The gel was dried onto 3 MM blotting paper and used to expose X-ray film for overnight at −70 °C. For supershift assays, 1 μl of antiserum recognizing each of the NF-κB subunits was added to the EMSA reaction 30 min before electrophoresis.

Western Blot Analysis

To obtain the whole-cell lysates, samples containing 1×10^7 cells were pelleted, washed twice with ice-cold PBS, and then lysed in 150 μl of modified RIPA buffer [50 mM Tris–Cl, 1 % (v/v) NP-40, 0.35 % (w/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4] supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A for 20 min at 4 °C. Supernatants after centrifugation at 14,000 g for 15 min at 4 °C were collected. Alternatively, cytoplasmic extracts were prepared. Samples containing 30–50 μg of protein were separated on SDS-polyacrylamide gel and then transferred onto nitrocellulose membrane (0.45 μm, Bio-Rad). Membranes were immunoblotted with primary antibodies and followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000) and visualized by ECL (Amersham Biosciences) according to the manufacturer's instructions.

IKK Assay

Whole-cell lysates (500 mg) were collected in modified RIPA buffer without sodium

deoxycholate, and cellular debris was removed by high-speed centrifugation. Lysates were precleared by incubation with 0.25 µg of the appropriate control IgG together with 20 µl of protein A/G plus (25 %, v/v) agarose conjugate for 30 min at 4 °C, followed by centrifugation. Supernatants were then incubated with 1 µg of anti-IKKα/β for 2 h at 4 °C, and then 20 µl of protein A/G plus agarose was added and incubated at 4 °C on a rocker platform overnight. After several washes with IP buffer and PBS, beads containing IKKα/β were incubated with 0.5 µg GST-Iκ $B\alpha$ substrate and 200 µM ATP in 20 µl kinase buffer (50 mM Tris–Cl, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.5 mM DTT, 1 mM benzamidine, 10 µg/ml aprotinin, and 1 µg/ml leupeptin) at 30 °C for 30 min. Kinase reactions were stopped by the addition of 5 µl 5 × Laemmli's loading buffer and heated at 100 °C for 5 min. The samples were resolved by 8 % SDS-PAGE, electro-transferred to nitrocellulose membrane, and probed with anti-phosphor-Iκ $B\alpha$ (Ser^{32}) antibody (1:1,000). Membranes were re-probed with anti-IKK to ensure equal loading and the presence of total IKK protein.

Plasmids, Transfection, and NF-κB-Dependent Luciferase Reporter Assay

To measure the effect of honokiol on TNF-α-induced NF-κB-dependent gene reporter transcription, HeLa cells were seeded into 24-well plates at a density 1.6×10^5 cells/well for 24 h. Subsequently, cells were transiently transfected with p3EnhConA-Luc or pControl-Luc (0.75 µg) using Lipofectamine 2000 (Invitrogen). To normalize the transfection efficiency, cells were co-transfected with 0.25 µg of β-galactosidase control vector. After overnight incubation, cells were pretreated with honokiol for 12 h following by 5 ng/ml TNF-α for 15 h and then harvested with 1 × reporter lysis buffer (Promega, Madison, Wis., USA). Relative luciferase activity was measured with a Bright-GLO luciferase assay system using POLARStar OPTIMA luminometer (BMG Labtechnologies). Luciferase activity was normalized with β-galactosidase activity, as measured by the Beta-GLO luciferase assay system according to the manufacturer's instructions.

To measure the effect of honokiol on NF-κB-dependent gene reporter transcription induced by various kinases, HeLa cells were transfected with p3EnhConA-Luc and β-galactosidase control vector together with 0.2 µg of expression vectors. After 5 h of incubation, cells were treated with honokiol for 24 h and then harvested and assayed as described above.

Evaluation

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Two compounds (A and B) were considered enhancing each other's actions if the effect of the combined treatment (AB) was larger than the sum of their individual effects (AB > A + B) after subtraction of the respective background control values.

Modifications of the Method

Mortellaro et al. (1999) reported that the immunosuppressive drug PNU156804 blocks IL-2-dependent proliferation and NF-κB and AP-1 activation in human primary T lymphocytes.

Spencer et al. (1999) found in murine NIH3T3 fibroblasts and primate COS-7 cells that taxol selectively blocks microtubule-dependent NF-κB activation by phorbol ester via inhibition of Iκ $B\alpha$ phosphorylation and degradation.

Yan and Polk (1999) reported that aminosalicylic acid inhibits IκB kinase α phosphorylation of Iκ $B\alpha$ in mouse intestinal epithelial cells.

Acarin et al. (2000) found that oral administration of the anti-inflammatory substance triflusal results in the downregulation of constitutive transcription factor NF-κB in the postnatal rat brain.

Eberhardt et al. (2002) studied the involvement of nuclear factor-κB and Ets transcription factors in glucocorticoid-mediated suppression of cytokine-induced matrix metalloprotease-9 expression in rat mesangial cells.

Kang et al. (2002) showed that inhaled nitric oxide attenuated acute lipopolysaccharide-induced lung injury in rabbits via inhibition of nuclear factor-κB.

Macotela et al. (2002) found on rat pulmonary fibroblasts that 16 K prolactin induces NF-κB activation.

Roshak et al. (2002) reported small-molecule inhibitors of NF-κB for the treatment of inflammatory joint disease.

Burke et al. (2003) found that BMS-345541 is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice.

Castro et al. (2003) described β-carbolines as inhibitors of the NF-κB kinase.

Clarke et al. (2003) reported that two distinct phases of virus-induced nuclear factor-κB regulation enhance tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in virus-infected cells.

Murata et al. (2003) described discovery of selective IKK-β serine–threonine protein kinase inhibitors.

Yadav et al. (2003) reported that a diarylheptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, P44/42, and transcription factor nuclear factor-κB.

HMG-CoA reductase inhibitors (statins) inhibited the binding of nuclear proteins to both NF-κB and AP-1 DNA consensus oligonucleotides in human endothelial and vascular smooth muscle cells as assessed by EMSA (Dichtl et al. 2003).

Gupta et al. (2004) discussed the essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor-*kappa*B and induction of apoptosis.

Mühlbauer et al. (2004) studied differential effects of deoxycholic acid and taurodeoxycholic acid on NF-κB signal transduction and IL-8 gene expression in human colonic epithelial cells.

Syrovets et al. (2005) found that acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF-α induction by direct interaction with IκB kinases.

Eberhardt et al. (2005) found that dissociated glucocorticoids equipotently inhibit cytokine- and cAMP-induced matrix-degrading proteases in rat mesangial cells.

Matsubara et al. (2005) found that a histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine production through inhibition of Ca^{2+}-dependent protein kinase C and Raf/MEK/ERK and IKK/Iκ B/NF-κB signal cascades.

Mendoza-Milla et al. (2005) reported that NF-κB activation but not PIK/Akt is required for dexamethasone-dependent protection against TNF-α cytotoxicity in L929 cells.

Appendix

Electrophoretic Mobility Shift Assay (EMSA)

Most papers mentioned above used the electrophoretic mobility shift assay (EMSA) for determination of nuclear factor-κB. This method is one of the most sensitive ones for studying the DNA-binding properties of a protein. It can be used to deduce the binding parameters and relative affinities of a protein for one or more DNA sites or for comparing the affinities of different proteins to the same sites (Fried 1989). It is also useful for studying higher-order complexes containing several proteins, observed as a "supershift assay." EMSA can also be used to study protein- or sequence-dependent DNA bending (Crothers et al. 1991).

In an EMSA, or simple "gel shift," a ^{32}P-labeled DNA fragment containing a specific DNA site is incubated with a candidate DNA-binding protein. The protein–DNA complexes are separated from free (unbound) DNA by electrophoresis through a non-denaturing polyacrylamide gel. The protein retards the mobility of the DNA fragments to which it binds; thus, the free DNA migrates faster through the gel than does the DNA–protein complex. An image of the gel reveals the positions of the free and bound ^{32}P-labeled DNA.

A protocol of the detailed procedure was described by Carey and Smale (2000).

Procedure

1. Prepare a 40 ml 4.5 % native acrylamide gel (using 1–1.5 mm spacers)

Acrylamide mix (30 %:29:1 acrylamide/bisacrylamide)	6 ml
5 × Tris–borate–EDTA (TBE) buffer	4 ml
Glycerol (20 % vol/vol)	2 ml
Water	28 ml
Ammonium persulfate (10 % solution in water)	300 µl
N,N,N,N-Tetramethylenediamine (add just before pouring the gel)	30 µl

Pre-run the gel for 2 h at 10 mA.

2. Set up binding reactions in 0.5-ml siliconized microcentrifuge tubes.

Recombinant protein (0.5–100 ng)	1.00 µl
^{32}P-labeled DNA template (ideally 1 fmol)	1.00 µl
Poly(dI:dC) (1 µg/µl)	0.20 µl
Dimethylsulfoxide (0.1 M)	0.10 µl
$MgCl_2$ (0.1 M)	0.75 µl
Buffer D	6.70 µl
Water	to 10 µl

Buffer D is 20 mM HEPES-KOH (pH 7.9), 20 % glycerol (vol/vol), 0.2 mM EDTA, 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM DTT. Add PMSF and DTT just before use.

3. Incubate the reaction at 30 °C (incubate at 15–25 °C or on ice for crude extracts) for 1 h.
4. Load the samples directly (with no dye) onto gel. Carefully layer the mix onto the bottom of the well and observe the schlieren line form at the glycerol–buffer interface.
5. Run the gel for desired time at 10 mA; for a 30-bp fragment, allow the bromophenol blue dye to migrate about two-thirds of the way down the gel.
6. When the electrophoresis run is complete, carefully pour out the buffer into the sink and remove the gel from the apparatus. Remove the comb and split the plates, leaving the gel attached to one plate.
7. (Optional) Fix the gel in gel-fixing solution (200 ml methanol, 100 ml acetic acid, 700 ml water) at 15–25 °C for 15 min.
8. Place the gel on two sheets of Whatman 3MM paper. Cover the other side of the gel with Saran Wrap and dry on a gel dryer at 70 °C for 1 h.
9. Expose the gel to autoradiography film or to phosphorimager screen overnight.

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TNF-α Antagonism

General Considerations

TNF-α has been cloned and identified by Beutler et al. (1985). It is primarily produced in macrophages, lymphocytes, neutrophils, endothelial

cells, keratinocytes, and fibroblasts during acute inflammatory reactions. Tumor necrosis factors or the TNF family refer to a group of cytokines. This cytokine is mainly secreted by macrophages and can cause cell death or apoptosis. The first two members of the family are tumor necrosis factors (TNF), formerly known as TNF-α or TNF-alpha (cachectin) and tumor necrosis factor-beta (TNF-β) (lymphotoxin-alpha). These cytokines can bind to and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR and are involved in the regulation of a wide spectrum of various cellular biological processes including apoptosis, coagulation, differentiation, cell proliferation, differentiation, apoptosis, lipid metabolism, etc. They are known for their involvement in a variety of diseases including autoimmune disease, cancer, and insulin resistance. TNF-α is a member of a large family of proteins and receptors that are involved in immune regulation such as kinases, including nuclear factor kappa B (NF-κ B), p38 MAP kinase, JUN kinases, and others. Therefore, it is a therapeutic target for immune-mediated inflammatory diseases (Pfizenmaier et al. 1996; Van Deventer 1997; Rath and Aggarwal 1999; Feldmann et al. 2001; Furst et al. 2001; Taylor 2001; Doggrell 2002; Braun et al. 2003; Sharma and Anker 2002; Louie et al. 2003; Nanes 2003; Peng et al. 2003; Chen et al. 2003; Taylor et al. 2004; Gupta and Gollapudi 2005; Pfeifer et al. 2006; Reber et al. 2006; Wagner and Laufer 2006). This may be achieved by small molecular anti-cytokine agents inhibiting cytokine production, which target p38 mitogen-activated protein (MAP) kinase, TNF-α-converting enzyme (TACE), or IL-1β-converting enzyme (ICE).

Several so-called biologicals are in clinical use:

Etanercept (Enbrel), a fully human soluble TNF receptor fusion protein consisting of the extracellular ligand-binding domain of the 75-kDa receptor for human tumor necrosis factor-α and the constant portion of human IgG1 (Jarvis and Faulds 1999; Pugsley 2001; Scallon et al. 2002; Agnholt et al. 2003; Cole and Rabasseda 2004; Goffe 2004; Moe et al. 2004; Vallejo et al. 2005). The compound has been approved for treatment of psoriasis, psoriatic arthritis, ankylosing spondylitis, and rheumatoid arthritis.

Infliximab (Remicade), a chimeric antihuman TNF-α monoclonal antibody (Scallon et al. 2002; Agnholt et al. 2003; Di Sabatino et al. 2004; Wagner et al. 2004; Panaccione et al. 2005; Shen et al. 2005; Pfeifer et al. 2006). The compound is used for treatment of rheumatoid arthritis and Crohn's disease.

Adalimumab (HUMIRA), a recombinant human antihuman TNF-α monoclonal antibody (Gordon et al. 2005; Aggarwal et al. 2006; Scheinfeld 2006; Shen et al. 2006). The compound is used for treatment of rheumatoid arthritis and psoriatic arthritis.

Imatinib mesylate (STI571, Gleevec), a kinase inhibitor of TNF-α production (Kilic et al. 2000; Traxler et al. 2001; Dietz et al. 2004; Kaelin 2004; Lassila et al. 2005; Wolf et al. 2005; Adcock et al. 2006). The compound has been found to be active in the treatment of chronic myelogenous leukemia, gastrointestinal stromal tumors, eosinophilic disorders, and systemic mast cell disease.

Omalizumab (Xolair), a recombinant humanized monoclonal antibody which specifically binds the Cε3 domain of IgE, the site of high-affinity IgE receptor binding (Easthope and Jarvis 2001; Anonymous 2002; Johansson et al. 2002; Davis 2004; Richards et al. 2004; Belliveau 2005; D'Amato 2006). The compound is used for treatment of bronchial asthma and allergic rhinitis.

Anakinra (Kineret) is a specific recombinant human interleukin-1 receptor antagonist that differs from naturally occurring IL-1 receptor antagonist by the presence of a methionine group (Cvetkovic and Keating 2002; Fleischmann et al. 2004; Le and Abbenante 2005; Waugh and Perry 2005). The compound is effective in patients with active rheumatoid arthritis, either when given alone or in combination with methotrexate.

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Inhibition of TNF-α Release

Purpose and Rationale

There are two distinct types of tumor necrosis factors, TNF-alpha (cachectin) and TNF-beta (lymphotoxin), with biological activities going beyond the necrosis of tumor cells. Some of the known activities include the induction of interleukin 1, activation of PMNs, modulation of endothelial cell functions, and augmentation of specific immune functions. The complex sequence of hemodynamic and metabolic collapse, which leads to shock and death during lethal endotoxinemia, appears to represent the response of the infected host to the acute, systemic release of TNF-alpha. Thus, drugs that antagonize the activity of this mediator could be of clinical value in combating its fatal effects.

Procedure

Twenty hours before the initiation of the experiments, L 929 cells are harvested from stock cultures and are plated in 96-well culture plates (2×10^4 cells/well) and incubated at 37 °C and 5 % CO_2 in air. For each group 6 wells are set up. The cells are then preincubated for 30 min with test substances or solvent before TNF-alpha is added (between 1 and 10 IU/well). After an additional incubation time of 20 h, the culture plates are flicked out, and the remaining living cells are lysed by the addition of bidistilled water (100 µl). After 30 min incubation at room temperature, 100 µl of LDH reagent is given to each culture well. After 15 min, the enzyme activity is determined photometrically at 490 nm.

Evaluation

The percent inhibition is calculated according to the formula:

$$\%\ \text{inhibition} = 100\ \% - \frac{\text{ext. test group} - \text{ext. spontaneous lysis}}{\text{ext. positive control} - \text{ext. spontaneous lysis}}.$$

The positive control is the group which receives vehicle and TNF-alpha. The spontaneous lysis is based on cultures which receive vehicle without TNF-alpha.

Modifications of the Method

Maloff and Delmendo (1991) measured the binding of tumor necrosis factor (TNF-α) to the human TNF receptor. Membranes were prepared from HeLa S3 human cervical epithelioid carcinoma cells. An aliquot of 0.2 mg of membrane preparation was incubated with 62 pM [^{125}I]TNF-α for 3 h at 4 °C. Nonspecific binding was measured in the presence of 50 nM TNF-α. Membranes were filtered and washed three times, and the filters were counted to determine the bound [^{125}I]TNF-α.

Golebiowski et al. (2005) tested pyrazolone-based cytokine synthesis inhibitors for the inhibition of TNF-α production using lipopolysaccharide-stimulated monocytic cells. Duplicate cultures of human monocytic cells (2.0×10^6/well) were incubated for 15 min in the presence or absence of

various concentrations of inhibitor before the stimulation of cytokine release by the addition of lipopolysaccharide (1 μg/ml). The amount of TNF-α released was measured 4 h later using an ELISA system.

Kumar et al. (1997) described the homologues of CSBP/p38 MAP kinase, their activation, as well as substrate specificity and sensitivity to inhibition by pyridyl imidazoles.

McLay et al. (2001) reported the discovery of a p38 MAP kinase inhibitor displaying a good oral antiarthritic efficacy.

Ignar et al. (2003) described the regulation of TNF-α secretion by a specific melanocortin-1 receptor peptide agonist.

Kinase Assay

The p38 enzyme assay is carried out at room temperature for 1 h, using 40 ng/well of the mouse enzyme. The substrate, 50 μg/ml ATF-2 transformation factor, is coated onto 96-well plates, and the assay is carried out in 25 mM HEPES buffer, pH 7.7, containing 25 mM magnesium chloride, 2 mM dithiothreitol, 1 mM sodium orthovanadate, and 100 μM ATP. Phosphorylated ATF-2 is quantitated using a phospho-specific ATF-2 primary antibody (rabbit antihuman) followed by a europium-labeled secondary antibody (sheep anti-rabbit IgG) with addition of the DELFIA enhancement solution resulting in fluorescence. ERK was measured using a [^{33}P] ATP filtration assay format for substrate myelin basic protein. ZAP-70, Syk, and Lck kinase activities were measured using the homogeneous time-resolved fluorescence methodology (HTRF) with the catalytic domains of each of the tyrosine kinases, biotinylated, specific peptide substrates, streptavidin-linked APC, and europium cryptate-conjugated anti-phosphotyrosine antibody.

Monocyte TNF-α Release Assay

Adherent human monocytes (100,000 cells/well) were incubated with LPS (10 ng/ml) in the absence and presence of compound for 18 h. Individual experiments were carried out in quadruplicate samples. TNF-α was measured by sandwich ELISA and IC_{50} values calculated for the activity of individual compounds. IC_{50} values shown from repeat experiments are means ± SEM.

Mouse TNF-α Release Assay

Compound was administered orally to BALB/c mice 30 min prior to LPS (0.1 mg/kg i.p.) challenge. Serum TNF-α levels were determined 90 min after LPS insult. Results represent means ± SEM.

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Effect of TNF-α Binding

Purpose and Rationale

Several authors compared the effects of the prototypes of the tumor necrosis factor (TNF) antagonists infliximab or adalimumab and etanercept (Vuoltteenaho et al. 2002; Kirchner et al. 2004; Shen et al. 2005).

Scallon et al. (2002) compared the binding and functional properties of the two prototypes of the TNF antagonists infliximab and etanercept.

Although both infliximab and etanercept are potent neutralizers of TNF bioactivity, there are fundamental differences in their molecular structures, their binding specificities, and the manner in which they neutralize TNF. Infliximab is a chimeric monoclonal antibody (mAb) with murine variable regions and human IgG1 and κ constant regions (Knight et al. 1993). The size (149 kDa) and structure of infliximab are therefore similar to those of naturally occurring antibodies. Etanercept is a fusion protein made up of the extracellular domain of the p75 TNF receptor (CD120b) and the hinge and Fc domains of human IgG1 (Mohler et al. 1993), a structure distinct from any known naturally occurring molecule. Importantly, infliximab is not known to bind to any antigen other than TNF, whereas etanercept binds equally well to both TNF and lymphotoxin α (LT α), consistent with observations reported for the cellular p75 TNF receptor (Schall et al. 1990; Smith et al. 1990). Each infliximab molecule is capable of binding to two TNF molecules, and up to three infliximab molecules can bind to each TNF homotrimer, thereby blocking all receptor binding sites on TNF. In contrast, it is believed that the bivalent etanercept molecule forms a 1:1 complex with the TNF trimer in which two of the three receptor binding sites on TNF are occupied by etanercept and the third receptor binding site is open. In addition, the p75 TNF receptor is known to have fast rates of association and dissociation with TNF (Evans et al. 1994), which suggests that etanercept may only transiently neutralize the activity of an individual TNF molecule.

Procedure

Cell Culture

KYM-1D4 cells that endogenously express TNF receptors (Butler et al. 1994) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10 % FBS. Human umbilical vein endothelial (HUVE) cells from Cell Systems (Seattle, Wash., USA) were maintained in HUVE cell medium supplied by Cell Systems. K2 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 % FBS, 2 mM L-glutamine, 0.5 μg/ml mycophenolic acid, 2.5 μg/ml hypoxanthine, and 50 μg/ml xanthine. All cells were cultured in a humidified incubator maintained at 37 °C and 5 % CO_2.

Binding to Monomer Subunits of TNF

Dimethylsulfoxide (DMSO) was added to ^{125}I-TNF (40–60 μCi/μg; 1.48–2.2 MBq/μg) to a final concentration of 10 % DMSO and incubated at 20 °C for 30 min to allow dissociation of TNF trimers. The mixture was passed over a 10 × 300-mm Superose 12 column equilibrated with PBS, and ^{125}I-TNF trimer and monomer were collected separately. Polystyrene 96-well microtiter plates were coated by incubating 50 μl of 1 μg/ml of infliximab, etanercept, or an isotype-matched negative control antibody (cM-T412) in the wells overnight at 4 °C. After washing with PBS-0.05 % Tween 20 (PBS-T), all wells were blocked for 1 h at 37 °C with PBS-1 % bovine serum albumin and washed three times with PBS-T. Triplicate wells were then incubated with ^{125}I-TNF trimer [0.4 μCi (14.8 kBq), 10 ng/ml] or ^{125}I-TNF monomer [0.1 μCi (3.7 kBq), 2.5 ng/ml] alone or with 5 μg/ml unlabeled TNF. After 1 h at 37 °C, the wells were washed with PBS-T and counted for ^{125}I.

Binding Assay to Measure Stability of Complexes with Soluble TNF

Each well of a 96-well enzyme immunoassay plate was incubated overnight at 4 °C with 100 μl of 0.1 M carbonate, pH 9.6, containing 10 μg/ml goat antihuman γ Fc antibody. Plates were washed three times with PBS-T and then

incubated for 1 h at 37 °C in blocking buffer (10 mM HEPES, pH 7.5, containing 0.1 % porcine gelatin, 150 µl/well). Wells were incubated for 1 h at 37 °C with 100 µl/well of blocking buffer containing 1 µg/ml infliximab or etanercept. Plates were washed three times with PBS-T, and then all TNF binding sites were saturated by incubating the wells for 1 h at 37 °C in 100 µl/well of blocking buffer containing 10 ng/ml ^{125}I-TNF (40–60 µCi/µg). Wells were washed three times with PBS-T and then filled with 100 µl/well of blocking buffer alone or containing an excess of soluble, unlabeled competitor such as infliximab, etanercept, or human TNF, and subsequently incubated at 37 °C. At the indicated time points, triplicate wells were washed three times with PBS-T to remove free ^{125}I-labeled TNF. The last wash was aspirated and replaced with 50 µl of scintillation fluid and the entire plate counted in a Packard TopCount gamma counter.

Assay for Bioactivity of Dissociated Soluble TNF

Microtiter plates were coated with goat antihuman γ Fc antibody and used to capture etanercept as described above. Wells were washed three times with PBS-T and incubated with 100 µl of 10 ng/ml unlabeled human TNF in 100 µl/well of blocking buffer for 1 h at 37 °C. Wells were washed three times with KYM media, filled with 100 µl of KYM media, and 500 ng/ml mouse TNF was added to each well as a competitor. After a 1-h incubation at 37 °C, the soluble fraction was removed and preincubated for 1 h in fresh wells with either no mAb, 10 µg/ml antihuman TNF mAb (infliximab), 85 µg/ml anti-mouse TNF mAb (cV1q huG3), or a combination of 10 µg/ml antihuman TNF and 85 µg/ml anti-mouse TNF mAb. After the preincubation, the soluble fractions were added to cultures of KYM-1D4 cells (50,000 cells/well in a 96-well plate) and the cells incubated for 16 h at 37 °C in the presence of 0.5 µg/ml actinomycin D. To quantitate cell viability, MTT dye was added to a final concentration 0.5 mg/ml and the cells incubated at 37 °C for 4 h. The medium was aspirated and 100 µl of 100 % DMSO was added to the cells. The difference between the absorbance at 550 and 650 nm was then determined.

HUVE Cell Assay to Measure Stability of Complexes with Soluble TNF

Infliximab or etanercept was mixed with 1 µg/ml human TNF at 10:1 or 30:1 M ratios in HUVE cell medium and incubated for 30 min at 37 °C. Serial dilutions of the preformed complexes were then added to confluent HUVE cells cultured in 96-well plates. Cells were incubated with the preformed complexes in 100 µl of HUVE cell medium for 4 h at 37 °C and then washed three times with HBSS. The cells were then incubated for 1 h at 37 °C in HBSS containing 1 µg/ml ^{125}I-labeled anti-E-selectin (20 µCi/µg). Cells were washed three times with HBSS, the last wash was aspirated and replaced with 30 µl of scintillation fluid, and the entire plate was counted in a Packard TopCount gamma counter.

Binding Assay to Measure Stability of Complexes with Transmembrane TNF

K2 cells, which stably express an uncleavable and thus permanently transmembrane (tm) form of TNF, were seeded at a density of 5×10^4 cells/well in a 96-well round-bottom plate in 100 µl of IMDM, 5 % FBS. Subsequently, ^{125}I-labeled infliximab or ^{125}I-labeled etanercept (both at 8.5 µCi/µg) was added to a final concentration of 0.5 µg/ml (enough to saturate all TNF binding sites on the cells). After a 1-h incubation at 25 °C, unbound infliximab and etanercept were removed by washing three times with IMDM medium. Fresh IMDM, 5 % FBS medium (100 µl) alone, or containing 50 µg/ml of an unlabeled soluble competitor, was added to the cells. Soluble competitors were either infliximab or etanercept for samples treated with radiolabeled infliximab and one of infliximab, etanercept, or human LTα for samples treated with radiolabeled etanercept. The cells were then incubated at 37 °C in 5 % CO_2. At different time points, cells in selected wells were washed with PBS, and the number of counts bound to the cells determined using a gamma counter (PerkinElmer Wallac, Wellesley, Mass., USA).

Characterization of Infliximab and Etanercept Binding to tmTNF

K2 cells or TNF-negative Sp2/0 control cells were seeded in 96-well round-bottom plates at a density of 5×10^4 cells/well in IMDM, 5 % FBS. Varying amounts of ^{125}I-labeled infliximab (23.4 μCi/μg) or ^{125}I-labeled etanercept (22.4 μCi/μg) were added to the cells. After a 16-h incubation at 4 °C, cells were washed four times with culture medium (IMDM, 5 % FBS), the last wash was aspirated, and 50 μl of culture medium was added to each well. The cells were then removed with cotton swabs, and the number of counts per well was determined using a gamma counter (PerkinElmer Wallac). The resulting binding data were analyzed by nonlinear regression using Prism software (GraphPad Software, San Diego, Calif., USA).

HUVE Cell Assay to Compare Ability to Inhibit tmTNF Bioactivity

K2 cells or Sp2/0 control cells were seeded in 96-well round-bottom plates at a density of 1×10^5 cells/well in IMDM, 5 % FBS. Varying amounts of infliximab or etanercept in IMDM, 5 % FBS were added and the mixture incubated for 1 h at 37 °C. This mixture was then added to confluent cultures of HUVE cells in 96-well plates. The resulting cell–cell mixture was incubated for an additional 4 h at 37 °C in a 5 % CO_2 incubator. Cells were then washed three times with HBSS and incubated for 1 h with 1 μg/ml ^{125}I-anti-E-selectin (20 μCi/μg). Cells were washed three times with HBSS, the last wash was aspirated and replaced with 30 μl of scintillation fluid, and the plate counted in a Packard TopCount gamma counter.

Evaluation

Data were analyzed using a paired Student's *t*-test to determine whether there was a statistically significant difference between the capacities of infliximab and etanercept to block the bioactivity of tmTNF.

Modifications of the Method

Maloff and Delmendo (1991) developed high-throughput radioligand binding assays for interleukin 1-α (IL-1-α) and tumor necrosis factor (TNF-α) in isolated membrane preparations.

Zhang et al. (2002) described the identification and characterization of a dual tumor necrosis factor converting enzyme/matrix metalloprotease inhibitor for the treatment of rheumatoid arthritis.

Transgenic mice expressing human tumor necrosis factor develop severe polyarthritis (Keffer et al. 1991; Kollias et al. 1999; Kontoyiannis et al. 1999; Mijatovic et al. 2000; Akassoglou et al. 2003; Li and Schwarz 2003). A targeting vector containing a genomic fragment encoding the entire TNF-α with the ARE-containing 3′ UTR was replaced with the 3′ UTR from the β-globin gene. This mutation increases the stability and translational efficiency of TNF-α mRNA and thus results in chronic TNF-α overexpression that leads to severe erosive polyarthritis. Administration of anti-TNF-α antibodies completely prevents the disease.

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Binding to Interferon Receptors

Purpose and Rationale

The interferons (IFNs) were discovered in 1957 as biological agents interfering with virus replication. Interferons are protein molecules. Interferon was named for its ability to interfere with viral proliferation. Three forms of interferon – alpha

(α), beta (β), and gamma (γ) – have been recognized. These interferons have been classified into two types: type I includes the alpha and beta forms, and type II includes the gamma form. Type I interferons are produced upon stimulation by a virus by almost any cell, and their primary function is to induce viral resistance in cells. Type II interferon is secreted by natural killer cells and T lymphocytes, and their purpose is to signal the immune system to respond to infectious agents or cancerous growth.

IFNs are a family of secreted proteins occurring in vertebrates and can be classified as cytokines. The IFNs are multifunctional and are components of the host defense against viral and parasitic infections and certain tumors. They affect the functioning of the immune system in various ways and also affect cell proliferation and differentiation.

IFNs were initially classified by their sources as leukocyte, fibroblast, and immune IFNs. Leukocyte and fibroblast IFNs, together, were also designated as type 1 IFNs and immune IFN as type 2 IFN. The recent nomenclature designates leukocyte IFNs as IFN-α and IFN-ω (earlier α-1 and α-2, respectively) fibroblast IFN as IFN-β, and immune IFN as IFN-γ.

Interferons bind to receptors on the cell surface and induce the synthesis of specific proteins. Littman et al. (1985) found that recombinant IFN-γ produced in bacteria, which is not glycosylated, binds to cellular receptors with an affinity similar to that of natural IFN-γ.

Procedure

Human lymphoblastoid cells (Daudi, MOLT-4 and Raji) are grown in stationary cultures in Dulbecco's medium with 10 % heat-inactivated horse serum. HeLa cells are grown in Eagle's medium with 7 % horse serum.

The following interferons are used: purified recombinant interferon-γ (rIFN-γ) (Genentech, antiviral activity 1.2×10^7 units/mg), natural human INF-β (Interferon Working Group of the NCI, antiviral activity 2×10^5 roentgen units/mg), rIFN-2α (Schering, antiviral activity 2×10^8 reference units/mg), and rIFN-β (Cetus Corp., antiviral activity 2.6×10^8 reference units/mg).

Fifty micrograms of rIFN-γ is reacted for 2 h at 0 °C with 1 mCi of ^{125}I-Bolton–Hunter reagent (2,000 Ci/mmol) in 0.25 ml of sodium borate buffer, pH 8.0. The reaction is stopped by the addition of glycine to a final concentration of 0.2 M and applied to a 26 × 0.7 cm column of Sephadex G-75 equilibrated with phosphate-buffered saline, pH 7.4, containing 0.25 % gelatin. The reaction vial is washed with 20-µl aliquots of this buffer containing 40 % ethylene glycol and then with buffer alone. The washes are added to the column and 0.32 ml fractions are collected. The fractions containing ^{125}I-rIFN-γ are pooled and diluted with 1/10 volume of tenfold concentrated Eagle's medium containing 10 mg/ml bovine serum albumin and 0.1 mM dithiothreitol.

Cells harvested from exponentially growing cultures are centrifuged and resuspended at 8×10^6/ml in their own medium supplemented with 10 mM HEPES buffer, pH 7.4. Standard binding assays contain 3–5 × 10^6 cells and 0.46 nM ^{125}I-rIFN-γ. At the end of the reaction, the cells are centrifuged through 10 % sucrose at 10,000 rpm in microfuge tubes and the cell pellet is counted. A blank value is determined by incubating and processing in the same way an equal amount of ^{125}I-rIFN-γ in the absence of cells; this blank is subtracted from the cpm bound.

Evaluation

The binding data are analyzed using the LIGAND program developed by Munson and Rodbard (1980).

Modifications of the Method

Blatt et al. (1996) described the biological activity of consensus interferon, a wholly synthetic type I interferon, developed by scanning several interferon-alpha nonallelic subtypes and assigning the most frequently observed amino acid in each position.

IFN-τ, a new class of type I interferon was described by Pontzer et al. (1994), Alexenko et al. (1997, 1999), Martal et al. (1998), and Swann et al. (1999).

Thiam et al. (1998) reported the agonist activities of a lipopeptide derived from INF-γ on murine and human cells by analysis and

quantification of cell surface markers using flow cytometry and cell-ELISA.

Bosio et al. (1999) reported efficacy of type I interferon in cytomegalovirus infections in vivo. Oral administration of type I interferons (murine INF-α and INF-β) reduced early replication of murine cytomegalovirus in both the spleen and liver of infected BLB/c mice.

Tovey and Maury (1999) found a marked antiviral activity of murine interferon-α/β or individual recombinant species of murine INF-α, INF-β, or INF-γ or recombinant human INF-α1–8 in mice challenged systemically with a lethal dose of encephalomyocarditis virus, vesicular stomatitis virus, or varicella zoster virus. Oromucosal administration of INF-α also exerted a marked antitumor activity in mice injected i.v. with highly malignant Friend erythroleukemia cells or other transplantable tumors, such as L1210 leukemia, the EL4 tumor, or the highly metastatic B16 melanoma.

To gain more insight into similarities of different INF-α species, Viscomi et al. (1999) evaluated neutralization and immunoactivity of a variety of INF preparations with various monoclonal antibodies obtained through immunization with recombinant, lymphoblastoid, and leukocyte INF-α.

Reporter transgenic mice expressing the luciferase gene under the control of separate TCR-response elements from the INF-γ promoter or expressing the green fluorescent protein gene under the control of an INF-γ minigene were employed by Zhang et al. (1999) to explore the basis for IL-12 regulation of INF-γ gene transcription.

Poynter and Daynes (1999) studied the influence of constitutively expressed INF-γ on age-associated alterations in inducible nitric oxide synthase regulation using cell cultures from mouse spleen for nitrite and cytokine analysis.

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Chemokine Antagonism

Purpose and Rationale

The human chemokine system comprises about 50 distinct chemokines and 20 G-protein-coupled chemokine receptors (Rossi and Zlotnik 2000; Sallusto et al. 2000; Zlotnik and Yoshie 2000; Fernandez and Lolis 2002; D'Ambrosio et al. 2003; Houshmand and Zlotnik 2003; Ono et al. 2003; Proudfoot et al. 2003; Chen et al. 2004; Haringman and Tak 2004; Cunha et al. 2005). The biological activities of chemokines range from the control of leukocyte trafficking in basal and inflammatory conditions to regulation of hematopoiesis, angiogenesis, tissue architecture, and organogenesis. Grainger and Reckless (2005) studied the anti-inflammatory effects of broad-spectrum chemokine inhibitors. Several groups described CC chemokine receptor-1 antagonists (Liang et al. 2000a, b; Naya et al. 2001; Eltayeb et al. 2003; Gladue et al. 2006). CC chemokine receptor-3 (CCR3) antagonists were reported by De Lucca et al. (2005) and Fryer et al. (2006). CC chemokine receptor-5 (CCR5) antagonists were described by Rosi et al. (2005) and Saita et al. (2005).

De Lucca et al. (2005) described the discovery of CC chemokine receptor-3 (CCR3) antagonists with picomolar potency.

Procedure

Biological Assays

CCR3 Receptor Binding

Millipore filter plates (no. MABVN1250) are treated with 5 μg/ml protamine in phosphate-buffered saline, pH 7.2, for 10 min at room temperature. Plates are washed three times with phosphate-buffered saline and incubated with phosphate-buffered saline for 30 min at room temperature. For binding, 50 μl of binding buffer (0.5 % bovine serum albumen, 20 mM HEPES buffer, and 5 mM magnesium chloride in RPMI 1640 media) with or without a test concentration of a compound present at a known concentration is combined with 50 μl of ^{125}I-labeled human eotaxin (to give a final concentration of 150 pM radioligand) and 50 μl of cell suspension in binding buffer containing 5×10^5 total cells. Cells used for the binding assay are CHO cell lines transfected with a gene expressing human CCR3 (Daugherty et al. 1996). The mixture of compound, cells, and radioligand is incubated at room temperature for 30 min. Plates are placed onto a vacuum manifold, vacuum is applied, and the plates are washed three times with binding buffer with 0.5 M NaCl added. The plastic skirt is removed from the plate, and the plate is allowed to air-dry; the wells are punched out, and the radioactivity counted (cpm).

Evaluation

The percent inhibition of binding is calculated using the total count obtained in the absence of any competing compound or chemokine ligand, and the background binding determined by addition of 100 nM eotaxin in place of the test compound.

Human Eosinophil Chemotaxis Assay

Neuroprobe MBA96 96-well chemotaxis chambers with Neuroprobe poly(vinylpyrrolidone)-free polycarbonate PFD5 5-μm filters in place

are warmed in a 37 °C incubator prior to the assay. Freshly isolated human eosinophils are suspended in RPMI 1640 with 0.1 % bovine serum albumin at 1×10^6 cells/ml and warmed in a 37 °C incubator prior to the assay. A 20 nM solution of human eotaxin in RPMI 1640 with 0.1 % bovine serum albumin is warmed in a 37 °C incubator prior to the assay. The eosinophil suspension and the 20 nM eotaxin solution are each mixed 1:1 with prewarmed RPMI 1640 with 0.1 % bovine serum albumin with or without a dilution of a test compound that is at twofold the desired final concentration. The filter is separated, and the eotaxin/compound mixture is placed into the bottom part of the chemotaxis chamber. The filter and upper chamber are assembled, and 200 μL of the cell suspension/compound mixture is added to the appropriate wells of the upper chamber. The upper chamber is covered with a plate sealer, and the assembled unit is placed in a 37 °C incubator for 45 min. After incubation, the plate sealer is removed and all remaining cell suspension is aspirated off. The chamber is disassembled, and unmigrated cells are washed away with phosphate-buffered saline, and then the filter is wiped with a rubber-tipped squeegee. The filter is allowed to completely dry and stained with Wright–Giemsa. Migrated cells are enumerated by microscopy.

Calcium Mobilization Assay

Intracellular calcium flux was measured as the increase in fluorescence emitted by the calcium-binding fluorophore, fluo-3, when preloaded cells were stimulated with CCR3 ligand. Freshly isolated eosinophils were loaded with fluorophore by resuspending them in a HEPES-buffered PBS solution containing 5 μM fluo-3 and incubating for 60 min at 37 °C. After being washed twice to remove excess fluorophore, cells were resuspended in binding buffer (without phenol red) and plated into 96-well plates at 2×10^5/well. Plates were placed individually in a FLIPR-1 (Molecular Devices) that uses an argon-ion laser to excite the cells and robotically adds reagents while monitoring changes in fluorescence in all wells simultaneously. To determine the IC_{50}, compound or buffer alone was added and cells were incubated for 5 min; eotaxin was then added to a final concentration of 10 nM. The fluorescence shift was monitored, and the base-to-peak excursion was computed automatically. All conditions were tested in duplicate, and the mean shift per condition was determined. The inhibition achieved by graded concentrations of compound was calculated as a percentage of the compound-free eotaxin control.

Modifications of the Method

Chen et al. (1998) reported in vivo inhibition of CC and CX_3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar–Kyoto (WKY) rats by the viral protein vMIP-II.

Ruth et al. (2001) investigated fractalkine, a chemokine, in rheumatoid arthritis and in rat adjuvant-induced arthritis. Fractalkine in vascular biology was discussed by Umehara et al. (2004).

Laudanna and Constanin (2003) described new models of intravital microscopy for analysis of chemokine receptor-mediated leukocyte vascular recognition.

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Influence of Peroxisome Proliferator-Activated Receptors (PPARs) on Inflammation

Purpose and Rationale

Peroxisome proliferator-activated receptors (PPARs) play an important role not only in lipid metabolism and diabetes but also in the inflammation process (Devchand et al. 1996; Delerive et al. 2001; Cabrero et al. 2002; Clark 2002; Blanquart et al. 2003; Moller and Berger 2004; Nencioni et al. 2003; Tai et al. 2003; Woerly et al. 2003; Diep et al. 2004).

Jiang et al. (1998) reported that PPAR-γ antagonists inhibit the production of monocyte inflammatory cytokines.

Procedure

Monocyte Preparation

Human monocytes are isolated from freshly collected buffy-coat preparations of whole human blood. The mononuclear cell fraction is prepared by dilution with an equal volume of phosphate-buffered saline (PBS) at room temperature and layered over a solution containing 3 ml Ficoll–Hypaque per 10 ml blood and PBS and centrifuged for 10 min at 900 g. The mononuclear cell layer is transferred to a fresh tube, mixed with three vols of PBS, and centrifuged for 10 min at 400 g. The supernatant is removed, and the dilution and centrifugation are repeated three times. Mononuclear cells are resuspended in RPMI medium 1640, counted, and diluted to 5×10^6 cell per ml, after which 1 ml is transferred to each well of a 24-well tissue culture plate and incubated for 37 °C in a 5 % CO_2 humidified incubator. The nonadherent cells are removed and the monocytes washed once with PBS before adding 1 ml of fresh RPMI medium 1640 with 10 % fetal bovine serum. Experiments are initiated on the day blood is collected, and all manipulations are carried out under endotoxin-free conditions.

Cytokine Assay

Monocytes in fresh medium are treated with inducers and candidate induction inhibitors at the time of culture initiation. Medium is collected from triplicate wells 18–20 h after the test compounds are added. Supernatant concentrations of TNF-α, IL-6, and IL-1β are measured by ELISA.

RNA Blot

Total RNA of human monocyte cultures is isolated 15 h after stimulation with 25 nM PMA in the absence or presence of PPARγ agonists. RNA blot analyses are performed with standard procedures and labeled probes prepared from human TNF-α and GAPDH cDNA with the Megaprime DNA labeling Kit (Amersham Life Science).

Luciferase Assay

U937 cells (2×10^7) are transfected with 10–20 µg luciferase reporter DNA by electrophoresis at 875 V cm^{-1}, 960 µF (Bio-Rad Laboratories). Transfected cells are allowed to recover for 1 h, and triplicate samples are either untreated or treated with 25 nM PMA in the absence or presence of indicated drugs. Luciferase activity is measured 18–36 h later using the dual-luciferase reporter assay system (Promega) with the pRL-TK vector as an internal reporter control.

Evaluation

To obtain IC_{50} values, data are fitted to a four-parameter exponential and the derived parameters used to calculate the concentration at which 50 % of the maximal activity is observed.

Modifications of the Method

Kojo et al. (2003) evaluated human PPAR subtype selectivity of a variety of anti-inflammatory drugs based on a novel assay for PPARδ (β).

Procedure

Plasmids

The cDNAs for human PPAR α, δ, and γ were synthesized using the polymerase chain reaction (PCR) with human liver cDNA for human PPARα, human heart cDNA for human PPARδ (β), and human fat cell cDNA for human PPARγ 1 as templates. Retinoid X receptor (RXR) α expression plasmids were constructed by inserting a coding sequence of RXRα into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, Calif., USA). The cDNA for human RXRα was synthesized with human kidney cDNA as a template. The template cDNAs used were purchased from Clontech Laboratories (Palo Alto, Calif., USA). The amplified cDNA fragments were cloned into pCRII (Invitrogen), and the nucleotide sequences of the cDNAs were determined by the dideoxy chain termination method using an automated laser fluorescent DNA sequencer. Multiple clones of each cDNA were sequenced, and artifacts generated by the *Taq* polymerase used in the PCR were revised by replacement of the specified region among the clones. The PPAR and RXRα expression plasmids were constructed by inserting each full-length cDNA at a multiple cloning site of the mammalian expression vector pCDM8, pcDNAI, or pcDNA3.1 (+) (Invitrogen). Coactivator expression plasmids pcDNA3.1-CBP and pcDNA3.1-SRC-1 were provided by Fujimura and Aramori of the Pharmacological Research Laboratories of Fujisawa Pharm. The human RXRγ expression plasmid was purchased from Invitrogen.

The reporter gene plasmid pGVPPREluc-1 was constructed as follows. Three copies of a 33-bp PPRE identical to that of acyl CoA oxidase were first cloned at the *Sal*I site of pBLCAT-2 to generate pBLPPRECAT-1. The luciferase reporter plasmid pGVPPRELuc was constructed by inserting the PPRE-containing fragment of pBLPPRECAT-1 at a multiple cloning site of PGV-P2 (Wako Pure Chemical, Osaka).

Gal4-PPARδ (β) fusion expression plasmid for one-hybrid assay was constructed as follows: full-length PPARδ (β) cDNA and PPARδ (β)-LBD cDNA were prepared by PCR amplification using pCDM8-hPPARδ as a template, and the amplified cDNA was cloned into a multiple cloning site of the pBIND vector (Promega, Madison, Wis., USA). The nucleotide sequence of each fusion expression plasmid was checked with an automated DNA sequencer. Reporter plasmid pG5luc for the one-hybrid assay was purchased from Promega.

Transient Transfection Assay

The African green monkey fibroblast cell line CV-1 was obtained from ATCC (Manassas, Va., USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, Md., USA) supplemented with penicillin, streptomycin, and 10 % heat-inactivated fetal calf serum. Cells were seeded at 2×10^5 per well of 6-well culture dishes and after overnight culture transiently transfected with 1 μg each of pGVPPRELuc luciferase reporter plasmid, PPAR expression plasmid, and RXRα expression plasmid together with the control *Renilla* luciferase expression plasmid RL-TK (Promega) using Lipofectamine 2000 (Gibco-BRL). Coactivator expression plasmid was also included when a transfection was performed for the PPARδ assay. For the one-hybrid assay, cells were transfected with 1 μg each of pG5luc reporter plasmid and Gal4-PPARδ fusion expression plasmid with RL-TK control plasmid. Cells were harvested 4 h after transfection and plated again at 1.6×10^4 per well onto 96-well plates. The drugs dissolved in dimethyl sulfoxide were added to the culture, and the cells were incubated at 37 °C for 24 h. After being washed with PBS(-),

cells were lysed with PLB (passive lysis buffer) (Promega) and the lysates were used for reporter assays. Expression of the reporter was measured by the activity of firefly luciferase using the dual luciferase reporter assay system (Promega) and ARVO HTS 1420 multilabel counter (Amersham Biosciences) as a luminometer. Firefly luciferase activity was corrected for transfection efficiency based on the activity of internal control *Renilla* luciferase.

Bishop-Bailey and Warner (2003) found that PPARγ ligands induce prostaglandin production in vascular smooth muscle cells and concluded that indomethacin acts as a peroxisome proliferator-activated receptor-γ antagonist.

Fahmi et al. (2001) reported that peroxisome proliferator-activated receptor γ activators, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2, inhibit interleukin-1β-induced nitric oxide and metalloproteinase 13 production in human chondrocytes.

Cheng et al. (2004) found that peroxisome proliferator-activated receptor γ, which is activated by ligands such as troglitazone or 15-deoxy-$\Delta^{12,14}$-PGJ_2, inhibits interleukin-1β-induced membrane-associated prostaglandin E_2 synthase-1 expression in human synovial fibroblasts by interfering with the early growth response protein Egr-1.

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Binding to Histamine H_4 Receptor

Purpose and Rationale

Histamine plays an important role in many types of allergic and inflammatory processes, which include both acute and delayed hypersensitivity reactions. Tissue mast cells are the source of histamine in such occasions. The magnitude of such problems depends on the route of exposure (local vs. systemic), sites of exposure (e.g., inhaled vs. cutaneous), dose of allergen, and the degree of previous sensitization to the allergen. Clinical manifestations of histamine release can vary from life-threatening anaphylactic reactions, to urticaria (hives), to local wheal and flare reactions. Four histamine receptors have been identified(H1, H2, H3, and H4), all of which are G protein-coupled receptors, and the receptors are seen in smooth muscle, endothelial cells, gastric parietal cells, central nervous system, mast cells, eosinophils, T cells, dendritic cells, etc.

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H_1 receptor couples mainly to $G_{q/11}$, thereby stimulating phospholipase C, whereas the H_2 receptor interacts with G_s to activate adenylyl cyclase. The histamine H_3 and H_4 receptors couple to G_i proteins to inhibit adenylyl cyclase and to stimulate MAPK (Hough 2001).

The H_4 receptor is highly expressed in peripheral leukocytes and intestinal tissue, making this receptor an interesting target in inflammatory diseases (Fung-Leung et al. 2004; De Esch et al. 2005; Lim et al. 2006; Zhang et al. 2006). The new receptor was cloned and characterized by Oda et al. (2000). Liu et al. (2001b) reported comparison of histamine H_4 receptors in several species. Gbahou et al. (2006) compared the pharmacology of human histamine H_3 and H_4 receptors and described the structure–activity relationships of histamine derivatives. Thurmond et al. (2004) described a potent and selective histamine H_4 antagonist with anti-inflammatory properties. Lim et al. (2005) evaluated histamine H_1-, H_2-, and H_3-receptor ligands at the human H_4 receptor and identified 4-methylhistamine as the first potent and selective H_4 receptor agonist.

Procedure

Cell Culture

SK-N-MC cell lines, which stably express either the human H_3R (SK-N-MC/hH_3) or H_4R (SK-N-MC/hH_4) as well as a cAMP-responsive-element (CRE)-driven β-galactosidase reporter gene SK-N-MC/hH_3 or SK-N-MC/hH_4 cells (Lovenberg et al. 1999; Liu et al. 2001a), were cultured in Eagle's minimum essential medium supplemented with 5 % fetal calf serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 600 µg/ml G418 at 37 °C in 5 % CO_2 and 95 % humidity.

Radioligand Binding Assays

The SK-N-MC/hH_3 cell homogenates were incubated for 40 min at 25 °C with approximately 1 nM [^{3}H]*N*-methylhistamine in 25 mM KPO_4 buffer and 140 mM NaCl (pH 7.4 at 25 °C), with or without competing ligands, whereas the SK-N-MC/hH_4 cell homogenates were incubated 1 h at 37 °C in 10 nM [^{3}H]histamine and 50 mM Tris–HCl (pH 7.4 at 37 °C), with or without competing ligands. Bound radioligands were collected on 0.3 % polyethyleneimine-pretreated Whatman GF/C and washed three times with 3 ml of ice-cold washing buffer (4 °C) containing 25 mM Tris–HCl and 140 mM NaCl (pH 7.4 at 4 °C) for the hH_3R and 50 mM Tris–HCl (pH 7.4 at 4 °C) for the hH_4R.

Evaluation

Binding analysis of 10 nM [^{3}H]JNJ 7777120 (test compound) and 0.1 nM [^{125}I]iodophenpropit to the hH_4R was performed with the same conditions as described for [^{3}H]histamine. In saturation binding

analysis, the nonspecific binding of [^{3}H]histamine or [^{3}H]JNJ 7777120 was determined with 1 μM clobenpropit. The binding analysis of [^{3}H]mepyramine and [^{125}I]iodoaminopotentidine binding to human H_1R and human H_2R, respectively, was performed according to Bakker et al. (2004). The binding data were analyzed with Prism 4.0 (GraphPad Software, San Diego, Calif., USA), and data are presented as mean ± SEM. Mouse and rat H_4R radioligand binding assays were performed according to Liu et al. (2001b).

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In Vivo Methods for Anti-inflammatory Activity

General Considerations

The inflammatory process involves a series of events that can be elicited by numerous stimuli, e.g., infectious agents, ischemia, antigen–antibody

interactions, and chemical, thermal, or mechanical injury. The response is accompanied by the clinical signs of erythema, edema, hyperalgesia, and pain. Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms:

- An acute, transient phase, characterized by local vasodilatation and increased capillary permeability
- A subacute phase, characterized by infiltration of leukocytes and phagocytic cells
- A chronic proliferative phase, in which tissue degeneration and fibrosis occur

According to these phases, pharmacological methods have been developed.

Methods for testing acute and subacute inflammation are:

- UV erythema in guinea pigs
- Vascular permeability
- Oxazolone-induced ear edema in mice
- Croton oil ear edema in rats and mice
- Paw edema in rats (various modifications and various irritants)
- Pleurisy tests
- Granuloma pouch technique (various modifications and various irritants)

The proliferative phase is measured by methods for testing granuloma formation, such as:

- Cotton-wool granuloma
- Glass rod granuloma
- PVC sponge granuloma

Furthermore, methods for testing immunological factors have been developed, such as:

- Adjuvant arthritis in rats (various modifications)
- Experimental allergic encephalomyelitis
- Schultz–Dale reaction
- Passive cutaneous anaphylaxis
- Arthus type immediate hypersensitivity
- Delayed type hypersensitivity (see Part IX, "Antiarthrotic and Immunomodulatory Activity")

Methods for Testing Acute and Subacute Inflammation

Ultraviolet Erythema in Guinea Pigs

Purpose and Rationale

The test was first described by Wilhelmi (1949) who was able to delay the development of ultraviolet erythema on albino guinea pig skin by systemic pretreatment with clinically equivalent doses of phenylbutazone and other nonsteroidal anti-inflammatory agents. The test procedure was further developed by Winder et al. (1958) and since that time modified by various investigators.

Procedure

Albino guinea pigs (Pirbright white strain) of both sexes with an average weight of 350 g are used. Eighteen hours prior testing, the animals are shaved on both flanks and on the back. Then they are chemically depilated by a commercial depilation product or by a suspension of barium sulfide. Twenty minutes later, the depilation paste and the fur are rinsed off in running warm water. On the next day, the test compound is dissolved (or suspended) in the vehicle, and half the dose of the test compound is administered by gavage (at 10 ml/kg) 30 min before ultraviolet exposure. Control animals are treated with the vehicle alone. Four animals are used for each treatment group and control. The guinea pigs are placed in a leather cuff with a hole of 1.5 × 2.5 cm size punched in it, allowing the ultraviolet radiation to reach only this area. An original Hanau ultraviolet burner Q 600 is warmed up for about 30 min prior to use and placed at a constant distance (20 cm) above the animal. Following a 2 min ultraviolet exposure, the remaining half of the test compound is administered. The investigator has to protect himself/herself by gloves and ultraviolet glasses. The erythema is scored 2 and 4 h after exposure.

Evaluation

The degree of erythema is evaluated visually by two different investigators in a double-blinded manner. The following scores are given:

- 0 = no erythema
- 1 = weak erythema
- 2 = strong erythema
- 4 = very strong erythema

Animals with a score of 0 or 1 are considered to be protected. The scoring after 2 and after 4 h gives some indication of the duration of the effect. ED_{50} values can be calculated. Doses of 1.5 mg/kg indomethacin p.o., 4 mg/kg phenylbutazone p.o., and 60 mg/kg acetylsalicylic acid p.o. have been found to be effective.

Critical Assessment

The test has the advantage of simplicity but needs training of the investigators. Attempts to use reflection photometers in order to eliminate subjective scoring were unsuccessful. Corticosteroids after systemic application are rather ineffective in this test and, however, can be evaluated after topical administration. The test is not particularly useful to study the duration of the anti-inflammatory effect.

Modifications of the Test

Yawalkar et al. (1991) tested several steroids after local application in the ultraviolet-induced dermatitis inhibition in guinea pigs. Clobetasol propionate was more effective than hydrocortisone, and halobetasol propionate was superior to both corticosteroids.

Woodward and Owen (1979) used the albino guinea pig ear as the site of inflammation produced by UV radiation. Ear temperature, water content of the ear, and vascular permeability were measured. Indomethacin, phenylbutazone, and aspirin given subcutaneously were active but paracetamol was not.

Warren et al. (1993) studied the role of nitric oxide synthase and cyclooxygenase in the skin blood flow to UVB irradiation in the shaved dorsal skin of anesthetized male **Sprague–Dawley rats** with a laser Doppler flow probe. Topical application of clobetasol-17-propionate immediately after irradiation inhibited the 18-h UVB response in a dose-dependent manner.

Gloxhuber (1976) measured skin thickness using calipers in **hairless mice** after UV irradiation of the back and treatment with anti-inflammatory drugs.

Woodbury et al. (1994) and Kligman (1994) described a rapid assay of the anti-inflammatory activity of topical corticosteroids by inhibition of a UVA-induced neutrophil infiltration in hairless mouse skin. Skh hairless mice were irradiated with UVA light on an area of 2 × 2 cm square on the dorsal trunk for 200 min in anesthesia. Steroid treatment was once daily for 7 days. Irradiation was on the eighth day. Neutrophils were counted microscopically in punch biopsies.

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Vascular Permeability

Purpose and Rationale

The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by a phlogistic substance (Miles and Miles 1952). Mediators of inflammation, such as histamine, prostaglandins, and leukotrienes, are released following stimulation, e.g., of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma proteins are extravasated and edemas are formed. These effects are counteracted by H_1 antihistaminics, inhibitors of arachidonic acid metabolism, and by leukotriene receptor antagonists. In addition, membrane-stabilizing drugs are able to reduce capillary permeability. Vascular permeability is increased by intracutaneous injection of the mast cell-degranulating compound 48/80. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evans blue.

Procedure

Male Sprague–Dawley rats with a body weight between 160 and 200 g are used. The ventral sides of the animal are shaved. Five milliliter per kilogram of a 1 % solution of Evans blue is injected intravenously. One hour later the animals are dosed with the test compound orally or intraperitoneally or with the vehicle. Ten animals are used for each test group and the control. Thirty minutes later, the animals are briefly anesthetized with ether, and 0.05 ml of a 0.01 % solution of compound 48/80 is injected intracutaneously at three sites both at the left and ventral side. Ninety minutes after the injection of compound 48/80, the animals are sacrificed by ether anesthesia. The abdominal skin is removed and the dye-infiltrated areas of the skin are measured.

Evaluation

The diameter of the dye-infiltrated areas is measured in millimeters in two perpendicular directions, and the mean values of all injection sites in one animal are calculated. The percent inhibition in the treated animals as compared to the control group is calculated. A treated animal which shows values less than 50 % of controls can be considered as positive. ED_{50} values can be calculated in this way. Phenylephrine at a dose of 15 mg/kg has been found to be effective.

Critical Assessment of the Method

The test for vascular permeability is useful for characterization of a new anti-inflammatory compound.

Since compounds with sympathomimetic activity have a pronounced effect, this test cannot be regarded as a primary screening test for anti-inflammatory products. Together with an observation of writhing or "squirming" of mice, Whittle (1964) has proposed to be able to distinguish between narcotic and non-narcotic analgesics.

Modifications of the Method

Shionoya and Ohtake (1975) described a simple method for extraction of extravasated dye (Evans blue) in the skin.

Frimmer and Müller (1962) presented a critical survey on the use of dye methods for quantitative determination of increased capillary permeability following intracutaneous injection of active substances.

McClure et al. (1994) used the Olympus CUE-2 Image Analyzer to quantify vascular permeability in the Miles assay in guinea pigs.

Zentel and Töpert (1994) used oxazolone-induced Evans blue extravasation for preclinical evaluation of topical corticosteroids. Female **NMRI mice** were sensitized by topical application of 50 µl of 40 % oxazolone in ethanol to 4 cm^2 of the left flank. After 13 days the animals were injected intravenously with 0.2 ml of 0.5 % Evans blue in water, and 20 µl of 4 % oxazolone in ethanol was topically applied to 6 cm^2 of the right flank immediately after injection. Three hours later the challenged skin was treated with various corticosteroids in ointment. The animals were sacrificed 24 h after treatment and the challenged skin removed. Evans blue extravasation was measured spectrophotometrically at 623 nm.

Teixeira et al. (1993) studied acute inflammatory reactions in **guinea pig skin** measuring infiltration of ^{111}In-labeled eosinophils and neutrophils and edema formation by extravasation of ^{125}I-human serum albumin.

Fujii et al. (1996) quantified vascular permeability by the extravasation of pontamine sky blue in the skin of male ddY mice after subcutaneous injection of lipopolysaccharides.

Blackham and Woods (1986) measured the extravasation of pontamine sky blue in the **mouse** peritoneal cavity.

Cambridge et al. (1996) investigated 6-hydroxydopamine-induced plasma extravasation in **rat skin** after intravenous injection of ^{125}I-human serum albumin and Evans blue.

Rouleau et al. (1997) measured the inhibition of capsaicin-induced plasma extravasation by a histamine H_3 receptor agonist prodrug by analysis of extravasated Evans blue in the skin, eye conjunctiva, nasal mucosa, trachea, main bronchi, esophagus, and urinary bladder of rats.

Watanabe et al. (1984) used fluorescein isothiocyanate-labeled bovine serum albumin as tracer to measure vascular permeability in the **carrageenan air pouch of rats**.

Collins et al. (1993) studied the proinflammatory properties of the human recombinant vascular permeability factor containing 165 amino acids in **rabbits**.

Urinary bladder cystitis induced by cyclophosphamide was used as model of intestinal inflammation and pain by several authors (Ahluwalia et al. 1994; Bon et al. 1996; Boucher et al. 1997; Alfieri and Gardner 1997). Male Wistar rats weighing 300–400 g were treated first with test drug or saline subcutaneously or intraperitoneally and then injected 5 min later with 150 mg/kg i.p. cyclophosphamide. One hour later, anesthesia was induced by 40 mg/kg i.p. pentobarbitone, and 50 mg/kg Evans blue was injected into the jugular vein. Fifteen min later, the rat was exsanguinated by infusion of 50 ml saline into the left cardiac ventricle. The urinary bladder, the left kidney, the superior lobe of the left lung, and approximately 1-cm portions of the duodenum and jejunum were removed and blotted before dry weighing. The content of Evens blue dye was determined by spectrophotometry at 620 nm after extraction in known volumes of formamide at 60 °C for 60 h.

Ferrets were treated in the same way, but the dose of cyclophosphamide was 125 mg/kg, and the volume of exsanguination was 300 ml.

Hirota et al. (1995) induced **chemical peritonitis in rats** by applying 0.02 M HCl on the surface of the cecum or appendix and quantified the inflammation by measuring the extravasation of intravenously injected Evans blue bound to albumin extracted from those tissues.

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Inhibition of Leukocyte Adhesion to Rat Mesenteric Venules In Vivo

Purpose and Rationale

Reversible adherence of leukocytes to endothelium, basement membranes, and other surfaces is an essential event in the establishment of inflammation. Their entry into tissues is controlled by the dynamic interaction between adhesion molecules expressed by these cells and the endothelium. White cells circulating in the blood have the tendency to adhere to the walls of blood vessels, and this tendency is greatly increased in states of inflammation. Normally, when leukocytes collide with the vessel wall, the collision behaves elastically and the cells bounce off and back into the lumen. However, biochemical changes in inflamed tissues result in inelastic collisions of cells and an increase in their adhesion, thus initiating rolling of leukocytes along the endothelial surface. As adhesion further increases, rolling is slowed and may be followed by the cells coming to a complete stop and their migration out of the vessel. This can be observed by preparing a mesenteric venule of an anesthetized rat and following the flow and rolling of leukocytes by means of a microscope, thus allowing in vivo studies. In this test procedure, adhesion of leukocytes, to the vessel wall, is artificially induced by the application of the formyl-methionyl peptide fMet-Leu-Phe (FMLP). Formyl peptides are released from bacteria and mitochondria of damaged tissue, so these peptides provide a specific signal marking the presence of invading bacteria or tissue damage. The density of FMLP receptors ranges from 10^4 to 10^5 per cell, depending on the cell type. Activation of leukocytes through this receptor results in rapid expression of preformed L-selectin (LECAM-1) on the cell surface which causes the cells to roll along the endothelial surface. LECAM-1 is very rapidly shed from the surface of leukocytes, however, and integrins take over to maintain further adhesion and migration into the tissue.

Procedure

Sprague–Dawley rats are anesthetized by administration of Nembutal. The trachea, jugular vein, and carotid artery are prepared free, the abdominal cavity is opened, and a section of the ileum is pulled out and draped over a heated microscope table. Prior to test compound administration, the number of spontaneous adhering leukocytes is counted, every 5 min, in a defined section of a venule (covered with paraffin oil) during a 30-min period (control). Blood pressure, body temperature, and velocity of blood flow are also registered. The test compound is administered via continuous infusion during the entire test procedure beginning at $t = -30$ min. Following the determination of control values for spontaneous adhesion, FMLP (f-Met-Leu-Phe, 10^{-4} M) is dripped twice ($t = -30$ min and $t = 0$ min) on the preparation, and the number of adhering leukocytes is determined every 5 min over a 90 min period, beginning with the second application of FMLP ($t = 0$ min). Each test group consists of at least ten animals. The mean leukocyte count of every rat prior to FMLP and/or test substance application is taken as the 100 % value to obtain the baseline for further comparisons. The test compounds are dissolved in 0.9 % NaCl shortly before application.

Evaluation

Following the second topical application of FMLP (10^{-4} M), the number of adhering leukocytes in the mesenteric venule section is counted 30 min after the stimulus is given and again at the end of the observation period of 2 h. The influence of a continuous i.v. infusion of the test drug is compared with the positive control group (FMLP stimulation, without drug).

Modifications of the Method

A simple, rapid, in vitro assay for granulocyte adherence was developed by MacGregor et al. (1974). Heparinized whole blood is filtered through nylon fibers packed in Pasteur pipettes, and the percentage of granulocytes adhering was calculated.

Neutrophil adherence was tested in vitro by Burch et al. (1992). Human umbilical vein endothelial cells were plated at 5×10^4 cells/well into collagen-coated culture plates and grown to

confluence. Neutrophils were labeled with ^{51}Cr. Experimental agents were added to the neutrophils before their activation with FMLP. After 15 min incubation at 37 °C, the nonadherent leukocytes were removed by gentle aspiration followed by a wash with saline. The adherent neutrophils were lysed by 1 N NaOH and the radioactivity was quantitated.

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Oxazolone-Induced Ear Edema in Mice

Purpose and Rationale

The oxazolone-induced ear edema model as first described by Evans et al. (1971) in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration.

Procedure

Mice of either sex with a weight of 25 g are used. Before each use a fresh 2 % solution of oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) in acetone is prepared. The mice are sensitized by application of 0.1 ml on the shaved abdominal skin or 0.01 ml on the inside of both ears under halothane anesthesia. The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2 % oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is solved. Special pipettes of 0.1 or 0.01 ml are used. Groups of 10–15 animals are treated with the irritant alone or with the solution of the test compound. The left ear remains untreated. The maximum of inflammation occurs 24 h later. At this time the animals are sacrificed under anesthesia, and a disk of 8 mm diameter is punched from both sides. The disks are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema.

Evaluation

Average values of the increase of weight are calculated for each treated group and compared statistically with the control group. A 0.003 % solution of hydrocortisone and a 1 % solution of indomethacin were found to be active.

Critical Assessment of the Method

The method is suitable for both steroidal and nonsteroidal compounds as well as for the evaluation of various topical formulations.

Modifications of the Method

Griswold et al. (1974) applied a 3 % solution of oxazolone to the left paw of mice. The edema was assessed plethysmographically.

Various cutaneous models of inflammation for the evaluation of topical and systemic pharmacological agents have been discussed by Young and Young (1989).

Bailey et al. (1995) described a contact hypersensitivity model in mice for rank-ordering formulated corticosteroids. Male Swiss Webster mice were sensitized with 20 µl of 2 % oxazolone on the inner and outer aspects of each ear (10 µl each side). Mice were challenged 7 days later with 2 % oxazolone in acetone/olive oil (4:1) on both sides of the right ear. Animals were topically treated with

corticosteroids or nonsteroidal anti-inflammatory drugs or 20 mg formulated corticosteroids immediately after challenge. The mice were sacrificed after 24 h and edema and myeloperoxidase activity were determined. Edema was measured by taking the weight of 6 mm trephine punch biopsies of the right and left ears. Inhibition was calculated from change in ear weight of control or drug-treated ears versus placebo-treated ears. Myeloperoxidase activity was assessed spectrophotometrically (Williams et al. 1983) on tissue homogenates. In a delayed-type hypersensitivity model, animals were treated as in the contact hypersensitivity model, except that the mice were sensitized with 40 µl of 2 % oxazolone in acetone/olive oil (4:1) on the unshaved inguinal areas.

Meingassner et al. (1997) studied anti-inflammatory activity using allergic contact dermatitis in **mice**, **rats**, and **pigs**. Mice were sensitized on the shaved abdomen with 50 µl of 2 % oxazolone solution in acetone. After 7 days, they were challenged with 10 µl of 2 % (for topical testing) or 0.5 % (for systemic testing) oxazolone on the inner surface of the right ears. Pinnal weight was taken as a measure of inflammatory edema 24 h after challenge. Female Sprague–Dawley *rats* were sensitized by application of 80 µl of 2,4-dinitrofluorobenzene solution applied in 20 µl volumes to the inner surface of both ear lobes and to both shaved inguinal regions on day 1. Allergic contact dermatitis was elicited with 30 µl of 0.5 % 2,4-dinitrofluorobenzene applied to the test sites of ≈15 mm in diameter on both shaved flanks on day 12. Animals were treated by gavage 2 h before and immediately after challenge. Dermatitis was evaluated by measuring the thickness of the lifted skin fold at the test sites with a spring-loaded micrometer. Domestic **pigs** were sensitized with 400 µl of 10 % 2,4-dinitrofluorobenzene applied to four areas on both ears and groins. Challenge reactions were elicited 12 days later with 15 µl of 2,4-dinitrofluorobenzene (1 %) applied topically to test sites arranged in four craniocaudal lines on the dorsolateral shaved back (24 or 32 per pig). Test sites were treated twice either with 20 µl solution of test compound or with ≈50 mg of a cream formulation applied topically 30 min and 6 h after challenge. One day after challenge, the test sites were visually evaluated for intensity and extent of erythema and induration.

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Croton Oil Ear Edema in Rats and Mice

Purpose and Rationale

The method has been developed primarily as a bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied steroids by Tonelli et al. (1965).

Procedure

For tests in mice the irritant is composed as follows (v/v): 1 part croton oil, 10 parts ethanol, 20 parts pyridine, and 69 parts ethyl ether. For tests in rats the following mixture is prepared (v/v): 4 parts croton oil, 10 parts ethanol, 20 parts pyridine, and 66 parts ethyl ether. The standards and the test compounds are dissolved in this solution. For tests in mice male NMRI mice with a weight of 22 g are used, and for tests in rats male Sprague–Dawley rats with a weight of 70 g are used. Ten animals are used for controls and each test group. The test compounds are dissolved in a concentration of 0.03 mg/ml to 1 mg/ml for mice and in a three to ten times higher concentration for rats in the irritant solution. On both sides of the right ear, 0.01 ml in mice or 0.02 ml in rats is applied. Controls receive only the irritant solvent. The left ear remains untreated. The irritant is applied under ether anesthesia. Four hours after application the animals are sacrificed under anesthesia. Both ears are removed and disks of 8 mm diameter are punched. The disks are weighed immediately, and the weight difference between the treated and untreated ear is recorded indicating the degree of inflammatory edema. In the originally described method, the ears are removed by sharp, straight scissors 6 h after application and weighed as total. The animals were sacrificed 48 h after topical administration, and the thymus glands were removed, weighed, and expressed as mg thymus/100 g body weight.

Evaluation

The antiphlogistic effect can be determined by expressing the increase in weight of the treated ear as percentage of the weight of the contralateral control ear. The difference of both weights is divided by the weight of the contralateral ear times 100. Otherwise, the difference between both ears or excised disks is calculated as the average values for treated and control groups, and the effect is evaluated by statistical methods. Concentration of 0.5–1 mg/ml hydrocortisone has been proven to be effective.

Critical Assessment of the Method

The method is useful for evaluation of anti-inflammatory topical steroids especially in the modification when thymus weight is determined simultaneously. The method also can be used for topically applied nonsteroidal antiphlogistics.

Modifications of the Method

Wilhelmi and Domenjoz (1951) tested various drugs using croton oil-induced ear edema in mice and rabbits.

Tubaro et al. (1985) tested various anti-inflammatory drugs in the croton oil test in mice. Granulocyte infiltration in plugs taken from the inflamed ears was assessed by measuring peroxidase activity.

Zentel and Töpert (1994) used croton oil-induced ear edema in rats to evaluate topical corticosteroids. A plastic collar was fixed around the neck of Wistar rats of either sex (160–200 g body weight) to exclude oral uptake of the compounds. Fifty microliters of 5 % croton oil in ethanol or ethanol alone was topically applied to both ears. In the treatment groups drugs were coapplied with croton oil. Five hours after treatment the animals were sacrificed by CO_2 gas and the ears removed. Edema formation was measured by the increase in wet weight.

Iwasaki et al. (1995) measured the inhibition of croton oil-induced ear edema in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of simultaneously applied RU 486.

Weirich et al. (1977) measured skin temperature, ear thickness, and weight of excised punches after croton oil-induced edema in the ears of **white rabbits** and calculated phlogostasis values as the products of the percent reduction in skin temperature, auricular thickness, and tissue weight in relation to controls. The authors recommended this model for the primary evaluation of topical anti-inflammatory agents.

Colorado et al. (1991) described an apparatus to measure croton oil-induced ear edema in mice using precisely reproducible pressure on the ear. The device allows to follow the time course of inflammation by repeated measurements.

Akiyama et al. (1994) studied *Staphylococcus aureus* infection on experimental croton oil-inflamed skin in mice. *Staphylococcus aureus* cells were inoculated on the surface of the skin inflamed by application of croton oil in cyclophosphamide-treated mice. Skin specimens were taken at 1, 3, 6, 12, and 24 h after inoculation and examined by microscopy. The *Staphylococcus aureus* cells which attached to the surface of the skin immediately after inoculation had invaded the horny layer within 1 h. The cells gradually penetrated deeper into the epidermis. Application of corticosteroid ointments decreased the number of *Staphylococcus aureus* cells in the lesions.

Anderson and Groth (1984) induced **toxic contact reactions** to croton oil or dinitrochlorobenzene (DNCB) or **allergic contact reactions** to DNCB or oxazolone in guinea pig skin and tested the effect of various locally applied corticosteroids by macroscopic assessment and microscopic evaluation of cellular infiltrates.

Tarayre et al. (1984) used a 0.25 % solution of **cantharidin** in acetone and applied 0.025 ml to one mouse ear. Two phases of inflammation were observed. After local application nonsteroidal drugs showed effects in the first phase only, whereas steroids influenced both phases.

De Young et al. (1987) induced ear inflammation in rats by intradermal injection of 10 ng **recombinant human interleukin-1*β*** in 10 µl of saline.

Maloff et al. (1989) injected 20 µl of interleukin-1 solution into the left ear of mice and found a dose-dependent increase of ear thickness and myeloperoxidase activity which reached the maximum after 24 h. These effects were reduced by high doses of glucocorticoids but not by nonsteroidal anti-inflammatory drugs.

Chang et al. (1987) applied 4 µg **tetradecanoyl phorbol acetate** and test drugs dissolved in acetone to the right ear of mice. Ear edema was calculated by subtracting the thickness of the left ear (vehicle control) from the right ear (treated ear).

De Young et al. (1989) examined the temporal patterns of edema and accumulation of the polymorphic nuclear cell marker enzyme myeloperoxidase following application of tetradecanoyl phorbol acetate to mouse ears. Topical and oral corticosteroids inhibited both edema and myeloperoxidase accumulation, whereby clobetasol propionate was more effective than fluocinolone and dexamethasone. Cyclooxygenase and lipoxygenase inhibitors were very effective against myeloperoxidase accumulation but were inactive or moderately active versus edema.

Murakawa et al. (2006) studied the involvement of tumor necrosis factor-α in phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice.

Topical application of arachidonic acid to mouse ear has become a widely used test (Young et al. 1983, 1984; Opas et al. 1985; Crummey et al. 1987; Hensby et al. 1987; Tomchek et al. 1991). One mg arachidonic acid is applied to the right ear of mice and vehicle to the left ear of each animal. Drugs are topically applied in acetone to the ear 30 min prior to the arachidonic acid application. Ear swelling was measured using a caliper 1 h after arachidonic acid.

Griswold et al. (1995) induced inflammation in mice by local application of arachidonic acid or phorbol ester. Besides ear thickness, myeloperoxidase and DNA content were measured.

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Paw Edema

Purpose and Rationale

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, aerosil, sulfated polysaccharides like carrageenan or naphthoylheparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant, and the paw volume of the treated animals is compared to the controls. Many methods have been described on how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short-lasting inflammation, whereas other irritants cause the paw edema to continue over more than 24 h.

Procedure

Male or female Sprague–Dawley rats with a body weight between 100 and 150 g are used. The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1 % solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge.

Apparatus

Various devices have been developed for plethysmography of the paw. Winter et al. (1963) used mercury for immersion of the paw. A more sophisticated apparatus has been described by Hofrichter et al. (1969). Alpermann and Magerkurth (1972) described an apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer. Webb and Griswold (1984) reported a sensitive method of measuring mouse paw volume by interfacing a Mettler Delta Range top-loading balance with a microcomputer. Several authors used a commercially available plethysmometer from Ugo Basile, Varese, Italy (Damas and Remacle-Volon 1992; Braga da Motta et al. 1994; Legat et al. 1994; Griesbacher et al. 1994).

Evaluation

The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated. The difference at the various time intervals gives some hints for the duration of the anti-inflammatory effect. A dose–response curve is run for active drugs and ED_{50} values can be determined.

Modifications

Many agents can be used as irritants to induce paw edema in rats or mice. These are:

0.05 ml	Undiluted fresh egg white (Randall and Baruth 1976)
0.1 ml	Of 1 % ovalbumin solution
0.1 ml	Of 1 % formalin
0.1 ml	Of 0.2 % carrageenan solution (Schönhöfer 1967)
0.1 ml	Of 1 % carrageenan solution plus 100 ng PGE_2 or PGI_2 (Higgs et al. 1978; Portanova et al. 1996)
0.1 ml	Of 1–3 % dextran solution
0.1 ml	Of 2.5 % brewer's yeast powder suspension (Tsumuri et al. 1986)
0.1 ml	Of 0.5 % β-naphthoylheparamine solution (Peterfalvi et al. 1966)
0.1 ml	Of 0.1 % trypsin solution (Kalbhen and Smalla 1977)
0.1 ml	Of 0.1 % collagenase solution (Souza Pinto et al. 1995)

(*continued*)

0.1 ml	Of 0.1 % solution of collagenase from *Clostridium histolyticum* (Legat et al. 1994)
0.1 ml	Of solution of 100 IU hyaluronidase (Dewes 1955; Kalbhen and Smalla 1977)
0.1 ml	Of complete Freund's adjuvant
0.05 ml	Of 0.02 % serotonin solution (Kalbhen and Smalla 1977)
0.1 ml	Of 0.005 % bradykinin solution (Damas and Remacle-Volon 1992)
0.1 ml	Of 0.1 mg/ml prostaglandin E2 (Nikolov et al. 1978)
0.1 ml	Of 2.0 µg/ml prostaglandin E2 (repeated injections, Willis and Cornelsen 1973)
0.1 ml	Of 1 % concanavalin A solution (Lewis et al. 1976)
0.1 ml	Of 2.5 % suspension of aerosil
0.1 ml	Of 5 % suspension of kaolin (Lorenz 1961; Wagner-Jauregg et al. 1962)
0.05 ml	Of bentonite gel (Marek 1980)
0.1 ml	Of nystatin 15,000 units (Schiatti et al. 1970; Arrigoni-Martelli et al. 1971)
0.1 ml	Of 1 % phytohemagglutinin-P solution (Lewis et al. 1976)
0.01 ml	Of 0.5 % adriamycin (mouse paw) (Siegel et al. 1980)
0.1 ml	Of 0.001–0.1 % solutions of various phospholipases A2 (Cirino et al. 1989)
0.1 ml	Of 0.1 % zymosan solution (Gemmell et al. 1979)
0.1 ml	Of 0.05 % anti^{-}IgG solution (Gemmell et al. 1979)
0.1 ml	Of 2.5 % mustard powder suspension (Tsumuri et al. 1986)
0.1 ml	Of solution containing one unit of cobra venom factor(Leyck and Parnham 1990)
0.05 ml	Of 0.02–0.2 % sonic extract from *Porphyromonas gingivalis* (Griesbacher et al. 1994)
0.1 ml	Of 0.25 % suspension of papaya latex (Gupta et al. 1994)

The edema induced by the various irritants lasts for different times such as a few hours after serotonin and up to 2 days after aerosil or after kaolin. These irritants therefore are suitable to study not only the degree but also the duration of the anti-inflammatory action.

Standards

Depending on the irritant, steroidal and nonsteroidal anti-inflammatory drugs have a pronounced effect in the paw edema test. With carrageenan irritant doses of 50–100 mg/kg phenylbutazone p.o. have been found to be effective.

Critical Assessment of the Method

The paw edema method has been used by many investigators and has been proven to be suitable for screening purposes as well as for more in-depth evaluations. Dependent on the irritant, steroidal and nonsteroidal anti-inflammatory drugs, antihistaminics and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators, the results are often difficult to compare.

Further Modifications of the Method

Besides paw volume, Shirota et al. (1984) determined the surface temperature of the inflamed paw in rats using a special cage with rolling rods.

Kunz et al. (2004) assessed protein patterns in lumbar cord during a zymosan-induced paw inflammation in rats employing a two-dimensional gel electrophoresis revealing a time-dependent breakdown of scaffolding proteins such as neurofilament light chain protein. A calpain inhibitor prevented inflammation-induced neurofilament light chain breakdown in the spinal cord and reduced hyperalgesia.

Brooks et al. (1991) used anesthetized **dogs** and demonstrated that a significant inflammatory response can be elicited in the dog paw by subcutaneous injection of carrageenan. The increase in paw volume can be quantitatively measured as a pressure change recorded via a water-filled balloon fixed against the paw with nonexpandable tape. Effective doses of nonsteroidal anti-inflammatory drugs were closer to human therapeutic doses in dogs than in rats.

Oyanagui and Sato (1991) described an **ischemic paw assay in mice**. A commercial rubber ring (1×1 mm, $d = 42$ mm) was bound 14 times to the right hind leg of mice just above the articulation. After 20 min of ischemia, the rubber was cut off with scissors. Paw swelling was measured after another 20 min of natural blood recirculation.

Wirth et al. (1992) described a **thermic edema** which was induced in anesthetized

Sprague–Dawley rats by immersing paws of the right and left hind limb into water of 55 °C. Immediately thereafter, the rats received the test drug (the bradykinin antagonist Hoe 140) intravenously. Paw volume was measured at regular intervals by plethysmography.

Braga da Motta et al. (1994) described drug modulation of **antigen-induced paw edema in guinea pigs**. Male short-haired guinea pigs weighing 250–350 g received on day 0 a single dorsal s.c. injection of 1 ml of phosphate-buffered saline containing 20 μg of ovalbumin, dispersed in 1 mg $Al(OH)_3$. The animals were boosted with a similar injection of antigen on days 14, 21, and 28. Thirty-five days after the first injection of antigen or $Al(OH)_3$, the animals received an intraplantar injection of 0.5, 5, 50, or 200 μg ovalbumin, diluted in 100 μl of phosphate-buffered saline. Edema was measured 2, 4, 6, 8, 24, and 48 h after the challenge.

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Pleurisy Test

Purpose and Rationale

Pleurisy is a well-known phenomenon of exudative inflammation in man. In experimental animals pleurisy can be induced by several irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan (Survey by De Brito 1989). Carrageenan-induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration, and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate.

Procedure

Male Sprague–Dawley rats weighing 220–260 g are used. The animal is lightly anesthetized with ether, placed on its back, and the hair from skin over the ribs of the right side is removed using animal clippers. The region is swabbed with alcohol. A small incision is made into the skin under the right arm between the seventh and eighth rib. The wound is opened and a further shallow incision is made into the exposed intercostal muscle. 0.1 ml of 2 % carrageenan solution is injected into the pleural cavity through this incision. The injection needs to be made swiftly to avoid the risk of injuring the lung. The wound is closed with a Michel clip.

One hour before carrageenan injection and 24 and 48 h thereafter, groups of ten rats are treated with the standard or the test compound subcutaneously or orally. A control group receives only the vehicle of medication. The animals are sacrificed 72 h after carrageenan injection by ether inhalation. The animal is pinned on a dissection board with the forelimbs fully extended. An incision in the skin over the xiphisternal cartilage is made to free the cartilage from overlying connective tissue. The cartilage is lifted with a forceps, and a small cut is made with scissors in the body wall below to gain access into the pleural cavity. One ml of heparinized Hank's solution is injected into the pleural cavity through this cut. The cavity is gently massaged to mix its contents. The fluid is aspirated out of the cavity using a pipette. This is made easier if the dissection board is raised to an angle of 45–60°; the contents then pool in the corners of the cavity. The aspirated exudate is collected in a graduated plastic tube.

Evaluation

One milliliter (the added Hank's solution) is subtracted from the measured volume. The values of each experimental group are averaged and compared with the control group. ED_{50} values can be calculated using various doses. Several other parameters can be used:

- Measuring the white blood cell number in the exudate using a Coulter counter or a hematocytometer
- Determination of lysosomal enzyme activities
- Determination of fibronectin
- Determination of PgE_2

Critical Assessment of the Method

The pleurisy model has been accepted as a reliable method to study acute and subacute inflammation allowing the determination of several parameters simultaneously or successively. The activity of steroids as well as of nonsteroidal drugs can be measured (Tomlinson et al. 1994; Harada et al. 1996).

Modifications of the Method

The Evans blue–carrageenan-induced pleural effusion model has been proposed by Sancilio (1969, 1973) for screening of compounds with anti-inflammatory activity.

Meyers et al. (1993) tested the effect of treatment with interleukin-1 receptor antagonist on the

development of carrageenan-induced pleurisy in intact and adrenalectomized rats.

Fröde et al. (2001, 2002) tested the effects of TNF-α and IL-1β, IL-6, and IL-10 and their specific antibodies in the acute inflammatory response induced by carrageenan in a **mouse** model of pleurisy. Adult Swiss mice received a single intrapleural injection of 0.1 ml of sterile saline containing 1 % carrageenan. As the inflammatory response caused by carrageenan in the pleural space of the mice exhibits a biphasic response, peaking at 4 h, characterized primarily by neutrophils, and at 48 h due mainly to mononuclear cells, both interval points were studied. On the day of the experiment, different doses of TNF-α, IL-1β, IL-6, or IL-10 and their antibodies were injected into the pleural cavity of anesthetized mice. After 4 h or 48 h, the animals were sacrificed, the thorax was opened, and the pleural cavity was washed with 1 ml of sterile PBS containing 20 IU/ml heparin. All animals had been injected 60 min previously with a solution of Evans blue dye (25 mg/kg, 0.2 ml, i.v.) in order to evaluate the degree of exudation into the pleural space. Leukocytes were counted and evaluated microscopically. The amount of dye was estimated by colorimetry.

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Granuloma Pouch Technique

Purpose and Rationale

The method originally invented by Selye has been developed for screening by Robert and Nezamis (1957) using croton oil as irritant. An aseptic inflammation resulting in large volumes of hemorrhage exudate is elicited which resembles the subacute type of inflammation. Instead of croton oil, carrageenan can be used as irritant.

Procedure

Male or female Sprague–Dawley rats with a body weight between 150 and 200 g are used. Ten animals are taken for controls and for test groups. The back of the animals is shaved and disinfected. With a very thin needle, a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anesthesia. Into the resulting oval air pouch 0.5 ml of a 1 % solution of croton oil in sesame oil is injected avoiding any leakage of air. Forty-eight hours later the air is withdrawn from the pouch, and 72 h later any resulting adhesions are broken. Instead of croton oil 1 ml of a 20 % suspension of carrageenan in sesame oil can be used as irritant. Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard. For testing local activity, the test compound is injected directly into the air sac at the same time as the irritant. On the fourth or the fifth day, the animals are sacrificed under anesthesia. The pouch is opened and the exudate is collected in glass cylinders. Controls have an exudate volume between 6 and 12 ml, which is reduced dose dependently in the treated animals.

Evaluation

The average value of the exudate of the controls and the test groups is calculated. Comparison is made by statistical means. A clear dose response curve could be found by s.c. injection of 0.5, 1.0, and 2.0 mg hydrocortisone acetate/rat. Also doses of 1.5 mg/kg indomethacin were found to be active.

Critical Assessment of the Method

The method has been very useful to estimate the potency of anti-inflammatory corticosteroids both after local and after systemic application. By injection of a depot-preparation and induction of the granuloma pouch after various time intervals up to 4 weeks, the duration of action can also be determined (Vogel 1963, 1965).

Modifications of the Method

Carrageenan was used to induce exudate formation (Boris and Stevenson 1965).

Bobalik and Bastian (1967) developed a modified granuloma pouch technique in which *Mycobacterium butyricum* (adjuvant) was used as phlogistic agent.

Moreno (1993) sensitized rats by subcutaneous injection of methylated bovine serum albumin emulsified in complete Freund's adjuvant 1 week prior to the preparation of air pouches which were reinflated 4 days later. Seven days after formation of the air pouches, inflammation was induced in the pouches by injection of 1 mg methylated bovine serum albumin.

Martin et al. (1994) described an air pouch model in the 6-day-old rat by injection of carrageenan. Besides the usual parameters, leukocyte influx and the level of prostaglandin E_2 in the pouch exudate were measured.

In order to measure the effects of different classes of proteinase inhibitors, Karran and Harper (1995) studied collagen degradation in subcutaneous air pouches in rats. The air pouches were formed in the dorsal region and were inflamed 6–8 days later by injecting λ-type carrageenan. Degradation of ^{14}C-collagen was followed in the inflammatory exudate fluid of the air pouches.

Sugio and Tsurufuji (1981) reevaluated the vascular constriction hypothesis as the mechanism of anti-inflammatory action of glucocorticoids. Rats were injected with 8 ml of air subcutaneously on the dorsal surface under light ether anesthesia to make an oval air sac. After 24 h, 4.0 ml of 2 % heat-sterilized solution of carrageenan in 0.9 % NaCl solution was injected into the air sac (day 0). Drug effects were tested on day 7. Vascular permeability in the granuloma pouch was measured using ^{125}I-HSA and ^{131}I-HSA. About 1 μCi of purified ^{125}I-HSA in 0.2 ml saline was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was withdrawn to measure the leakage of ^{125}I-HSA into the pouch fluid. After administration of the drug, about 1 μCi of purified ^{131}I-HSA was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was again withdrawn to measure the concentration of ^{131}I-HSA. The ratio of ^{125}I-HSA/^{131}I-HSA was taken as an index of vascular permeability change induced by drug treatment.

Atkinson et al. (1962) implanted compressed pellets of carrageenan subcutaneously to rats and measured the effects of some anti-inflammatory substances on wet weight of the pellets.

Bowers et al. (1985) described a method to induce a **granuloma in the rat lung** by instillation of a 2 % carrageenan solution into one lower lobe of the lung via the trachea. No respiratory impairment was noticed during this procedure.

Further phlogistic agents inducing specific inflammatory cascades such as zymosan (complement activation) or lipopolysaccharide (cytokine release) have been used for pharmacological evaluation of anti-inflammatory agents (Erdö et al. 1994; Miller et al. 1997).

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Urate-Induced Synovitis

Purpose and Rationale

The importance of urate in gout and the deposition of sodium urate in gouty tophi are well known. Faires and McCarty (1962) reported that they themselves were the subjects for a study injecting 20 mg sodium urate crystal suspension in their own knee joint. They experienced severe pain and prostration which resembled an acute gouty attack. Based on this experience they developed an experimental model in dogs for testing anti-inflammatory compounds (McCarty and Faires 1963; McCarty et al. 1966).

Procedure

Preparation of Sodium Urate Crystals

0.4 g (0.01 Mol) sodium hydroxide pellets is dissolved in 400 ml distilled water in a glass beaker; 1.68 g (0.01 Mol) uric acid is added. The resultant opaque preparation is allowed to remain overnight at room temperature. The next morning, the crystals are harvested by decanting the supernatant solution and are then washed three times in cold saline, resuspended in saline, and sterilized in an autoclave. Suspensions for injections are kept in rubber-stoppered, multi-dose vials containing 15–24 mg of urate per ml.

Unanesthetized healthy dogs weighing between 18 and 25 kg are used. They are trained to lie quietly on their backs in a dog cradle under light restraint. The skin above one knee is shaved and disinfected and a sterile 21-gauge needle inserted into the joint. Slight aspiration produces a small amount of clear, viscous synovial fluid, indicating entry into the joint. The needle is left in place, a syringe containing the urate suspension is attached, and volumes from 0.1 to 0.5 ml are injected into the joint (approximately 2–10 mg urate).

One hour before the injection of urate crystals, the animals are treated with the test compound or the standard. Experiments are designed so that a pair of dogs is tested on each of 2 days. On the first day, only one dog receives the drug. One week later the opposite knee of each dog is injected, but the other dog is treated.

Evaluation

A scoring system is adopted in which inflammatory symptoms ranging from tenderness, limping, and occasional 3-legged gait to complete 3-legged gait are scored from 1+ to 4+.

Critical Assessment of the Method

In spite of the fact that the experiment originally has been performed in human volunteers and that the method closely resembles pathological conditions in man, due to animal protection law conditions, the method can be recommended only for special investigations.

Modifications of the Method

Carlson et al. (1986) developed an automated microcomputer-based system for determining canine paw pressure quantitatively in the dog synovitis model.

According to Phelps and McCarty (1967), dogs are anesthetized and placed on their sides with the hind leg firmly fixed with tape so that the femur and tibia form a 90° angle. The knee is punctured with a needle. When a few drops of synovial fluid can be withdrawn indicating a correct puncture of the joint, 6–10 ml of saline is injected to distend the joint, and a polyethylene catheter is inserted through the needle, which is then withdrawn. 0.5 ml of a 0.02 % sodium urate suspension is injected into the joint. The catheter is attached to a pressure transducer. Constant pressure recordings can be taken during the acute phase of inflammation. Pressure changes are plotted against time, whereby each dog is compared

with his own control. Treatment with nonsteroidal anti-inflammatory drugs, such as indomethacin, shows a considerable reduction of intra-articular pressure.

Fujihira et al. (1971) injected the urate suspension into the knee joint of a hind leg of well-trained Beagle dogs. They were placed on three weighing machines whereby both forelegs rested on one balance and the hind legs individually on other balances. In this way, the relative change of weight on each hind leg after intra-articular injection of urate suspension can be measured, indicating a decrease of weight in the injected leg, counterbalanced to the other leg. Time response curves could be found after nonsteroidal anti-inflammatory drugs.

Rosenthale et al. (1972) found a long-lasting inflammatory effect of prostaglandins PGE_1 and PGE_2 after injection in the knee joint of dogs.

Schaible and Schmidt (1985) induced an acute experimental arthritis in the knee joint of anesthetized **cats** by intra-articular injection of a 4 % kaolin suspension and recorded the activity of single fine afferent units from filaments of the saphenous nerve.

Perkins and Campell (1992) injected either sodium urate crystals or Freund's complete adjuvant into one knee of **rats**. The maximum tolerated pressure was determined with or without treatment by analgesic drugs after 18–24 h (urate injections) or 64–70 h (Freund's complete adjuvant).

Daniel and Jouvin (1984) induced inflammation of the **guinea pig palatal mucosa** by injection of a microcrystalline suspension of monosodium urate.

Botrel et al. (1994) induced chronic inflammation in the knee joint of Beagle dogs by intra-articular injection of Freund's complete adjuvant. Besides body temperature, differences in skin temperature, and difference in stifle diameter, the vertical force exerted by the arthritic hind limb measured by a force plate was chosen as parameter.

Schött et al. (1994) induced monoarthritis in rats by injection of 300 µg carrageenan in 50 µl saline into the right tibiotarsal joint. Weight bearing was found to be an objective measure of arthritic pain.

Carleson et al. (1996) induced acute inflammation in the temporomandibular joint of rats by intra-articular injection of substance P and measured neurokinin A, calcitonin gene-related peptide, and neuropeptide Y in the perfusate.

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Methods for Testing the Proliferative Phase (Granuloma Formation)

Cotton-Wool Granuloma

Purpose and Rationale

The method has been described first by Meier et al. (1950) who showed that foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan.

Procedure

Male Wistar rats with an average weight of 200 g are anesthetized with ether. The back skin is shaved and disinfected with 70 % ethanol. An incision is made in the lumbar region. By a blunted forceps subcutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region. The pellets are either standardized for use in dentistry weighing 20 mg or pellets formed from raw cotton which produce a more pronounced inflammation than bleached cotton. The animals are treated for 7 days subcutaneously or orally. Then, the animals are sacrificed, and the pellets prepared and dried until the weight remains constant. The net dry weight, i.e., after subtracting the weight of the cotton pellet, is determined.

Evaluation

The average weight of the pellets of the control group as well as of the test group is calculated. The percent change of granuloma weight relative to vehicle control group is determined.

Critical Assessment of the Method

The method has been useful for the evaluation of steroidal and nonsteroidal anti-inflammatory

drugs. For testing corticosteroids, the test can be performed in adrenalectomized rats.

Modifications of the Method

Bush and Alexander (1960) produced granulomata in rats by means of cotton-wool pellets which have been impregnated with carrageenan.

Tanaka et al. (1960) implanted filter paper pellets soaked with 7 % formalin solution in rats.

Hicks (1969) implanted pellets impregnated with irritant substances, such as capsicum oleoresin.

Instead of cotton pellets, paper disks have been implanted (Tsurumi et al. 1986).

Roszkowski et al. (1971) immersed the cotton pellets in a 1 % carrageenan solution, dried overnight and soaked in a 0.25 oxytetracycline solution before implantation.

Rudas (1960) described a method for quantitative evaluation of the granulation tissue formed in experimental wounds. Plastic rings were incorporated into the wounds on the back of rats inhibiting contraction of the wound edges and epithelialization of the wound. The growth of granulation tissue inside the rings was measured.

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Sponge Implantation Technique

Purpose and Rationale

The sponge implantation technique was described first by Saxena (1960) for short-term experiments but was used subsequently to study the formation of granulomata using long-term implantation.

Procedure

Sponges used for implantation are prepared from polyvinyl foam sheets (thickness 5 mm). Disks are punched out to a standard size and weight (10.0 ± 0.02 mg) using a 13-mm cork borer. The sponges are then soaked in 70 % v/v ethanol for 30 min, rinsed four times with distilled water, and heated at 80 °C for 2 h. Prior to implantation in the animal, the sponges are soaked in sterile 0.9 % saline in which either drugs, antigens, or irritants have been suspended. Typical examples include 1 % carrageenan, 1 % yeast, 1 % zymosan A, 6 % dextran, heat-killed *Bordetella pertussis* (4×10^9 to 5×10^{10} organisms/ml), or 0.5 % heat-killed *Mycobacterium tuberculosis*.

Sponges are implanted in female Wistar rats weighing 150–200 g under ether anesthesia. A 20-mm dorsal incision is made and the dermis separated from the underlying muscle layer by insertion of blunt forceps to form separate cavities into which sponges are inserted. Up to eight sponges may be implanted per rat. The

dorsal incision is closed with Michel clips and the animals are maintained at a constant temperature of 24 °C.

For short-term experiments, the animals are treated with test drug or standard once before implantation orally or subcutaneously. For long-term experiments, the rats are treated daily up to 3 weeks.

Evaluation

For estimation of the fluid phase of sponge exudates, e.g., protein content, enzyme levels, and biological mediators such as prostaglandins as well as for leukocyte migration, the sponges are removed already after 9 h.

For studying the chronic phase of inflammation besides dry weight DNA, indicating cell content, hexosamine, indicating glycosaminoglycan content, and hydroxyproline, indicating collagen content, can be determined.

Critical Assessment of the Method

The sponge implantation technique has been proven to be a versatile method which was used and modified by many investigators.

Modifications of the Method

Boucek and Noble (1955) implanted polyvinyl sponges in rats, hamsters, rabbits, and humans.

Holm-Pedersen and Zederfeldt (1971) implanted two cubes 10 × 10 × 10 mm of cellulose sponge connected with a silk suture. After various implantation periods, the sponges were dissected free, and the strength of the connection between the two parts of the sponge was determined after removal of the connecting suture.

Paulini et al. (1974, 1976) implanted polyester–polyurethane sponges which were inserted at both ends of a 15-mm-long PVC tube separated by a cotton-wool plug.

Bonta et al. (1979) used polyether sponges measuring 4 × 1.5 × 0.5 cm. A thin polyethylene cannula is inserted into a hole of the sponge and fixed with two stitches. After implantation of the sponge, the cannula is pulled through a subdermal tunnel to a neck incision where about 1.5 cm is exteriorized and closed with a tube sealer. One ml of a 2 % carrageenan solution is injected into the sponge via the cannula. To study the local effect of drugs, the test compounds can be injected together with the carrageenan. The drugs can be administered repeatedly at any time.

The cannulated sponge method was further modified by Bragt et al. (1980) using a subdermally implanted Teflon cylinder. This cylinder is provided with holes to ensure contact and exchange between the inner chamber and the surrounding tissue and with two cannulae allowing injection of material and withdrawal of exudate at any given stage of granuloma development.

Damas and Remacle-Volon (1992) implanted in rats sterilized polyester sponges which were removed after 4 h and weighed.

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Glass Rod Granuloma

Purpose and Rationale

The glass rod granuloma as first described by Vogel (1970) reflects the chronic proliferative inflammation. Of the newly formed connective tissue, not only wet and dry weight but also chemical composition and mechanical properties can be measured.

Procedure

Glass rods with a diameter of 6 mm are cut to a length of 40 mm and the ends rounded off by flame melting. They are sterilized before implantation by boiling in water. Male Sprague–Dawley rats with an initial weight of 130 g are anesthetized with ether, and the back skin is shaved and disinfected. From an incision in the caudal region, a subcutaneous tunnel is formed in cranial direction with a closed blunted forceps. One glass rod is introduced into this tunnel finally lying on the back of the animal. The incision wound is closed by sutures. The animals are kept in separate cages. The rods remain in situ for 20 or 40 days. Treatment with drugs is either during the whole period or only during the last 10 or 2 days. At the end the animals are sacrificed under CO_2 anesthesia. The glass rods are prepared together with the surrounding connective tissue which forms a tube around the glass rod. By incision at one end, the glass rod is extracted and the granuloma sac inverted forming a plain piece of pure connective tissue. Wet weight of the granuloma tissue is recorded. The specimens are kept in a humid chamber until further analysis. For measurement of the mechanical properties, the specimens are fixed into the clamps of the Instron$^{(R)}$ instrument allowing a gauge length of 30 mm. The load until break is recorded with a crosshead speed of 50 mm/min. In order to calculate tensile strength (N/mm^2), the value of load at rupture (N) is divided by cross-sectional area (measured as volume = wet weight divided by length). Finally, the granuloma tissue is dried and the dry weight is recorded. In addition, biochemical analyses, such as determination of collagen and glycosaminoglycans, can be performed.

Evaluation

Several parameters can be determined by this method. Granuloma weight was reduced by corticosteroids depending on the dose and time of administration and was also diminished after treatment with nonsteroidal anti-inflammatory agents and lathyrogenic compounds. Furthermore, antiproliferative terpenoids reduced the granuloma weight. The mechanical parameters showed different results after these drugs indicating a different mode of action. Treatment with corticosteroids increased tensile strength. Only after long-term treatment with toxic doses a decrease was found. Anti-inflammatory compounds, such as acetylsalicylic acid or indomethacin and antiproliferative terpenoids, showed an increase of strength at medium and high doses.

Critical Assessment of the Method

In contrast to most other granuloma methods, the glass rod granuloma measures the late proliferative phase of inflammation. Since the newly formed connective tissue is not contaminated with the irritant, biochemical analyses can be performed. The peculiar feature is the possibility to study the mechanical properties of newly formed proliferative connective tissue.

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See Vogel (2006).

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Influence of Cytokines

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Methods for Testing the Proliferative Phase (Granuloma Formation)

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Anti-Pyretic Activity

Vino Daniel

Contents

General Considerations

Treatment with antipyretics has been very important in the pre-antibiotic era. Nevertheless, for treatment of acute viral diseases and for treatment of protozoal infections like malaria, the reduction of elevated body temperature by antipyretics is still necessary. For anti-inflammatory compounds, an antipyretic activity is regarded as a positive side effect. To evaluate these properties, fever is induced in rabbits or rats by injection of lipopolysaccharides or brewer's yeast.

Antipyretic Testing in Rats

Purpose and Rationale

The subcutaneous injection of brewer's yeast suspension is known to produce fever in rats. A decrease in temperature can be achieved by the administration of compounds with antipyretic activity.

Procedure

A 15 % suspension of brewer's yeast in 0.9 % saline is prepared. Groups of six male or female Wistar rats with a body weight of 150 g are used. By the insertion of a thermocouple to a depth of 2 cm into the rectum, the initial rectal temperatures are recorded. The animals are fevered by an

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injection of 10 ml/kg of brewer's yeast suspension subcutaneously in the back below the nape of the neck. The site of injection is massaged in order to spread the suspension beneath the skin. The room temperature is kept at 22–24 °C. Immediately after yeast administration, food is withdrawn. Eighteen hours post challenge, the rise in rectal temperature is recorded. The measurement is repeated after 30 min. Only animals with a body temperature of at least 38 °C are taken into the test. The animals receive the test compound or the standard drug by oral administration. Rectal temperatures are recorded again 30, 60, 120, and 180 min post dosing.

Evaluation

The differences between the actual values and the starting values are registered for each time interval. The maximum reduction in rectal temperature in comparison to the control group is calculated. The results are compared with the effect of standard drugs, e.g., aminophenazone 100 mg/kg p.o. or phenacetin 100 mg/kg p.o.

Critical Assessment of the Method

The antipyresis test in rats can be regarded as a classical method in pharmacology.

Modifications of the Method

Stitt and Shimada (1991) and Shimada et al. (1994) induced fever in rats by microinjecting 20 ng PGE_1 directly into one of the brain's circumventricular organs of the rat known as the organum vasculosum laminae terminalis.

Luheshi et al. (1996) induced fever by intraperitoneal injection of 100 µg/kg lipopolysaccharide into rats and measured the inhibition of fever by an interleukin-1 receptor antagonist.

Telemetry has been used to record body temperature in animals (Riley et al. 1978; Gallaher et al. 1985; Clement et al. 1989; Guillet et al. 1990; Kluger et al. 1990; Bejanian et al. 1991; Watkinson et al. 1996; Miller et al. 1997.

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Antipyretic Testing in Rabbits

Purpose and Rationale

Lipopolysaccharides from Gram-negative bacteria, e.g., *E. coli*, induce fever in rabbits after intravenous injection. Only lipopolysaccharide fractions are suitable, which cause after 60 min an increase of body temperature of 1 °C or more at a dose between 0.1 and 0.2 μg/kg. In the rabbit, two maxima of temperature increases are observed. The first maximum occurs after 70 min, the second after 3 h.

Procedure

Rabbits of both sexes and of various strains with a body weight between 3 and 5 kg can be used. The animals are placed into suitable cages and thermocouples connected with an automatic recorder are introduced into the rectum. The animals are allowed to adapt to the cages for 60 min. Then, 0.2 ml/kg containing 0.2 μg lipopolysaccharide is injected intravenously into the rabbit ear. Sixty minute later, the test compound is administered either subcutaneously or orally. Body temperature is monitored for at least 3 h.

Evaluation

A decrease of body temperature for at least 0.5 °C for more than 30 min as compared with the temperature value before administration of the test compound is regarded as positive effect. This result has been found after 45 mg/kg phenylbutazone s.c. or 2.5 mg/kg indomethacin s.c.

Critical Assessment of the Method

The measurement of body temperature in rabbits with polysaccharide-induced fever is a more sensitive test than the yeast fever in rats. Furthermore, the method is used as a decisive test for the absence of pyrogens in parenteral drugs by several pharmacopoeias such as USP 23 (1995).

Modifications of the Method

Cashin and Heading (1968) described a simple and reliable assay for antipyretic drugs in **mice**, using an intracerebral injection of pyrogens.

Davidson et al. (1991) tested the effect of human recombinant lipocortin on the pyrogenic action of the synthetic polyribonucleotide polyinosinic-polycytidylic acid in rabbits.

Yeast-induced pyrexia in **rats** has been used for antipyretic efficacy testing by Loux et al. (1972) and Cashin et al. (1977).

van Miert et al. (1977) studied the effects of antipyretic agents on fever and ruminal stasis induced by endotoxins in **conscious goats**.

Petrova et al. (1978) used turpentine-induced fever in rabbits to study antipyretic effects of dipyrone and acetylsalicylic acid.

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Shimada et al. (1994) studied the mechanism of action of the mild analgesic dipyrone preventing fever induced by injection of prostaglandin E_1 or interleukin-1β into the organum vasculosum terminalis of the rat brain.

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Part IX

Antiarthrotic and Immunmodulatory Activity

Antiarthrotic Activity

Hans Gerhard Vogel and Martin Braddock

Contents

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General Considerations

Multifactorial causes can lead to osteoarthritis (OA), and its pathogenesis is not clearly understood yet (Berenbaum 2013; Santos et al. 2014; Liu-Bryan and Terkeltaub 2015). The main characteristics of OA are the slowly progressing deterioration of the articular cartilage, accompanied by intermitted painful inflammatory episodes, and a continuous subchondral bone remodeling, often resulting in osteophyte formation in non-weight-bearing joint areas. Because of the lack of innervation and vascularization of the cartilage, the destruction of this specific tissue remains unnoticed until other joint compartments are involved such as the synovial membranes, answering with reactive synovitis to cartilage debris, or mechanoreception changes in the underlying bone, or until the diminution of the articular cartilage results in a radiographically detectable joint space narrowing.

Therefore, analgesic and anti-inflammatory therapy has been the major treatment for OA in the past, and NSAIDs still constitute the majority of the drugs used against OA according to guidelines (Stanos 2013). Intra-articular injection of corticosteroids is also applied, although with geographically variable emphasis. Nevertheless, they are still considered a useful tool in severe cases. Both classes of drugs are now reviewed more carefully with regard to their potentially harmful effects on cartilage maintenance and chondrocyte function – which leads to a more critical approach

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in drug selection. A variety of chondroprotective drugs (Verbruggen 2006), mainly sulfated polysaccharides, may play a certain role in the pharmacotherapy of OA; however, an unequivocal clinical benefit may await further study and evaluation (Van Vijen et al. 2012; Junior and Inacio 2013).

Antioxidative enzymes or drugs such as superoxide dismutase or diacerein are also considered to influence osteoarthritic conditions, and a number of nutraceuticals have been identified which may confer chondroprotection in nonclinical and clinical studies (Jerosch 2011; Leong et al. 2013; de Campos 2014). Recently, injection of a platelet-rich plasma formulation has shown benefit in patients (Mangone et al. 2014), and further studies are awaited. Matrix metalloproteinase (MMP) inhibitors originally designed to inhibit tumor cell invasion have shown promising results in counteracting the progressive enzymatic cartilage degradation in nonclinical studies, and despite a lack of clinical success, some alternative approaches to targeting MMPs are being considered for this indication. A further treatment of interest is hyaluronic acid or derivatives, which are applied intra-articularly in a series of injections. Their mechanism of action, however, is not yet clear, and clinical benefit appears not to be equivocal (Wang et al. 2004; Petrella 2005; Currain 2010; Tashiro et al. 2012; Ishijima et al. 2014; Mladenovic et al. 2014), with most preparations having been filed as "devices" rather than "drugs," claiming a viscosupplementation with anti-inflammatory, analgesic, and chondroprotective properties.

In Vitro Methods for Anti-osteoarthritic Activity

General Considerations

Since most of the drugs in use for OA were originally selected for other (e.g., arthritic) indications and only subsequently claimed to be effective in OA, they have not been primarily selected and optimized by in vitro assays specific for this condition. Thus, the indication lacks commonly agreed upon in vitro assays as well as clearly defined standard drugs to evaluate such models. Correspondingly, the variety of assay systems used to test compounds for their effect on cartilage maintenance and/or degradation is large, and the list below reflects this multitude.

The in vitro systems applied to assess drug effects upon chondrocytes range from homogenates of cartilage (Yu et al. 1991; Vignon et al. 1991; Zafarullah et al. 1992; Schlichtling et al. 2014) over chondrocyte monolayers at different culture conditions and passages, suspensions of aggregated chondrocytes or clusters, cells cultured in or over an artificial matrix like agarose to cartilage explants, models of degradation (Grenier et al. 2014), mechanical compression (de Isla et al. 2012), injury (Lee et al. 2013), three-dimensional culture (Lozito et al. 2013), and even organ cultures (Korver et al. 1989; Ono et al. 2014) like mouse patellas (Verschure et al. 1994). The species used in these studies vary over an equally wide range from mouse (Mohamed-Ali 1992), rat (Ismaiel et al. 1991; Seed et al. 1993; Seong et al. 1994; Srinivas et al. 1994), rabbit (Akatsuka et al. 1993; Collier and Ghosh 1991; Shimazu et al. 1993), dog (Venn et al. 1990), cattle, to human tissues (Bulstra et al. 1992; Green et al. 1995; Chu et al. 2008) derived from normal as well as osteoarthritic conditions. The chondrocyte and cartilage explant culture systems used for several years are described in more detail below with special emphasis on comparability and standardization.

Purpose and Rationale

Articular chondrocytes not only control the regular balance of matrix synthesis and degradation in healthy cartilage turnover but are also regarded as key players in the enhanced degradation and finally reduced synthesis of matrix components in pathological conditions like OA. The two main cartilage constituents are collagen type II fibrillar network and proteoglycans attached to hyaluronic acid filaments, also termed aggrecans. The latter are the more sensitive and the first ones to change in cartilage degradation. Therefore, the in vitro assays are performed mainly with chondrocytes, and the

parameters measured focus on proteoglycan synthesis and/or degradation.

The exact mechanisms of cartilage pathophysiology are not yet elucidated, but enzymatic degradation involving metalloproteinases is considered the main event. In vitro, interleukin-1 and retinoic acid induce enhanced matrix degradation as well as reduced matrix synthesis, as observed in OA. Their role in the actual disease process in vivo, however, remains obscure. They therefore are used rather as tools to induce a disease-relevant condition in vitro than being subject of direct pharmacological intervention. Thus, the in vitro assays described here are suitable to compare and select a variety of drugs for their effect upon the main biological activity of articular chondrocytes. To address the respective mechanisms of drug action, more specific follow-up assays like enzyme inhibition or cytokine release or inhibition tests should be applied.

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Modulation of Cellular Proteoglycan Metabolism

Purpose and Rationale

In primary cultures, articular chondrocytes grown in an artificial matrix after digestion of the original bone maintain their characteristic synthesis and turnover rate of cartilage matrix macromolecules for a long time. These metabolic processes can be influenced pharmacologically. In the following assay, compounds are tested for their effect upon the normal turnover of cartilage matrix by chondrocytes. The test is used to detect stimulation of matrix formation, but also to check for potential impairment of cartilage function. Specific matrix staining reveals the amount of newly formed matrix remaining around the cells at the end of treatment. Alternatively, incorporation of radiolabeled sulfate into the newly formed proteoglycans allows to quantitate the anabolic activity at the end of the experiment.

Procedure

Reagents

A 1 % (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

A 0.025 % (w/v) solution of collagenase type II, activity 242 U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

A 2 % solution of low melting agarose from SeaPlaque in 0.9 % NaCl is prepared by heating in a microwave and stored in a water bath at about 50 °C.

A buffer of 25 mM sodium acetate (2.051 g/l) with 0.4 M magnesium chloride 6-hydrate (81.32 g/l) is prepared and adjusted with acetic acid to pH 5.6.

A staining solution is prepared with 0.1 g of Alcian Blue, obtained from Sigma, in 67.5 ml buffer, filtered, and supplemented with 10 ml of a 25 % solution of glutaraldehyde.

An 8 M guanidinium hydrochloride solution is prepared.

Tissue and Cell Preparation

Fetlocks of freshly slaughtered steers (age 18–20 months) are skinned, and the metacarpophalangeal joint is opened under semi-sterile conditions. With a sterile scalpel, articular cartilage is

then carefully removed from the underlying bone from all accessible cartilaginous regions and transferred into a sterile Ham's F12 solution at +4 °C. The tissue is washed with Ham's F12 to remove adherent synovial fluid. The pieces are then transferred into a 150 ml trypsinizing flask, containing the Pronase solution including the added serum, and incubated with gentle stirring for 1 h at 37 °C and 95 % humidity. The fluid is then removed, and the collagenase solution including the added serum is incubated with gentle stirring overnight. The resulting cell suspension is first filtered through a 90 μm and then a 50 μm Nylon filter and then centrifuged at 800 rpm for 10 min. Resuspension and washing are performed with HBSS, and cells are counted and checked for vitality under the microscope using the eosin staining. The vitality level should reach at least 95 %. A cell suspension is prepared of 4×10^6 cells/ml Ham's F12 supplemented with 20 % FCS.

To prepare the agarose cell cultures, 24-well plates are coated with 0.2 ml/well of a 1:1 mixture of the 2 % agarose solution with preheated Ham's F12 and left at room temperature to gel. Then 0.1 ml/well (0.2 ml/well for radiolabeling) of a 1:1 mixture of the above described cell suspension with the 2 % agarose solution is added. Care has to be taken to maintain an even cell suspension and not to overheat the cells during this procedure. After gel formation at room temperature, the multiwell plates are placed in the incubator, and 0.5 ml/well medium is added either 4 h later or the following day. The medium consists of Ham's F12 supplemented with 5 % FCS and 25 μg/ml ascorbic acid and is changed every second day.

Assay

The assay starts 5 days after cell preparation. Compounds are added to the medium in a final concentration of 10 μM with 6–8 replicae per compound and added anew with each change of medium over a total period of 8 days. The concentration can be varied according to the expected potency of the drug studied. An untreated control group as well as standard compound groups are always included. As standard compound, pentosan polysulfate to check for matrix increase, or retinoic acid to cause matrix decrease, can be applied.

At the end of treatment, the medium is removed, the wells are washed 3× with 500 μl of the medium without supplements, and 1 ml/well of the staining solution is added for 48 h. After removal of the supernatant, the following washing steps are performed for 10 min each:

- 3 × 500 μl/well 3 % acetic acid
- 1 × 500 μl/well 3 % acetic acid in 25 % ethanol
- 2 × 500 μl/well 50 % ethanol
- 1 × 500 μl/well 70 % ethanol

With 500 μl/well of 8 M guanidinium hydrochloride solution, the bound stain is then extracted for 24 h at +4 °C. After shaking the plates gently for 10 min, 100 μl/well of each supernatant is then transferred to round-bottom microtiter plates, and the extinction is photometrically assessed in the plate reader at a wavelength of 610 nm.

Evaluation

The extinction is expressed in percentage as staining density with the control values defined as 100 %. Values $\geq$110 % are interpreted as stimulation of matrix formation and values lower than 80 % as inhibition of matrix formation. Experiments with 8 wells/treatment usually exhibit a standard deviation below 7 %.

Critical Assessment of the Test

The described method is suitable to compare up to 50 drugs in one experiment. The price for this is the limited quantification, as the staining is not strictly stoichiometric, and it does not allow the distinction between matrix synthesis and degradation. For more detailed assessment, radiolabeling is the better choice. The limitation of these primary culture assays lies in the elaborate preparation and isolation of the chondrocytes. Several attempts to immortalize this differentiated mesenchymal cell type have resulted in the loss of cartilage-specific properties. A new cell line

developed by MB Goldring (Green et al. 1995) might overcome this difficulty but has not yet been reported to be modified pharmacologically.

Modifications of the Test

The agarose culture system for chondrocytes, originally described by Benya and Schaffer (1982), has been well characterized by Aydelotte et al. (1988, 1992), and the effects of different agarose densities have been studied by Verbruggen et al. (1990).

Instead of agarose gel cultures, some authors use 3D chondrocyte clusters in suspension (Bassleer et al. 1990, 1992; Henrotin et al. 1992) or suspensions over agarose (Archer et al. 1990) or embedded in collagen gels (Malemud et al. 1994).

Alternatively, encapsulation in alginate beads, either directly after isolation (Guo et al. 1989), even as primary culture for several months (Häuselmann et al. 1994), or after expansion in monolayers (Bonaventure et al. 1994), offers the opportunity to recover the chondrocytes later by depolymerization of the alginate.

Monolayers of articular chondrocytes can be used as well, but preferentially short-term (up to 3 days of culture), as under this culture condition chondrocytes tend to dedifferentiate to a fibroblast-like appearance and metabolic program. Authors using this modification are, e.g., Kolibas and Goldberg (1989), Lane et al. (1992), and McCollum et al. (1991). The importance of culture conditions is addressed in the study by Seid et al. (1993) and that of culture duration in the paper by van der Kraan et al. (1992).

A dot blot assay by cuprolinic blue precipitation has been described by Jortikka et al. (1993), which is restricted to serum-free conditions. Instead of matrix staining, radiolabeling can be applied as described in the next assay. In this case, the amount of cells should be doubled per well to assure sufficient label incorporation. Sauerland et al. (2003) showed that the pressure loading regime and frequency controls the metabolic activities of chondrocytes from cartilage explants and this may be relevant to human tendinopathies (Parkinson et al. 2010a, b).

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Cellular Chondrocytic Chondrolysis

Purpose and Rationale

In this assay, a disease-relevant situation is achieved by adding interleukin-1 (IL-1) to articular chondrocytes grown in agarose gel. IL-1 suppresses proteoglycan (PG) synthesis as well as increases their degradation, and thus results in a process which is also observed in degradative joint diseases in vivo. This process is termed chondrocytic chondrolysis. The test is used to detect the potential interference of a drug with this pathological process. Effect upon PG synthesis is studied by radiolabeling at the end of the experiment and measuring the amount of incorporated sulfate. The effect upon PG degradation, and its release from the cellular environment, can be examined by prelabeling with $Na_2{}^{35}SO_4$ and following the amount of released incorporated sulfate over time from the supernatant with each or every second medium change.

Procedure

Reagents

A 1 % (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

A 0.025 % (w/v) solution of collagenase type II, activity 242 U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

A 2 % solution of low melting agarose from SeaPlaque in 0.9 % NaCl is prepared under heating and microwave application and stored in a water bath at about 50 °C.

Human recombinant interleukin-1 (IL-1) α or β from Genzyme is stored in aliquots at –20 °C.

For radiolabeling, $Na_2{}^{35}SO_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium hydrochloride (GuHCl) solution is prepared.

Tissue and Cell Preparation

Tissue and cell preparations are performed as for the previous assay, except the initial number of cells/well should be 400,000, corresponding to a cell-containing gel volume of 0.2 ml/well, and the medium added is supplemented with 10 % FCS and 25 μg/ml ascorbic acid. It is changed every second day.

Assay

The assay starts 6 days after cell preparation. Except for the control group, interleukin-1α is added in a concentration of 3 U/well and added anew with each consecutive medium change. Except for the IL-1 control group, compounds are added to the medium in a final concentration of 10 μM with 4 replicae per compound and with the subsequent medium changes. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8-day treatment, the medium is replaced by a medium containing 1 μCi $Na_2{}^{35}SO_4$/well and incubated for 24 h. The supernatant is then removed, mixed 1:1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The multiwell plates with the remaining gels are deep frozen for at least 24 h to facilitate solubilization, thawed, and then extracted with 500 μ l/well 8 M GuHCl supplemented with inhibitors. The content of each well is then centrifuged at 13,000 rpm for 30 min. After this step, the supernatant contains the matrix trapped around the cells. This is equally separated by a PD10-Sephadex column into free versus incorporated radiolabel. The probes containing the incorporated sulfate and derived from both fractions (medium and gel) are then mixed with scintillating fluid and assessed in a β-scintillation counter.

Evaluation

Counts per minute (cpm) from medium and gel fraction are calculated and related to the total well content. They are added if total incorporation is measured or left separately in case the ratio between released versus retained label is of interest. The data are converted into percent incorporation, with the values of the untreated control group or those of the IL-1 control group serving as 100 %.

Critical Assessment of the Test

This is a sensitive test, in which an adequate labeling protocol can provide detailed information. The time- and material-consuming separation of free from incorporated radiolabel at the end, however, limits the size of the experiments and number of compounds to be studied.

In both cellular tests, drug effects should be checked for (anti)proliferative activity in a separate proliferation test.

Modifications of the Test

If the catabolic response to a drug is of more interest than the anabolic one, the radiolabeling can be shifted to a time point prior to treatment, and the release of incorporated sulfate into the supernatant will allow to follow the time course and amount of PG degradation. This prelabeling should not start earlier than 4 days after plating to assure a comparable matrix synthesis rate. Because IL-1 suppresses PG synthesis at a lower

concentration than it stimulates its degradation, a double to triple amount of IL-1 should be used in this modification.

Instead of bovine, human chondrocytes can be used (Raiss et al. 1992). One should allow 4 days of adjustment in the agarose system before starting treatment with human cells. If available, human serum gives a higher PG synthesis rate than fetal calf serum (Oestensen et al. 1991). The heterogeneity of responses depends on the individual source and should be considered (Verbruggen et al. 1989).

When using human instead of bovine cells, the stimulation of degradation and inhibition of synthesis is more effective with IL-1β than IL-1α: a concentration of 0.1 U/ml results in a reduction of PG synthesis of ca 50 % (Raiss et al. 1995). When incubating with radiolabeled sulfate, the exposure time should be doubled to 48 h to yield sufficient incorporation.

Cartilage chondrolysis has also been achieved with fibronectin fragments (Homandberg et al. 1997). Chu et al. (2008) have explored the effects of the anesthetic bupivacaine on chondrocyte viability in vitro, and bupivacaine has been shown to induce chondrocyte cell death and hyaluronan was shown to confer protection in this assay system (Liu et al. 2012). This assay system has been used to explore the effects of oxygen tension on markers of catabolism (Parker et al. 2013).

To exclude direct interference of a drug with interleukin-1, all-trans retinoic acid can be used instead of this cytokine.

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Cartilage Explant Chondrolysis

Purpose and Rationale

Chondrocytes vary in their metabolic activity, and cytokine response depends on the relative location within the joint (surficial vs. deep, weight-bearing vs. non-weight-bearing, etc.). Therefore, the cellular assays are a homogeneous mixture of an otherwise heterogeneous cell population. Two reasons suggest a

verification of the cellular results obtained in tissue culture assays: First, chondrocytes are more reactive after isolation compared to those in tissue culture, which may lead to false-positive results. Second, intact cartilage matrix acts as barrier for certain compounds of high molecular size and fixed charge, so that they may not reach their target cells. Therefore, explant assays are recommended as a follow-up to the cellular tests.

Procedure

Reagents

Ham's F12 medium is supplemented with 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

Human recombinant interleukin-1 (IL-1) α or β from Genzyme is stored in aliquots at –20 °C.

For radiolabeling, $Na_2{}^{35}SO_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium hydrochloride (GuHCl) solution is prepared.

Tissue Preparation

Fetlocks of freshly slaughtered steers (age 18–20 months) are skinned, and the metacarpophalangeal joint is opened under semi-sterile conditions. With a sterile punch (as used for obtaining skin biopsies), full-thickness disks of cartilage are obtained from all accessible cartilaginous areas, and their wet weight is assessed. In each well, 1 ml of medium is added, consisting of Ham's F12 supplemented with 10 % FCS and 25 µg/ml ascorbic acid, and approximately 30 mg wet weight of cartilage is transferred corresponding to 3 disks of 4 mm in diameter.

Assay

The assay is started 1–2 days after tissue preparation. Except for a control group, interleukin-1α is added in a concentration of 8 U/well and with each of the following medium changes (every second day). Except for an IL-1 control group, compounds are added to the medium in a final concentration of 10 µM with 6–8 replicae per compound, which are also added with each medium change. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8-day treatment, the medium is replaced by a medium containing 15 µCi $Na_2{}^{35}SO_4$/well and incubated for 24 h. The supernatant is removed, mixed 1:1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The explants are washed three times with Ham's F12 at +4 °C and extracted with 1 ml/well of 4MGuHCl supplemented with inhibitors for 48 h, and then a second time with 0.5 ml/well for 24 h. Both fractions are mixed and separated with a PD10-Sephadex column into free versus incorporated sulfate. The samples containing the incorporated sulfate derived from the medium as well as explant extraction are then mixed with scintillating fluid and assessed in a β-scintillation counter.

Evaluation

Counts per minute (cpm) from medium and explant fractions are calculated related to mg wet weight of cartilage of the respective wells. They are either added if total incorporation is measured or left separately, in case the ratio between released versus retained matrix is of interest. The data are converted into percent incorporation in comparison with the values of the untreated control or of the IL-1 control group serving as 100 %.

Critical Assessment of the Test

Punched disks of similar size standardize the surface/volume ratio and give more reproducible results than chips of cartilage obtained by scalpel dissection. A disadvantage is the greater amount of cartilage needed, which makes it unsuitable for human tissue obtained from joint replacement surgery. When using human tissue, interpretation and comparison of results should be restricted to the same source: striking differences occur between specimens from surgery or postmortem, between different joints (hip vs. knee), and different ages of the donor (young or adolescent vs. 35 years and older), as well as different stages of severity and the duration of degenerative joint diseases in cases of surgical specimens.

Modifications of the Test

The effect of serum concentrations on proteoglycan synthesis has been studied by McQuillan et al. (1986), and the effect of different concentrations of DMSO and glycerol, of importance for cryopreservation, has been examined on human fetal hip cartilage by Yang and Zhang (1991).

Some authors (Nixon et al. 1991) use bovine nasal septum as cartilage source, but the convenient homogeneity and mass of this tissue is outweighed by a matrix composition and biomechanical properties clearly distinct from the articular cartilage.

Several authors use human cartilage from joint replacement surgery (e.g., Pelletier et al. 1989; Pelletier and Martel-Pelletier 1989), and some compare drug effects upon visually normal cartilage to those with fibrillated or osteoarthritic cartilage (Lafeber et al. 1992, 1993; Verbruggen et al. 1989, 1990).

A step toward organ culture represents the culture of full-thickness cartilage with the subchondral bone, cultured for 24 h on moist lens tissue (Chayen et al. 1994).

Bordji et al. (2000) published evidence for the presence of peroxisome proliferator-activated receptor (PPAR) α and γ and retinoid Z receptor in cartilage.

Bondeson et al. (2006) established a model of cultures of synovial cells from digested osteoarthritis synovium derived from patients undergoing knee or hip arthroplasties. In addition to investigation of chondroprotective activity in tissue culture studies in cartilage explants, human or bovine has been used to determine chondroprotectivity. Ono et al. (2014) showed a positive effect of kartogenin in both culture chondrocytes and cartilage explants, Culley et al. (2013) showed a role from chromatin modulation via histone deacetylation inhibition, and Assirelli et al. (2014) showed attenuation of chondroprotective IL-4.

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Influence on Matrix Metalloproteinases

Purpose and Rationale

Matrix metalloproteinases (MMPs) form a multigene family of more than 20 secreted and membrane-tethered zinc-dependent endopeptidases, which are classified according to their structures and substrate specificities (Nicholson et al. 2005). Members of the family include collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, MMP-12), and membrane-type matrix metalloproteinase (MT1-MMP to MT6-MMP). They are regulated by natural inhibitors, such as α_2-macroglobulin, and the tissue inhibitors of metalloproteinases (TIMPs). The catalytic domains of the MMPs have an ellipsoid shape with a small active cleft. This cleft contains the catalytic zinc ion, which is essential for catalysis. MMPs can degrade all components of the extracellular matrix and have been implicated in a number of pathological conditions. They are produced in response to the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) and are found in excess in the arthritic joint (Borkakoti 1998, 2004; Close 2001; Rosenblum et al. 2003; Mott and Werb 2004). MMPs belong to the metzincin superfamily of metalloproteinases, which also includes astacins, ADAMs (proteins with a disintegrin and metalloproteinase domain), and ADAMTS (an ADAM with a thrombospondin-like motif). There is a clear role for MMPs in OA (Li et al. 2011; Galasso et al. 2012; Wang et al. 2013; Jackson et al. 2014), and a number of molecules showed promise based on preclinical evaluation. Despite very large efforts to develop MMP inhibitors as drugs, clinical trial outcomes have been disappointing as the clinical utility of both narrow- and broad-spectrum MMP inhibitors appears to have been restricted by dose- and duration-dependent musculoskeletal adverse events in humans and an incomplete understanding of complex biology (Levin et al. 1998; Bigg and Rowan 2001; Martel-Pelletier et al. 2001; Matter et al. 2002; Nelson et al. 2002; Aranapakam et al. 2003; Skotnicki et al. 2003; Zask et al. 2003; Matter and Schudok 2004; Skiles et al. 2004; Li et al. 2011; Martel-Pelletier et al. 2012). New strategies for disease modification in OA are needed (Barr and Conaghan 2013), and a number of novel approaches are revisiting the potential of targeting MMP activity in both OA and other indications (Chang and Werb 2001; Devy and Dransfield 2011; Devel et al. 2012; Meszaros and Malemud 2012, Vandenbroucke and Libert 2014).

Procedure

In Vitro Assays

The influence of test substances on recombinant MMP-2, MMP-3, and MMP-9 activity was determined using the fluorescent quenched substrate Mca-Pro-Leu-Gly-LeuDpa-Leu-Ala-Arg [where Mca = (7-methoxycoumarin-4-yl) acetyl and Dpa = 3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl].

Each enzyme was active site titrated using a standard preparation of human recombinant TIMP-1 for MMP-1, TIMP-2 for MMP-2, and TIMP-9 for MMP-9. Then, 200 pM of MMP-2 or MMP-9, 400 pM of MMP-3, or 3 nM of MMP-1 was mixed with increasing concentrations of test substances in a 50 mM HEPES buffer, pH 7.6, containing 150 mM of NaCl and 5 mM of $CaCl_2$, and the assay was initiated by adding 2 μM of substrate. The rate of substrate hydrolysis was linear up to 30 min at 22 °C. The reaction was stopped by adding 10 mM EDTA. The rate of the reaction was measured in triplicate for each test drug concentration examined using the Perkin Elmer LS50B spectrofluorometer with excitation and emission wavelength of 325 and 375 nm, respectively. Less than 5 % of the substrate was hydrolyzed during the rate measurements.

Evaluation

IC50 (μM) values were determined by plotting V_i/V_0, where V_i is the rate of substrate hydrolysis in the presence of inhibitor and V_0 is the rate in its absence, as a function of test drug concentrations and nonlinear regression analysis.

Modifications of the Method

Similar procedures were used in the following studies:

Lewis et al. (1997) to study an orally active collagenase inhibitor

Billinghorst et al. (2000) and Dahlberg et al. (2000) to compare the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1

Berton et al. (2001) to study the inhibition of matrix metalloproteinase activities by long-chain fatty acids

Matter et al. (2002) to investigate structure–activity relationship of tetrahydroisoquinoline-3-carboxylate-based matrix metalloproteinase inhibitors

Bottomley et al. (1997) studied the inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors.

The potency of the inhibitors against human collagenase 1 (MMP-1), stromelysin (MMP-3), gelatinase B (MMP-9), and aggrecan metabolism was tested. Medium from human dermal fibroblasts (CCD45) cultured in the presence of recombinant human IL-1α (25 ng/ml) was used as source of collagenase. Collagenase activity was determined by measuring the degradation of ^{14}C-labeled collagen fibrils. Human prostromelysin-1 was antibody affinity purified from conditioned human fibroblast culture medium. Progelatinase B was purified by gelatin–agarose affinity chromatography from human neutrophils. Both prostromelysin and procollagenase B were activated by treatment with trypsin. Stromelysin and gelatinase B activities were determined by measuring the cleavage of the fluorogenic substrate Mca-Pro-Lys-Pro-Leu-Gly-Leu-Dpa-NH_2; assays were performed using 50 mM borate buffer/1 mM $CaCl_2$ containing 0.05 % Brij-35, at a substrate concentration of 2 μM. Assays were started by the addition of enzyme to a mixture of substrate and an inhibitor and incubated for 4 h at 37 °C; the assay was stopped by the addition of acetic acid to a final concentration of 0.17 mM. The fluorescence of the product Mca-Pro-Lys-Pro-Leu-Gly (λ_{ex} 325 nm; λ_{em} 395 nm) was measured with a Hitachi F-4500 fluorescence spectrophotometer.

A similar method was used by Reichelt et al. (2002) for the design, synthesis, and evaluation of matrix metalloproteinase inhibitors bearing cyclopentane-derived peptidomimetics as P1′ and P2′ replacements.

Perlman et al. (2003) found that IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in human synovial fibroblasts.

A fluorescent screening assay for collagenase using collagen labeled with 2-methoxy-2,4-diphenyl-3(2*H*)-furanone was recommended by O'Grady et al. (1984).

Zhang et al. (2004) identified and characterized a dual tumor necrosis factor-α-converting enzyme (**TACE**)/matrix metalloproteinase inhibitor for the treatment of rheumatoid arthritis.

Assay of TNF-α-Converting Enzyme (TACE)

A synthetic peptide of pro-TNF-α containing the minimal TACE cleavage sequence, Abz-LAQAVRSSSR-Dpa, was used as a substrate. A segment of the extracellular portion of the human TACE that comprises the catalytic domain, the disintegrin domain, the epidermal growth factor-like domain, and the Crambin-like domain was used. The protein was expressed in Chinese hamster ovary (CHO) cells and purified by nickel–nitrilotriacetic acid and preparative size exclusion chromatography to near homogeneity. Compounds were tested for their ability to inhibit the cleavage of the substrate by the purified enzyme in a fluorescence-based fluorescence resonance energy transfer (FRTE) assay. The human TACE protein (1 μg/ml) was pretreated with the inhibitors at various concentrations for 10 min at room temperature. The reaction was initiated by the addition of pro-TNF-α peptide to the TACE protein, and the increase in fluorescence was monitored at an excitation wavelength of 320 nm and emission wavelength of 420 nm over a period of 10 min (Jin et al. 2002).

MMP Enzymatic Assays

A continuous assay was used in which the substrate is a synthetic peptide containing a fluorescent group (7-methoxycoumarin), which is quenched by energy transfer to a 2,4-dinitrophenyl group. When the peptide was cleaved by MMPs, an increase in fluorescence was observed. The substrate used was 7-methoxycoumarin-PQGL-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-AR-OH. The assays were carried out at room temperature in a buffer containing 50 mM HEPES, pH 7.4; 100 mM NaCl; 5 mM $CaCl_2$; and 0.005 % Brij-35 (Knight et al. 1992). The enzyme reaction was initiated by adding the substrate to a final concentration of 20 μM. The initial rate of the cleavage reaction was determined immediately after substrate addition.

Sadowski and Steinmeyer (2001) studied the effects of nonsteroidal anti-inflammatory drugs and dexamethasone on the activity and expression of MMP-1, MMP-3, and tissue inhibitor of metalloproteinases-1. Bovine chondrocytes were cultured in alginate gel beads. Cells were treated with IL-1α in the presence of vehicle or drugs at various concentrations. After 48 h, mRNA expression of MMP-1, MMP-3, and the tissue inhibitor of metalloproteinases (TIMP-1) was analyzed by RT-PCR-ELISA. The protein synthesis of TIMP-1 and MMP-3 was determined by immunoprecipitation. The activity of enzymes and inhibitors was measured by functional assays (Yoshioka et al. 1987; Steinmeyer et al. 1998).

The synthesis and biological activity of **TACE inhibitors** are described by Beck et al. (2002), by Letavic et al. (2002, 2003), and by Tsukida et al. (2004).

Downs et al. (2001) used **ELISA** for the analysis of collagenase cleavage of type II collagen to the C-terminal neoepitope. Valleala et al. (2003) described an ELISA assay for MMP-9 (gelatinase B).

Microtiter plates were coated with 100 μl of 5 μg/ml MMP-9-specific monoclonal antibody (TNO-S22.2) in PBS overnight at 4 °C. After three washes in PBS containing 0.05 % Tween 20(PBS-T), 100 μl of purified MMP-9 or cell supernatant was added. After overnight incubation at 4 °C, the plates were washed and incubated for 1 h at 37 °C with 100 μl of biotin-labeled anti-MMP-9 polyclonal antibody (TNO-B21) diluted in PBS-T/EDTA +0.1 % casein (PBS-T/DTA/C) (0.8 μg/ml). After washing, bound polyclonal antibody was assessed by incubation with 100 μl of avidin/HRP at 1:10,000 in PBS-T/EDTA/C. Non-bound conjugate was washed away after 1 h at 37 °C, and the chromogene 3,3′ 5,5′-tetramethylbenzidine in the presence of H_2O_2 was added. The reaction was stopped after 20 min with 2 M H_2SO_4, and the absorption was measured at 450 nm in a Titertek Multiscan spectrophotometer.

Peppard et al. (2003) developed an assay suitable for high-throughput screening to measure matrix metalloproteinase activity.

Sabatini et al. (2005) studied the effect of inhibition of matrix metalloproteinases on cartilage loss in vitro and in a guinea pig model of osteoarthritis.

Zymography was used by several authors to measure the activity of metalloproteinases (Maquoi et al. 1998, 2002; Hattori et al. 2002; Sartor et al. 2002; Sato et al. 2002; Kaji et al. 2003; Kerkvliet et al. 2003; Kim and Kim 2004; Liu et al. 2004; Martin-Chouly et al. 2004; Naqvi et al. 2005).

Samples were subjected to electrophoresis on a 4.5 % acrylamide stacking gel/7 % acrylamide separating gel containing 1 mg/ml gelatin, in the presence of sodium dodecyl sulfate, under nonreducing conditions. After electrophoresis, gels were washed twice with 2.5 % Triton X-100, rinsed with water, and incubated at 37 °C overnight in 50 mM Tris, 5 mM $CaCl_2$, and 2 mM $ZnCl_2$, pH 8.0. The gels were stained with Coomassie brilliant blue and destained in a solution of 25 % ethanol and 10 % acetic acid. Gelatinase activities appeared as clear bands against a blue background. To determine the inhibition profile of the enzyme activities, gels were incubated in the presence of one of the following inhibitors in the activation buffer: 10 mM EDTA as an inhibitor of MMPs or 10 mM PMSF (phenylmethylsulfonyl fluoride) as an inhibitor of serine proteases. The molecular weight of gelatinolytic bands was estimated using recombinant protein molecular weight markers. Images of zymograms were acquired with the Gel Doc 1000 gel documentation system. The amount of enzyme was quantified by measuring the intensity of the negative bands using a densitometer analyzer with Quantity One software. Results were expressed as arbitrary units of relative intensity.

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Aggrecanase Inhibition

Purpose and Rationale

Degenerative joint diseases are characterized by cartilage extracellular matrix degeneration, where loss of aggrecan, an aggregating proteoglycan, is an early event in the destruction of articular cartilage. Aggrecan, a multidomain proteoglycan, is a major component of cartilage and provides compressive resistance to articular cartilage. During the early stages of osteoarthritis and then throughout the disease, there is increased loss of glycosaminoglycan-rich aggrecan fragments via proteolysis attributable to "aggrecanase" activity.

Aggrecanase-1 and aggrecanase-2 are members of the ADAMTS (a disintegrin and metalloproteinase possessing thrombospondin domain) family of zinc-containing metalloproteinases, responsible for the cleavage of the aggrecan interglobular domain (IGD) at the Glu^{373}-Ala^{374} peptide bond, a unique site untouched by any previously identified enzyme (Arner et al. 1998; Abbaszade et al. 1999; Arner et al. 2002; Little et al. 1999; Tortorella et al. 1999, 2001; Patwari et al. 2005; Stanton et al. 2005; Wight 2005; Song et al. 2007).

Inhibition of aggrecanase may impart overall cartilage protection and offer a potential therapy to alter the progression of osteoarthritis. Besides endogenous inhibitors of aggrecanase (Bonassar et al. 1997; Sandy et al. 1998; Hashimoto et al. 2001, 2004; Little et al. 2002a; Malfait et al. 2002; Munteanu et al. 2002; Gendron et al. 2003; Pratta et al. 2003; Tortorella et al. 2004), also synthetic inhibitors are described (Bottomley et al. 1997; Munteanu et al. 2000; Little et al. 2002b; Sabatini et al. 2002; 2005; Sawa et al. 2002; Wada et al. 2002; Cherney et al. 2003; Vankemmelbeke et al. 2003; Noe et al. 2004; Liacini et al. 2005; Simpson 2011; Gilbert et al. 2011).

Xiang et al. (2006) described the synthesis and biological evaluation of biphenylsulfonamide carboxylate aggrecanase-1 inhibitors and Nuti et al. (2013) synthesis and biological evaluation of arylsulfonamide inhibitors of aggrecanase-1 and aggrecanase-2.

Procedure

Bovine carpal joints were obtained from young (1–2-week-old) animals. Full-depth articular cartilage plugs were harvested using a cork borer and then sliced on a custom dice to generate individual disks 6 mm wide, 1 mm thick, and 30 mg in weight.

Cartilage explants were cultured at 37 °C for 5 days in a humidified atmosphere of 5 % CO_2 in air in cartilage explant media (CEM) consisting of Dulbecco's modified Eagle's medium containing 1 % antimycotic/antibiotic, 2 mM glutamine, 10 mM HEPES, and 50 μg/ml of ascorbate (all from Sigma, St. Louis, MO, USA). The explants were washed with CEM, and one weighed disk per well was placed in a 96-well culture dish with 0.2 ml medium and six to eight replicates per treatment and cultured for 3 days in the presence or absence of recombinant human IL-1α (rhIL-1, 5 ng/ml, Sigma) and the presence or absence of small molecule compound. Media were replaced every day. The proteoglycan content in the medium was measured as sulfated glycosaminoglycan (GAG) by a colorimetric assay (Farndale et al. 1982) using dimethylmethylene blue (DMMB) and chondroitin sulfate C from shark cartilage (Sigma) as a standard. Treatment of cartilage with IL-1 results in the induction of catabolic enzymes including aggrecanases that degrade cartilage matrix proteoglycan. The cleaved proteoglycan is released from the matrix into the media. Addition of the compound together with IL-1 to the cartilage results in a

decrease of proteoglycan release, indicating the inhibition of proteoglycan degradation. During this early phase of proteoglycan degradation, aggrecanases are the predominant catabolic enzymes that cleave aggrecan with no significant role of other MMPs (Pratta et al. 2003).

Evaluation

Measured proteoglycan was expressed per weight of cartilage. IC_{50} values were calculated.

Modifications of the Method

Miller et al. (2003) reported a microplate assay specific for the enzyme aggrecanase. Peppard et al. (2003) developed a high-throughput screening assay for inhibitors of aggrecan cleavage using luminescent oxygen channeling (AlphaScreen).

A simplified assay was reported by Kashiwagi et al. (2001).

Activities of ADAMTS4 and ADAMTS5 were measured by incubating enzyme with purified bovine aggrecan (500 nM) in 100 µl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 10 mM $CaCl_2$, for 2 h at 37 °C and terminating the reaction with 10 mM EDTA. The digestion products were then deglycosylated with chondroitinase ABC (0.1 units/10 µg aggrecan) and then with keratinase (0.1 units/10 µg aggrecan) and keratinase II (0.002 units/10 µg aggrecan) for 2 h at 37 °C in 0.1 M Tris-HCl, pH 6.5, containing 50 mM sodium acetate. The enzymatically treated products were analyzed by Western blotting using BC-3 antibody or an antibody against the $GELE^{1480}$ neoepitope (Tortorella et al. 2000). To determine apparent inhibition constant $K_{i(app)}$ values for inhibitors against the aggrecanases, ADAMTS4 or ADAMTS5 (at a final concentration of 50 pM) was incubated with various concentrations of the inhibitor in 44 µl of the above buffer at room temperature for 30 min, and then a solution of bovine aggrecan (5.5 µl) was added. The reaction products were detected with anti-$GELE^{1480}$ antibody. The concentrations of ADAMTS4 and ADAMTS5 were confirmed by titration with recombinant N-TIMP-3.

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In Vivo Methods for Anti-osteoarthritic Activity

General Considerations

The current availability of animal models of osteoarthritis (OA) for pharmacological assessment is impeded in several aspects: firstly, the difficulty to address a generally slow progression of cartilage destruction and deterioration of joint function as encountered in human OA with an animal model achieving sufficient similarity to human pathology in an acceptable time frame; secondly, the discrepancy between the need of mild, reversible pathological changes, which can be modified therapeutically, and the paucity of reliable parameters with which to determine normal versus disease stages with a gradable range large enough to assess drug effects (this includes also the lack of validation (clinically as well as in animal models) of noninvasive methods to assess disease progression); thirdly, the lack of a true disease-modifying standard drug with which to validate the pharmacological effects in respect to the predicted clinical outcome.

This results in a situation that animal models closer to human pathology like the spontaneous OA in the Hartley strain of guinea pigs or the surgically inflicted joint instability in the Pond–Nuki dog model are too elaborate to be used routinely for drug selection. On the other hand, models like the chymopapain-induced cartilage degradation in rabbits are suitable to study drugs but are limited in their predictive value.

Some progress can be expected from the use of genetically modified animals (Rintala et al. 1997; Serra et al. 1997; Ameye and Young 2002; Han et al. 2002; van Lent et al. 2002; Johnson and Terkeltaub 2003; Scharstuhl et al. 2003; Glasson et al. 2004, 2005; Ueblacker et al. 2004; Ford-Hutchinson et al. 2005; Wadhwa et al. 2005; Xu et al. 2005; Zaka and Williams 2005; Zhang et al. 2005).

It should be noted that differences exist in the pathomechanisms of cartilage destruction between rheumatoid arthritis (RA) and OA. The destruction occurring in RA is closely linked to the inflammatory process, synovial tissue proliferation, and transgression across the cartilage surface, degrading cartilage proteoglycans and collagens simultaneously. In OA, inflammation is only an intermitted event, not instrumental in the degenerative cartilage destruction, in which proteoglycan degradation is the early event, and collagen loss occurs at a distinctly later stage. Therefore, animal models with a predominant inflammatory component as the air pouch model or other arthritis models, even those focusing on cartilage destruction, are not discussed in this chapter (see chapter, "▶ Methods for Testing Immunological Factors"). They are described in detail by Greenwald and Diamond (1988) and recently reviewed by Greenwald (1991, 1993).

Purpose and Rationale

As a multitude of different events can lead to OA, equally different techniques have been used to initiate osteoarthritic conditions in animal models: surgical methods are used to either stiffen the joint in a defined position (Palmoski and Brandt 1982; Konttinen et al. 1990; Meyer-Carrive and Ghosh 1992; Torelli et al. 2005) or inflict joint instability by partial meniscectomy or anterior crucial ligament (ACL) dissection. They are performed mainly in dogs, rabbits (Alam et al. 2011; Colombo et al. 1983; Moskowitz et al. 1973, 1979), guinea pigs (Schwartz 1985; Meacock et al. 1990), and mice (Kamekura et al. 2005; Loeser et al. 2013). Chemical modifications like intra-articular injections of iodoacetate, cytokines like IL-1, or enzymes like chymopapain or stromelysin are carried out mainly in rabbits (Williams et al. 1992; Regling et al. 1989), in chickens (Kalbhen 1983, 1987), and in rats (Combe et al. 2004). Mechanical forces are applied on bent or opened joints like impulse loading on sheep (Lindenhayn et al. 1984) or rabbit (Farkas et al. 1987; Mazières et al. 1984) knees, resulting in trauma models of OA. Spontaneously occurring OA is described in horses (Todhunter and Lust 1992; Haakenstad 1969), some breeds of dogs (Lust et al. 1985), rhesus macaques (Pritzker et al. 1989), guinea pigs (Bendele and Hulman 1988), and several strains of mice. For pharmacological purposes, only guinea pigs and STR/1 N

(Raiss et al. 1992), STR/ort (Dunham et al. 1989; Kyostio-Moore et al. 2011), and C57 black (Pataki et al. 1990) mice have been adapted. In all models mentioned (except horses), the relevant joint is the knee.

Since there exist recent extensive and critical reviews of OA models (Burton-Wurster et al. 1993; Pritzker 1994; Moskowitz 1990, 1992; Adams and Billingham 1982; Oegema and Visco 1999; Bendele 2001, 2002; Bonnet and Walsh 2005; Little and Smith 2008; Aigner et al. 2010; Gregory et al. 2012; Cohen-Solal et al. 2013; Fang and Beier 2014; Cook et al. 2014), also with respect to reversibility (Pita et al. 1986), with special emphasis on drug testing in the most appropriate model which relates human pathology to target to drug pharmacology (Hess and Herman 1986; Hinz and Brune 2004; Wieland et al. 2005; Poole et al. 2010; Little and Zaki 2012; Malfait et al. 2013; Teeple et al. 2013; Zhang et al. 2013), in perspective to cartilage research and markers (Carney 1991; Malemud 1993; Kyostio-Moore et al. 2011; Thiede et al. 2012) in a model of type 2 diabetes mellitus (Onur et al. 2014) and in progranulin-deficient mice (Zhao et al. 2014; Liu 2014), only some representative models of each category are described here.

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Canine Anterior Cruciate Ligament (ACL) Transection Model

Purpose and Rationale

Similar to human ACL ruptures (Kiapour and Murray 2014), ACL transection in the dog knee and the subsequent joint instability result in progressive cartilage erosion, fibrillation, and formation of osteophytes. This elaborate model is well characterized and regarded of high predictive

value. It is mostly known as the "Pond–Nuki dog model." These authors achieved the ACL transection originally through a lateral stab incision, whereas others also performed the ligament transection after opening the joint. Both versions lead to similar morphological and biochemical changes and are described and illustrated in detail by Adams and Pelletier (1988).

Procedure

Animals

Mongrels, beagles, greyhounds, and foxhounds are reported to be suitable, provided purebred strains are used. In general, younger animals display more repair phenomena, whereas the older ones seem to show more rapid degeneration. As the epiphyses fuse at the age of 13 months, and as pronounced changes require up to 4 months to fully develop, careful planning ahead is essential.

Operation

The dogs are initially anesthetized with 30 mg/kg sodium pentobarbital i.v., followed by a continuous inhalation of 1 % halothane with 1 l/min N_2O and 2 l/min O_2. After shaving and sterilizing the knee joint externally, it is fixed in a bent position at 90°, and a scalpel blade is inserted medially deep into the joint space diagonally posterior to the ACL and parallel to the lateral border of the patellar ligament. By rotation of the blade, the ACL is then dissected, the blade withdrawn, and the wound closed. In the contralateral knee, a sham operation is performed to inflict similar disturbance to the joint tissue, but without harming the ACL. The ACL dissection results after 8–12 weeks, in contrast to the sham-operated contralateral knee, in fibrillation and erosion of the cartilage, more pronounced on the tibial plateau than on the femoral condyles. Also observed histologically is a loss of metachromatic staining, and a fissured surface with cell clones appears. A marked osteophytosis and subchondral sclerosis have also developed at that time. Proteoglycan content and overall cartilage thickness, however, seem to remain stable (Pelletier and Martel-Pelletier 1985) or even increase not only for several months (McDevitt et al. 1977; Vignon et al. 1983; Brandt and Adams 1989) but for up to 3 years after transection, as Brandt, Myers et al. (1991) could show. At later stages, however, severe cartilage thinning and loss is recorded (Brandt et al. 1991a).

Evaluation

Macroscopic inspection of cartilage and osteophytes is recorded. Histological grading based on the Mankin score has been reported to be modified by drug treatment over 7 weeks (Abatangelo et al. 1989; Schiavinato et al. 1989). As levels and activity of neutral matrix metalloproteinases are elevated in the cartilage and synovium (Pelletier and Martel-Pelletier 1985), they might be additional parameters of interest to profile the test compounds.

Critical Assessment of the Test

In this instability model, a polysulfated glycosaminoglycan preparation (Arteparon), as well as intra-articular injections of a hyaluronic acid preparation, induced some morphological and biochemical changes, whereas low-dose prednisone had no effect. As there seems to prevail an anabolic response to the instability in the articular cartilage for quite a long time, the selected time points and parameters to assess disease progression and therapeutic success require careful consideration.

Modifications of the Test

Caron et al. (1996) and Pelletier et al. (1997) investigated the in vivo effect of the recombinant human interleukin-1 receptor antagonist on the development of lesions in the anterior cruciate ligament transection model in dogs.

Wenz et al. (1998) used the Pond–Nuki model in dogs to evaluate the effectiveness of intra-articular application of hyaluronic acid on the early forms of femoropatellar arthrosis. Pathomechanics of the canine Pond–Nuki model have been further explored by Pozi et al. (2013). Transection of the anterior cruciate ligament in dogs was also used in studies by Myers et al. (1999), Boileau et al. (2002, 2005), Smith et al. (1999, 2002), Behets et al. (2004), and Matyas et al. (2004).

Pelletier et al. (2005) and Moreau et al. (2011) studied the effects of licofelone, a 5-lipoxygenase/cyclooxygenase inhibitor, and tiludronate, a bisphosphonate, respectively, in the experimental anterior cruciate ligament dog model of osteoarthritis. Xie et al. (2013) investigated the effects of platelet-rich plasma on gene expression associated with healing of soft tissue.

ACL transection can be performed also in an arthrotomy operation as described by Adams and Pelletier (1988), and an instability can be achieved equally by meniscectomy (Hannan et al. 1987).

The **rabbit** is the main other species used for instability models, as described in detail for partial medial meniscectomy by DiPasquale et al. (1988), and for partial lateral meniscectomy in connection with ligament transection by Colombo (1988).

Obara et al. (1993) induced osteoarthritis by surgical dissection of the anterior cruciate ligament in rabbits and investigated fluorescence distribution after intra-articular administration of fluorescein-labeled sodium hyaluronate.

Anterior cruciate ligament transection in rabbits was used in several studies (Amiel et al. 2003; Kawano et al. 2003; Doschak et al. 2004; Zhang et al. 2004; Diaz-Gallego et al. 2005; Tiraloche et al. 2005).

Kobayashi et al. (2005) studied a vitamin B_1 derivate and its ability to enhance the chondroprotective effects of glucosamine hydrochloride and chondroitin sulfate in osteoarthritis created by partial medial meniscectomy of the knee joint in rabbits.

A partial medial meniscectomy with ligament transection is described for the **guinea pig** by Bendele (1987) and Schwartz (1988). This method has been used by Bendele et al. (1999) and by Sabatini et al. (2005).

Layton et al. (1987) produced biomechanical stress-induced hip osteoarthritis in guinea pigs by extra-articular myectomy and tendotomy.

The Hartley guinea pig strain spontaneously develops knee osteoarthritis whose manifestations are markedly similar to those of spontaneous knee osteoarthritis in humans (Bendele et al. 1989; Kraus et al. 2004).

Janusz et al. (2002) described a model of osteoarthritis in **rats** induced by surgically transecting the medial collateral ligament and meniscus and evaluated the effectiveness of a matrix metalloproteinase inhibitor in this model. Using this model, Moore et al. (2005) found that fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair. Pickarski et al. (2011) characterized the molecular changes in cartilage and subchondral bone in the rat model and provide data supporting potential exploratory biomarkers of disease progression.

Machner et al. (1999) investigated the influence of an altered sensible joint innervation on the development of knee osteoarthritis in Wistar rats. Partial sensible joint denervation was performed by injection of 100 μg capsaicin into a sponge placed on the sensible nerve trunk. Half of the rats underwent strenuous running exercises (20 km in a running wheel by intracranial self-stimulation). Under these conditions, severe osteoarthritis changes were observed, which were only mild without exercise.

Hayamai et al. (2004) studied cartilage degeneration and osteophyte formation in the anterior cruciate ligament transection model in rats, and Strassle et al. (2010) investigated the effects of zoledronate on osteoclast-mediated bone resorption in a monosodium iodoacetate rat model of degenerative joint disease. Naveen et al. (2014) reported on a comparison of degenerative structural changes observed in the monosodium iodoacetate model and in the rat ACL transaction model.

Wancket et al. (2005) used the medial meniscectomy model in Lewis rats to study the anatomical localization of cartilage degradation markers.

Ghosh et al. (1993), Appleyard et al. (1999), and Burkhardt et al. (2001) used an osteoarthritis model induced by lateral meniscectomy in **sheep**.

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Chymopapain-Induced Cartilage Degeneration in the Rabbit

Purpose and Rationale

Intra-articular injection of chymopapain into the rabbit knee joint results in cartilage degradation with rapid loss of proteoglycans. A transient inflammation shortly after injection normally subsides after 1–2 days. Severity and reversibility of the cartilage damage can be altered using different protocols (Williams et al. 1992).

Procedure

Most authors use New Zealand white rabbits at the age of 2–4 months. Male Chinchilla and Chinchilla Bastard rabbits are preferred, as they display a thicker cartilage than the New Zealand strain. The animals with an initial body weight of ca 2.5 kg are anesthetized with a continuous inhalation of 4 % halothane – 3 l/min N_2O – 1.8 l/min O_2. Both knee joints are carefully shaved and moisturized with 70 % alcohol. With a sterile syringe, a volume of 0.1 ml of the following solution is injected laterally into the joint space: 18 mg chymopapain (Sigma) is dissolved in 1 ml 0.9%NaCl, and 50 mg L-cysteine HCl is added for activation. The preparation is then passed under sterile conditions through a 0.22 μm Millipore filter and tested for activity prior to each operation. (Potential direct interference of the therapeutic agent to be tested with the chymopapain can also be addressed at that point.) One injection of 1.8 mg chymopapain in 0.1 ml per joint usually results in a proteoglycan loss/cartilage dry weight of ca 40 % after 10–12 days. Two to four animals per experiment receive chymopapain in 0.9 % NaCl in one joint and 0.9 % NaCl into the contralateral knee to assess the proteoglycan loss caused by the enzyme. Care is taken with all injections to apply the same volume to both joints, as the contralateral serves as internal control. If the drug is given orally, only one knee receives chymopapain treatment, whereas if the drug is applied intra-articularly into one knee (1–5 injections of 0.1 ml volume each, vehicle into contralateral joint, in a period of 5–10 days), both knees are treated with chymopapain. The animals are sacrificed after 10–14 days, and from defined regions of weight-bearing areas in the joint, full-thickness samples are obtained for histology and assessment of proteoglycan content.

Histology is performed on full-thickness sections of the articular cartilage, fixed with 3 %

formalin, embedded in paraffin, and stained routinely with safranin O/fast green or with toluidine blue (Romeis 1989).

The proteoglycan content is determined with the dimethylmethylene blue (DMMB) method modified after Farndale et al. (1986) and Chandrasekhar et al. (1987). The wet weight of the cartilage samples is determined immediately after preparation, and their dry weight is recorded after 3 days of drying at 60 °C. Samples are then soaked overnight in 1 ml buffer (containing 20 mM disodium hydrogen phosphate,1 mM EDTA, and 2 mM dithiothreitol in 500 ml aqua bidest at pH 6.8). Ten µl of papain, suspended in 0.05 M sodium acetate (Sigma 9001–73-4), is then added and incubated at 60 °C for 6 h. Ten µl of standard (shark chondroitin sulfate C (Sigma C4384) in a concentration range from 10 to 200 µg/ml buffer) and of each probe is then transferred into a 96-well microtiter plate. Two hundred µl of DMMB is added, and, after 1 min, the extinction is recorded photometrically at 690/540 nm. The DMMB solution is prepared by dissolving 16 mg DMMB (Serva 20335) in 2 ml methanol, adding 400 ml aqua bidest, 9.6 ml 1 N HCl solution, 3.04 g glycine, and 2.36 g NaCl, stirred and warmed until dissolved, and then diluted to a final volume of 1,000 ml with aqua bidest (pH 3.0). The results are recorded as µg chondroitin sulfate equivalents/mg cartilage dry weight.

Evaluation

The histological appearance of the articular cartilage is assessed with a modified Mankin score, emphasizing more earlier changes, and separating degradative aspects (e.g., loss of safranin staining) from repair aspects (e.g., the occurrence of pericellular staining). The proteoglycan content is expressed as % change to the contralateral knee or, comparing groups, to chymopapain-treated or untreated control groups. To relate the proteoglycan content to cartilage, dry weight has been found to be more reliable than choosing wet weight as baseline, as the cartilage water content is known to change in this model according to matrix composition.

Critical Assessment of the Test

In this model, hyaluronic acid preparations and some MMP inhibitors can be detected; NSAIDs do not change the parameters tested. The protocol can be modified to emphasize repair phenomena or to assure a more severe course of cartilage loss (Williams et al. 1993).

Modifications of the Model

Muehleman et al. (2002) investigated bone remodeling inhibition by a bisphosphonate in the rabbit model of cartilage matrix damage by chymopapain injection.

To avoid interaction of a drug with the enzymatic nature of the chymopapain, other degradation mediators can be applied instead, as, e.g., fibronectin fragments (Williams et al. 1988).

To ensure continuous synovial levels of a given drug, an osmotic minipump containing the drug can be implanted subcutaneously (Furman et al. 2014) in a mouse model of knee injury.

Kikuchi et al. (1998) induced experimental osteoarthritis in mature rabbits by intra-articular injection of collagenase.

Pomonis et al. (2005) developed a **rat model of osteoarthritis pain**. Male Sprague Dawley rats were anesthetized with isoflurane and received a single injection of iodoacetate (0.3, 1, or 3 mg), papain (0.5, 1, 2, or 3 %), or saline in a 50 µl volume into the left knee joint using a 27-gauge needle inserted through the patellar tendon. Assessment of pain-related behaviors was performed using a hind limb weight-bearing apparatus (Linton Incapacitance Tester Stoelting, Wood Dale, Ill., USA). Animals treated with intra-articular iodoacetate were evaluated for alterations in weight bearing after the acute and chronic administration of various drugs.

Osteoarthritic lesions in the knee joints of male C57bl10 mice were induced by a single intra-articular injection of bacterial collagenase (Van der Kraan et al. 1990; Van den Berg et al. 1993).

Van der Kraan et al. (1989) and Scharstuhl et al. (2002) induced arthritis in mice by a single intra-articular injection of papain in their knee joints. Khan et al. (2013) developed a rat model and scoring system potentially amenable to investigating the pathology of early OA.

Van Osch et al. (1995) developed a device to measure laxity of knee joints in mice. Reproducible, nonlinear S-shaped load displacement curves were determined from knee joints of normal mice. Parameters of anterior–posterior translation, varus–valgus rotation, and compliance were calculated from the curves. Laxity was markedly increased in animals with osteoarthritis induced by intra-articular injection of collagenase.

Saez-Llorens et al. (1991) and Cohen et al. (2004) described a model of **septic arthrosis** in rabbits.

Four-month-old female New Zealand rabbits were anesthetized with an intramuscular injection of 60 mg/kg ketamine and 6 mg/kg xylazine. The skin overlying the knees was shaved and cleansed with povidone-iodine and 70 % ethanol. The right knee was injected with 10^5–10^6 colony-forming units of *Staphylococcus aureus*, while the left knees were injected with sterile saline. Following injection, all rabbits were allowed normal cage activity. The rabbits were left either untreated or treated with antibiotic (40 mg/kg ceftriaxone) alone or additionally with different doses of test drug. Animals were anesthetized at 1, 4, and 7 days following initiation of treatment. Prior to aspiration of synovial fluid, the knee joint was inspected for evidence of effusion and range of motion testing. Following an arthrotomy, the gross appearance of the joint was examined for joint degradation and the fluid grossly inspected for purulence. Prior to sacrifice, an arterial blood sample was plated on agar plates, incubated for 48 h at 37 °C, and analyzed for colony growth. In the synovial fluid, the number of white blood cells was determined and bacterial growth examined in serial dilutions. Histology was performed in synovial tissue from the prepatellar fat pad and the medial femoral condyle.

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Spontaneous OA Model in STR/1 N Mice

Purpose and Rationale

This spontaneous model has the advantage of a gradually developing OA starting with cartilage surface erosion and fibrillation to osteophyte formation, subchondral bone remodeling, and finally eburnation, in a moderate time frame. All these steps, with only intermittent inflammatory flares, are similar to human pathology and develop without any interference. The disadvantages are, however, the restriction to systemic drug application, due to the relative small animal size, and the variance in onset and severity of the disease among the animals.

Procedure

The STR/1 N mouse strain, characterized extensively by Walton (1977–1979), can be obtained from the National Institutes of Health, Bethesda, MA, USA. It is not identical to the STR/ort strain as described by Benjamin et al. (1995), since it displays, in contrast to the ORT, no calcification in the ligaments or tendons. Male mice develop OA earlier and more consistently than females and, without dietary restriction, develop additionally obesity.

Beginning at the age of 10 weeks, male mice are trained to walk on a slowly rotating cylinder (or treadmill, manufactured by Ugo Basile, Italy) recording the mean walking time of each mouse. Mice with a moderate activity (neither dropping off too soon nor staying on for hours) are then selected to enter the experiment. In groups of 8–10 animals, the drug is applied systemically for 8 weeks, and the mobility of each animal is recorded once or twice a week on the rotating cylinder. The body weight is recorded regularly as well. At the end of the experiment, the animals are sacrificed, and both knee joints are dissected, fixed, decalcified, and embedded in defined orientation for histology.

Evaluation

The mean walking time decreases with age and disease progression and is recorded in a time-dependent graph over the treatment period.

The medial tibial plateau of the knee joints exhibits the most pronounced cartilage fibrillation and loss and is, therefore, selected for histological evaluation, based on a modified Mankin grade.

Critical Assessment of the Test

Since this model is best suitable for oral drug application, experience with disease-modifying drugs for osteoarthritis is limited. Drugs with anti-inflammatory properties increase walking time, but do not alter the morphological aspects of cartilage degradation in the joint. Analgesic drugs have no effect on either.

Modifications of the Test

Rudolphi et al. (2003) studied an inhibitor of interleukin-1β-converting enzyme in STR/1 N mice and in a collagenase-induced osteoarthritis model in BALB/c mice. Weakening of knee joint ligaments was achieved by intra-articular injection of highly purified bacterial collagenase leading to increased joint laxity which resulted in osteoarthritic lesions preferentially in the medial compartments of the injected knee joint (Van der Kraan et al. 1990).

Several authors (Nakamura 1990; Pataki et al. 1990; Wilhelmi and Meyer 1983) have used the C57black mouse which, however, is reported to vary considerably in the incidence and severity of the disease and also to develop the osteoarthritis only at older ages.

The STR/ort strain differs in its calcification of fibrous cartilage (Benjamin et al. 1995) and seems to change the orientation of the proteoglycans in the hyaline cartilage (Dunham et al. 1989). Kyostio-Moore et al. (2011) conducted an extensive analysis of potential biomarkers representative of spontaneous OA in this model. They included analyses of cytokines, markers of both oxidative stress and inflammation from hard to soft tissues, and compared the output with that obtained from samples analyzed in the CBA mouse, an OA-resistant mouse strain. Poulet et al. (2013) proposed that the development of spontaneous OA is likely not related to greater vulnerability to mechanical trauma as determined by histological analysis and FE geometric modeling.

Gaffen et al. (1997) found elevated aggrecan mRNA in STR/ort mice. Brewster et al. (1998) tested an orally active collagenase inhibitor in STR/ort mice. Microfocal X-ray-generated images of the hind limbs as well as histologic sections of the knees were scored for degradative changes and drug effects. Chambers et al. (2001) reported that matrix metalloproteinases and aggrecanases cleave aggrecan in different zones of normal cartilage but co-localize in the development of osteoarthritic lesions on STR/ort mice. Mason et al. (2001) reviewed the use of the STR/ort mouse as a model of osteoarthritis. Flannelly et al. (2002) studied the temporal expression of matrix metalloproteinases and tissue inhibitors in the murine STR/ort model of osteoarthritis. Price et al. (2002) compared collagenase-cleaved articular cartilage collagen in mice in the naturally occurring STR/ort model of osteoarthritis and in collagen-induced arthritis. Manion et al. (2011) found that administration of aspartyl-phenylalanine 1-methy ester in the diet delayed the progression of OA and prevented bone loss as late as 12–15 months into the study.

Glant et al. (1998) reported progressive polyarthritis induced in BALB/c mice by aggrecan from normal and osteoarthritic human cartilage.

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Transgenic Mice as Models of Osteoarthritis

Purpose and Rationale

Osteoarthritis comprises a group of diseases characterized by gradual degeneration of articular cartilage and a number of associated processes within the joint. Consequently, no single animal model based on one genetic alteration is likely to fulfill all the criteria of human osteoarthritis (Helminen et al. 2002; Säämänen and Vuorio 2004). Transgenic mice with **collagen mutations** include different types of collagen.

Several lines of transgenic mice have been produced harboring mutations in the type II collagen gene. These include a line expressing a human *COL2A1* transgene with a large internal deletion (Vandenberg et al. 1991) and another expressing a murine *Col2a1* transgene with a Gly58Cys substitution in the helical domain (Garofalo et al. 1991). An osteoarthritis phenotype can also be produced by increasing the expression rate of the normal *Col2a1* gene, which leads to disruption of the regulation of type II collagen fibril assembly (Garofalo et al. 1993). The transgenic mouse line Del1 harbors six copies of the *Col2a1* transgene with a 150-bp deletion of exon 7 and intron 7, removing sequences coding for the 15 amino acids at the amino terminal end of the triple helical domain (Metsäranta et al. 1992). Homozygous Del1 mice die at birth due to respiratory distress. Heterozygous Del1 mice develop early-onset human osteoarthritis-like lesions, usually confined to the knee joint (Säämänen et al. 2000).

Mice transgenic for type IX collagen mutations carrying a central in-frame deletion mutation that codes for truncated α1 (IX) chains develop mild chondrodysplasia and progressive osteoarthritis with ocular involvement (Nakata et al. 1993). Fässler et al. (1994) reported a non-inflammatory degenerative joint disease in mice lacking α1 (IX) collagen.

Xu et al. (2003) demonstrated osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of mice with the chondrodysplasia gene (*cho*). A mutation in the gene encoding the α1 chain of type XI collagen (*Col11a1)* was identified as the genetic cause of chondrodysplasia in mice.

Several transgenic models of osteoarthritis with mutations in non-collagenous molecules were described. Ameye et al. (2002) reported that abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. Ameye and Young (2002) recommended mice deficient in small leucine-rich proteoglycans as novel in vivo models of osteoporosis, osteoarthritis, Ehlers–Danlos syndrome, muscular dystrophy, and corneal diseases.

Zemmyo et al. (2003) described accelerated, aging-dependent development of osteoarthritis in α1 integrin-deficient mice. Morko et al. (2004) reported the upregulation of cathepsin K expression in articular chondrocytes in a transgenic mouse model of osteoarthritis.

Several studies report on the utility of inducible collagen transgenic mouse models. Kyrkanides et al. (2011) showed that experimental osteoarthritis driven in the collagen I-IL-1b^{XAT} transgenic model could induce neuroinflammation with pathology of Alzheimer's disease, offering support to the notion that peripheral inflammation may drive inflammation in the brain. Hosaka et al. (2013) studied the effect of Notch signaling in a col2a1 inducible transgenic mouse providing evidence of the Notch signaling pathway in endochondral ossification.

Involvement of **matrix metalloproteinases** has been demonstrated in the degradation of cartilage and the development of human osteoarthritic lesions. Holmbeck et al. (1999) reported that MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Neuhold

et al. (2001) found that postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. These mice develop cartilage lesions with histological similarities to human osteoarthritis. Salminen et al. (2002) reported differential expression patterns of matrix metalloproteinases and their inhibitors during development of osteoarthritis in the transgenic Del1 mouse model. Glasson et al. (2005) reported that deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis.

Huang et al. (2014) showed that enhanced synthesis of endogenous n-3 polyunsaturated fatty acids (PUFAs) can delay the onset of surgically induced OA in *fat-1* transgenic mice, where the fat-1 protein enhances the composition of n-3 to n-6 PUFAs.

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In Vitro Methods for Anti-Osteoarthritic Activity: General Considerations

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Canine Anterior Cruciate Ligament (ACL) Transection Model

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Methods for Testing Immunological Factors

Martin Braddock

Contents

Methods for Testing Immunological Factors

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Inhibition of Histamine Release from Mast Cells

Purpose and Rationale

Hypersensitivity reactions can be elicited by various factors: either immunologically induced, i.e., allergic reactions to natural or synthetic compounds mediated by IgE, or non-immunologically induced, i.e., activation of mediator release from cells through direct contact, without the induction of, or the mediation through immune responses. Mediators responsible for hypersensitivity reactions are released from mast cells. An important preformed mediator of allergic reactions found in these cells is histamine. Specific allergens or the calcium ionophore 48/80 induce release of histamine from mast cells. The histamine concentration can be determined with the *o*-phthalaldehyde reaction.

Procedure

Preparation of Mast Cell Suspension

Wistar rats are decapitated and exsanguinated. Fifty ml of Hank's balanced salt solution (HBSS) is injected into the peritoneal cavity, and following massage of the body, the abdominal wall is opened. The fluid containing peritoneal

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cells is collected in a centrifuge tube and centrifuged at 2,000 rpm. The cells are resuspended in HBSS. Then the cell suspension is brought to a final concentration of 10^5 mast cells/100 µl.

Test Compound Administration and Induction of Histamine Release

1 ml test drug (concentration range between 10^{-4} and 10^{-8} Mol) is added to the mast cell suspension (10^5 cells/100 ml) and the mixture is incubated at 37 °C for 15 min. The cells are made up to a volume of 3 ml with HBSS, an equal volume of calcium ionophore (10^{-6} g/ml), compound 48/80, or specific allergen is added. The suspension is incubated at 37 °C for 30 min followed by centrifugation at 2,500 rpm.

The Following Control Solutions Are Needed

- *Spontaneous histamine release*: contains only mast cells and solutions used to determine the baseline
- *Histamine release*: contains mast cells and solutions and calcium ionophore (10^{-6} g/ml)
- *Test compound control*: contains solutions and test compound to test the compound for native fluorescence
- *Solution control*: contains only solutions used in the test to determine the baseline

Extraction of Histamine

One ml of the top layer is transferred to a tube containing 300 mg NaCl and 1.25 ml butanol. The sample is alkalized to extract the histamine into butanol by adding 1 ml 3 N NaOH. Following mechanical shaking, the sample is centrifuged for 5 min. One ml of the top layer (butanol) is pipetted into a 5-ml tube containing 2 ml of n-heptane and 0.4 ml of 0.12 N HCl. The tube is mixed by inverting it several times. Following separation into aqueous and organic phases, 0.5 ml of the aqueous phase is transferred to another tube.

Induction of *o*-Phthalaldehyde Complexing Reaction

To each sample, 100 µl 1 N NaOH is added under constant stirring immediately followed by administration of 100 µl 0.2 % *o*-phthalaldehyde solution. After 2 min, the *o*-phthalaldehyde complexing reaction is stopped by addition of 50 µl 3 N HCl.

Determination of Histamine Release

The total sample is transferred to an autosampler vial, and the histamine concentration is determined by a fluorescence detector (using excitation and emission wave lengths of 350 and 450 nm, respectively).

Evaluation

Percent histamine release (hist. rel.) can be expressed by the following formula:

$$\frac{\text{Sample hist. rel.} - \text{Spontaneous hist. rel.}}{100\%\ \text{hist. rel.} - \text{Spontaneous hist. rel.}} \times 100$$

The statistical evaluation is carried out using the Student's *t*-test (comparison of 100 % control to experimental group).

Critical Assessment of the Method

Disodium cromoglycate has been reported to inhibit the release of histamine and the degranulation of rat mast cells (Orr and Cox 1969; Orr et al. 1971; Johnson and Bach 1975; Church and Young 1983). However, this effect of disodium cromoglycate and its analogues does not parallel the clinical efficacy (Kay et al. 1987).

Modifications of the Method

Johnston et al. (1978) studied the increased superoxide anion production by immunologically activated and chemically elicited macrophages.

Flint et al. (1985) found a significant inhibition of histamine release by disodium cromoglycate in human mast cells recovered by bronchoalveolar lavage.

Ali et al. (1985) investigated the histamine release from rat peritoneal mast cells, human basophil and neutrophil leukocytes, and mast cells from mesentery of the lung and heart of rats and guinea pigs by the skin irritating constituents thapsigargin and thapsigargicin from the resin of the umbelliferous plant *Thapsia garganica*.

Eady (1986) studied the reactivity of mast cells in bronchoalveolar lavage fluid of macaques repeatedly infected with *Ascaris suum*.

Wells et al. (1986) compared release of histamine, LTC_4, and PGD_2 from primate bronchoalveolar mast cells with that of rat peritoneal mast cells.

The release of β-hexosaminidase from mouse or rat bone marrow-derived mast cells and from rat peritoneal mast cells was studied by Broide et al. (1986).

Peretti et al. (1990) recommended flow cytometry to investigate mast cell degranulation. Peptides, including substance P and bradykinin analogues, release histamine from human skin mast cells (Lawrence et al. 1989).

Williams et al. (1991) studied the vancomycin-induced release of histamine from rat peritoneal mast cells and a rat basophil cell line (RBL-1).

Kase et al. (2009) studied the inhibitory action of roxithromycin on histamine release in mast cells and Yazid et al. (2013) provided further support for antiallergic activity of chromones.

A sensitive colorimetric assay for the release of tryptase from human lung mast cells in vitro has been described by Lavens et al. (1993).

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Mitogen-Induced Lymphocyte Proliferation

Purpose and Rationale

Cultured lymphocytes can be stimulated to a proliferative response and to DNA synthesis by various mitogens. Measurement of DNA synthesis can be accomplished by pulse-labeling the culture with tritiated thymidine (^{3}H-thymidine), a nucleoside which is incorporated into the newly synthesized DNA. Immunomodulating properties can be detected either by pretreatment of the animals in vivo or by adding the test drug to the cultured lymphocytes.

Procedure

Mice of NMRI strain weighing 18–20 g or rats of Lewis strain weighing 180–200 g are used.

Materials

Sheep red blood cell (SRBC)-specific antigen and/or the following mitogens:

- Lipopolysaccharide 10–0.1 μg/ml.
- Dextran sulfate 30–7.5 μg/ml.
- Phytohaemagglutinin 0.5–0.12 % stock solution.
- Concanavalin A 0.5–0.12 μg/ml.
- As standards, levamisole, cyclosporine A, prednisolone, or leflunomide are used.

Ex Vivo

Animals receive the test compound once a day for 5 days. Thereafter, they are sacrificed, spleens are removed, and a single cell suspension of 5×10^6 cells/ml is prepared. Mitogens are titrated (four replicates/group) in 0.1 ml/well and 0.1 ml of the cell suspension is added. Plates are incubated at 37 °C in 5 % CO_2 in air for 48–60 h and for another 8 h after addition of 0.25 μC ^{3}H-thymidine per well. Cells are harvested on glass fiber filters, and after drying the degree of radioactivity is determined using a β-counter.

In Vitro

Animals are sacrificed and their spleens removed. A single cell suspension of 10^7 cells/ml is prepared and 0.05 ml placed in each microtiter well (four replicates/group). Then the test compound (four times concentrated) is added in 0.05 ml. At last 0.1 ml of the double concentrated mitogen is added. Plates are incubated and processed as described above.

Evaluation

Stimulation index = proliferation ratio according to positive control, either with or without mean

spleen weight. Statistical evaluation is carried out using the Student's *t*-test (comparison of positive and/or negative control to experimental group).

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Inhibition of T Cell Proliferation

Purpose and Rationale

Activation and/or proliferation of clonal populations of T cells are critical for the initiation of an antigen-specific immune response. Thus, inhibition of T cell activation provides a potent means for suppressing specific immune response. A number of immunosuppressive agents exhibit the ability to suppress T cell activation.

Procedure

Purification of Peripheral Blood Leukocytes and T Cells

Peripheral blood leukocytes from normal donors are separated on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Leukocyte suspensions are washed in HBSS and are resuspended in RPMI 1664 medium (Gibco, Grand Island, NY) containing 10 % heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. Leukocyte suspensions are resuspended in RPMI 1664 containing 10 % heat-inactivated pooled human serum. Highly enriched T cells are obtained by passing leukocytes through a nylon wool column to remove macrophages and B cells and then depleted of NK and monocytes with anti-Leu 11 b (Becton Dickinson, Mountain View, CA) plus complement (Pel-Freez, Brown Deer, WI). These highly enriched T cells are approximately 95 % CD^{3+} cells, the remaining cells being B lymphocytes.

Mixed Lymphocyte Reaction

Peripheral blood leukocytes are incubated at 2×10^5/well with equal numbers of gamma-irradiated (3,000 rads) allogenic peripheral blood leukocytes and various concentrations of test compounds. Assays are performed in triplicate in 96-well, U-bottom plates. After 6 days of coculture, the cells are pulsed for 6 h with 1 μC of [^{3}H]thymidine per well. [^{3}H]Thymidine incorporation is then measured by scintillation counting. Data are presented as

$$\% \text{ inhibition} = \frac{\text{CPM}_{\text{expt}} - \text{CPM}_{\text{bckgrd}}}{\text{CPM}_{\text{ctrl}} - \text{CPM}_{\text{bckgrd}}} \times 100$$

where CPM_{expt} is mean counts per min of experimental cultures; $\text{CPM}_{\text{bckgrd}}$ is mean counts per min of background well, unstimulated cultures; and CPM_{ctrl} is mean counts per min of uninhibited, stimulated cultures.

Lymphocyte Stimulation and Proliferation

Peripheral blood leukocytes and isolated T cells are cultured with anti-CD3 (5 ng/ml) plus PMA (5 ng/ml), anti-CD28 (1:5,000 dilution) plus PMA (5 ng/ml), or 100 U/ml rhuIL-2 in RPMI 1644 containing 10 % fetal bovine serum. Peripheral blood leukocytes or T cells are cultured at 2×10^5 cell per well in a total volume of 200 µl/well. Assays are performed in quadruplicate in 96-well, U-bottom plates. [^{3}H]Thymidine (1 µC) is added to each well after 48 h of coculture, and after a 20 h pulse of [^{3}H] thymidine, the cells are harvested, and the amount of [^{3}H]thymidine uptake is quantitated on a scintillation counter.

ELISA Assays

Supernatants/well (100 ml) are harvested 24 h after initiation of cultures of peripheral blood leukocytes or T cells stimulated with anti-CD3 or anti-CD28 plus PMA. IL-2 in the coculture supernatant is quantitated using a commercially available IL-2 ELISA kit. All experiments are performed in duplicate.

IL-2R Assays

The expression of IL-2R on T cells stimulated for 48 h with anti-CD3 or anti-CD28 plus PMA is determined using FITC-conjugated anti-CD25 mABs (Becton Dickinson, Mountain View, CA). T cells are washed in HBSS and then stained with phycoerythrin-conjugated anti-CD3 mAB and fluorescein-conjugated antiCD25 mAB. The percent of cells coexpressing CD3+ and CD25+ is determined from 2,000 cells using an EPICS C flow cytometer (Coulter, Hialeah, FL).

Evaluation

Dose–response curves of inhibition of one-way mixed lymphocyte reaction and of IL-2 in the supernatant after stimulation with antiCD3 or anti-CD28 are established.

Modifications of the Method

Zielinski et al. (1993, 1994) studied the influence of leflunomide on expression of lymphocyte activation expression markers (IL-2 and transferrin receptors) as well as on cell cycle and on IL-2 receptor gene expression.

Calcineurin was found to be a key signaling enzyme in T lymphocyte activation and the target of immunosuppressive drugs (Clipstone and Crabtree 1993).

The viability and function of T lymphocytes has been explored using different cellular isolation techniques (Klein et al. 2006). A number of different vehicles have been shown to inhibit T cell proliferation which include the natural product silymarin (Morishima et al. 2010), heavy metals and polychlorinated biphenyls (Frouin et al. 2010), alternatively activated macrophages (Huber et al. 2010), type I interferon (Marshall et al. 2011), mesenchymal stem cells (Zinocker and Vaage 2012), and the programmed cell death-1 receptor (Patsoukis et al. 2015).

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Chemiluminescence in Macrophages

Purpose and Rationale

The stimulation of macrophages by antigen, complement, phorbolesters, etc., leads to elaboration of O_2^- and other oxygen metabolites. Superoxide ion (O_2^-) and other highly reactive oxygen metabolites (radicals) form the basis for an efficient microbicidal system in vivo. Yet, when these radicals are released in response to self-antigens, tissue damage is often the result. Inhibition of this process can be regarded as a measure for immunomodulating effects of compounds. The oxygen metabolites can produce light-emitting reactions (chemiluminescence), which is measurable if amplified with suitable agents, such as the cyclic hydrazide luminol.

Procedure

NMRI mice weighing 30 g or Sprague–Dawley rats weighing 250–300 g of either sex are used.

Positive Control

1. Sensitized mice, receiving vehicle
2. Mice, developing an autoimmune disease, receiving vehicle
3. Rats, developing adjuvant arthritis, receiving vehicle

Negative Control

1. Mice not sensitized, receiving vehicle
2. Mice, not developing an autoimmune disease, receiving vehicle
3. Rats without adjuvant arthritis

Materials

- 5×10^8 SRBC (sheep red blood cells)/0.5 ml 0.9 % NaCl solution (for sensitization)
- *Phorbolester:* Stock solution of 1 mg/ml phorbolmyristenacetate. This stock solution is diluted with Hank's balanced salt solution to a final concentration of 3.5 μM (working solution). For the induction of chemiluminescence,

the working solution is diluted in the test tube 1:4, resulting in a final phorbolester concentration of 0.875 µM.
- Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) final concentration 25 µg/ml

Ex Vivo Experiment

Groups of six animals are treated for 6 days orally or subcutaneously with test compound or the standard (prednisolone acetate or leflunomide). They are decapitated and exsanguinated. Macrophages are obtained by flushing the peritoneal cavity with 10 ml saline, containing 250 IU heparin. The cells are pooled, washed several times, and suspended again at a final concentration of 2×10^6/200 µl.

For measurement in the luminometer, the following mixture is prepared:

200 µl macrophages (2×10^6)
100 µl luminol solution (100 µg/ml)
100 µl phorbolmyristenacetate solution (3.5 µM)

Each sample is mixed thoroughly without the phorbolmyristenacetate solution, put into the luminometer, and counted at 2 min intervals for 10 s. The addition of the phorbolester induces the reaction.

In Vitro Experiment

To 100 µl of macrophage suspension (2×10^6 cells) is added 100 µl of the solution of the test compound and incubated for 15 min at 37 °C.

Then, 100 µl of luminol solution (100 µg/ml) and 100 µl of the 3.5 µM phorbolester solution are added and the luminescence measured in the luminometer.

Evaluation

The time of maximal counts for the positive control is recorded. For all groups, the ratio of counts per 10 s is determined at that time, compared to the positive control counts per 10 s, and the percent change is calculated. For statistical evaluation, the experimental group is compared with the positive control group using Student's *t*-test.

Modifications of the Method

Bird and Giroud (1985) described a technique of polymorphonuclear leukocyte chemiluminescence as a means to detect compounds with anti-inflammatory activity. Inflammatory polymorphonuclear leukocytes were obtained by injecting rats intrapleurally with 1 ml of a 1 % solution of calcium pyrophosphate and collection of the pleural exudate 4 h later. Chemiluminescence responses were measured using a Packard Picolite chemiluminometer and opsonized zymosan as the stimulus.

Seeds et al. (1985) found an independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes.

A microtechnique for studying chemiluminescence response of phagocytes using whole blood was described by Selvaraj et al. (1982).

Traykov et al. (1997) investigated the effects of phenothiazine compounds on activated macrophage-induced luminal-dependent chemiluminescence, and Szliszka et al. (2013) studied the anti-inflammatory activity of artepillin C, a constituent of the resinous green propolis. Van Dyke et al. (2003) explored the use of lucigenin-based chemiluminescence assay to interrogate various inflammatory stages.

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PFC (Plaque-Forming Colony) Test In Vitro

Purpose and Rationale

Identification of antibody-producing cells is based on the ability of the secreted IgM antibody to fix complement and thereby lyse the indicator erythrocytes. Spleen cells or peripheral blood lymphocytes, previously incubated with antigen, are mixed with sheep red blood cells (SRBC). After addition of complement and incubation, plaques (clear areas) caused by the lysis of SRBC appear in the otherwise cloudy layer. Antibody-forming cells can be detected by the appearance of plaques. The number of plaques obtained is proportional to the number of antibody-producing lymphocytes in the cell population.

Procedure

NMRI mice weighing 16–18 g or Lewis rats weighing 180–200 g of either sex are used.

Materials

- Absorbed guinea pig complement
- SRBC stored in Alsever's solution

Positive Control

Spleen cells incubated with antigen and medium

Negative Control

Spleen cells incubated with medium alone. The animals are decapitated and the spleens are removed from the peritoneal cavity. A single cell suspension of 15×10^6 cells/ml is prepared. For the induction of PFC, a 0.5 ml splenocyte suspension is added to 0.5 ml of a suspension of SRBC, previously washed in medium and diluted to 8×10^6 cells/ml. Thereafter, 1 ml of the solution of the test compound is added, and the limbrowells are incubated at 37 °C in a CO_2 incubator for 5 days. Per group 3 limbrowells are set up. On day 5, the three wells of each group are pooled and washed in medium, and the number of cells is determined. For each cell pellet, 875 µl of washed SRBC and 125 µl absorbed guinea pig complement are added. The suspension is mixed thoroughly and filled in chambers constructed of microslides. The chambers are placed in the incubator at 37 °C for 90–120 min. The plaque-forming colonies are counted immediately after incubation.

Evaluation

The activity of test compounds can be determined using the following formula:

1. PFC/3 wells:

$$x = \frac{\text{plaques} \times 100}{\mu\text{l}}$$

2. % change in the number of plaques:

$$x = \frac{\text{plaques} \times 100}{\text{plaquespos. control}}$$

$$d\% = x - 100$$

3. % change in number of cells:

$$x = \frac{\text{number of cells} \times 100}{\text{number of cells pos. control}}$$

$$d\% = x - 100$$

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Inhibition of Dihydroorotate Dehydrogenase

Purpose and Rationale

Dihydroorotate dehydrogenase catalyzes the fourth committed step in the de novo biosynthesis of pyrimidines. As rapidly proliferating human T cells have an exceptional requirement for de novo pyrimidine biosynthesis, small-molecule dihydroorotate dehydrogenase inhibitors constitute an attractive therapeutic approach to autoimmune diseases, immunosuppression, and cancer. The main mode of action of the immunosuppressive compound leflunomide and its active metabolites is considered to be the inhibition of the enzyme dihydroorotate dehydrogenase (Bruneau et al. 1998; Graul and Castañer 1998; Knecht and Löffler 1998; Rückemann et al. 1998; Schorlemmer et al. 1998; Herrmann et al. 2000; Liu et al. 2000).

Procedure

A fragment of human dihydroorotate dehydrogenase is expressed by means of the baculovirus expression vector system and purified to a specific activity greater than 50 U/mg (Knecht et al. 1996, 1997). Enzyme assays are performed with purified recombinant dihydroorotate dehydrogenase at 30 °C. The oxidation of the substrate dihydroorotate and the reduction of the co-substrate quinone is coupled to the reduction of the chromogen 2,6-dichlorophenolindophenol (DCIP). The reaction mixture contains 0.1 mM Q_D or 0.1 M Q_{10}, 1 mM L-dihydroorotate, 0.06 mM DCIP, 0.1 % Triton X-100 in 50 mM Tris–HCl buffer, 150 mM KCl, and pH 8.0. The reaction is started by addition of the enzyme. The loss of absorbance of the blue DCIP is monitored at 600 nm: $\in = 18.800\ \text{l mol}^{-1}\ \text{cm}^{-1}$. The enzyme activity in control assays without Q_D or Q_{10} which is approximately 1 % of maximum enzyme activity is subtracted from the activity values measured. Stock solutions of the test compounds are prepared in dimethyl sulfoxide with further dilutions in the buffer taken for the assays.

Evaluation

To determine the inhibitory potency of the agents, the initial velocity of dihydroorotate dehydrogenase reaction is measured at saturating substrate concentrations, 1 mM dihydroorotate and 100 μM Q_D, and varying concentrations of the drugs (1 nM through 100 μM). The equation is fitted to the initial velocities:

$$v = V/\{1 + [I]/IC_{50}\}$$

([I] is the inhibitor concentration) in order to find the concentration causing 50 % inhibition of the enzyme activity (IC_{50}). Both virtual (Diao et al. 2012) and high-throughput screening (Baldwin et al. 2005) and have been used to identify micromolar and sub-micromolar, respectively, inhibitors of DHODH activity. Recently, DHODH has emerged as a therapeutic target in bovine babesiosis (2014).

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Sphingosine 1-Phosphate

General Considerations

Sphingolipids have emerged as molecules whose metabolism is regulated to generation of bioactive products including ceramide, sphingosine, and sphingosine-1-phosphate. The balance between cellular levels of these bioactive products is recognized to be critical to cell regulation and may be a promising approach to tumor therapy and multiple sclerosis (Huwiler and Pfeilschifter 2006; Rosen et al. 2013; Blaho and Hla 2014), whereby ceramide and sphingosine cause apoptosis and growth arrest phenotypes and sphingosine-1-phosphate mediates proliferative and angiogenic responses. Sphingosine kinase is a key enzyme in modulating the levels of these lipids (Hannun and Obeid 1995; Hofmann and Dixit 1998; Mathias et al. 1998; Prieschl et al. 1999; Pyne and Pyne 2000; Cummings et al. 2002; MacKinnon et al. 2002; Rosen and Liao 2003; Chen et al. 2004; Deguchi et al. 2004; Lee et al. 2004; Peng et al. 2004; Cyster 2005; Kee et al. 2005; Watterson et al. 2005; Gardell et al. 2006; Taha et al. 2006). Ceramide formation and degradation are influenced by nitric oxide (NO) (Huwiler et al. 1999a, b; Franzen et al. 2002a, b).

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Binding to Sphingosine 1-Phosphate Receptors

Purpose and Rationale

At least five subtypes of the sphingosine 1-phosphate receptor with tissue specificity are known (Meyer zu Heringdorf et al. 1998; Kon et al. 1999; Im et al. 2000, 2001; Forrest et al. 2004; Hale et al. 2004a; Sanna et al. 2004; Zhou and Murthy 2004; Xin et al. 2004; Lepley et al. 2005; Kimura et al. 2006; Kitano et al. 2006).

The immunomodulator FTY720 is an agonist to sphingosine 1-phosphate receptors (Brinkmann et al. 2002, 2010; Brunkhorst et al. 2014; Chiba 2005; Chiba et al. 2011, 2014; Gräler and Goetzl 2004; Kunzendorf et al. 2004; Xin et al. 2004; Albert et al. 2005; Bandhuvula et al. 2005; Sawicka et al. 2003, 2005; Habicht et al. 2005; Takasugi et al. 2013; Xin et al. 2006; Zhang et al. 2013; Zhou et al. 2006). FTY720 is derived from ISP-1 (myriocin), a fungal metabolite that is an eternal youth nostrum in traditional Chinese herbal medicine (Fujita et al. 1994). The compound {2-amino-2-[2-(4-octophenyl) ethyl]propane-1,3-diol} is a highly potent immune modulating agent.

Further derivates such as sphingosine 1-phosphate receptor agonists (Hale et al. 2004b, c; Clemens et al. 2005; Foss et al. 2005; Galicia-Rosas et al. 2012; Guerrero et al. 2013; Kiuchi et al. 2005; Komiya et al. 2012; Jin et al. 2014; Jo et al. 2005; Li et al. 2005; Colandrea et al. 2006; Sanada et al. 2011; Satsu et al. 2013; Sobel et al. 2013; Ren et al. 2012; Yamamoto et al. 2014) and antagonists (Davis et al. 2005; Kennedy et al. 2011; Angst et al. 2012) have been described, and a patent review of sphingosine 1-phosphate receptors has been conducted (Roberts et al. 2013). Brinkmann et al. (2002) used the [γ-^{35}S] GTPS-binding assay to study the binding of the immune modulator FTY720 to sphingosine 1-phosphate receptors.

Forrest et al. (2004) studied the binding of sphingosine 1-phosphate agonists on distinct receptor subtypes.

Procedure

Receptors and Cell Lines

CHO cells stably expressing human $S1P_{1,2,3,4,5}$ were used (Mandala et al. 2002). cDNA sequences encoding rodent S1P receptors were cloned from genomic DNA by polymerase chain reaction using the following primers for each respective receptor:

5′-GAACCCGGGTGTCCACTAGCATCCCGG and 5′CCCGAATTCTTAGGAAGAAGAATTGACGTTTCC (mouse $S1P_1$), 5′-GAACCCGGGCGGCTTATACTCAGAGTACC and 5′-GGCGAATTCTCAGACCACTGTGTTACCCTC (mouse $S1P_2$), 5′-GAACCCGGGCAACCACGCATGCGCAGG and 5′-GTCGAATTCTCACTTGCAGAGGACCCCG (mouse $S1P_3$), 5′-GAACCCGGGAACATCAGTACCTGGTCCACGC and GCGGAATTCTAGGTGCTGCGGACGCTGG (mouse $S1P_4$), 5′-GAACCCGGGCTGCTGCGGCCGG and 5′-CGCGAATTCAGTCTGTAGCAGTAGGCACC (mouse $S1P_5$), 5′-GTAGGATCCGTGTCCTCCACCAGCATC and 5′GGCCGAATTCTTAAGAAGAAGAATTGACGTTTC (rat $S1P_1$), 5′-GAACCCGGGCATCCACGCATGCGCAG and 5′-GCCGAATTCTCACTTGCAGAGGACCCCATTCTG (rat $S1P_3$).

The polymerase chain reaction products were inserted in-frame after a FLAG tag using vector pCMV-Tag2 (Stratagene, La Jolla, Calif., USA). Stable lines were established by transfecting plasmids into CHO cells using Lipofectamine reagent, selecting for neomycin resistance, and screening single cell cultures for increased [^{33}P] S1P-specific binding. Membranes were prepared from positive clones and confirmed in [^{33}P]S1P and [^{35}S]GTPγS binding assays.

S1P Receptor Assays

Binding assays were conducted as described by Mandala et al. (2002). [^{33}P]S1P was sonicated with fatty-acid-free bovine serum albumin, added to test compounds diluted in dimethyl sulfoxide (DMSO), and mixed with membranes in 200 µl in 96-well plates with assay concentrations of 0.1 nM[^{33}P]S1P (22,000 dpm), 0.5 % bovine serum albumin, 50 mM HEPES-Na (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.3–0.7 µg of membrane protein. Binding was performed for 60 min at room temperature and terminated by collecting the membranes onto GF/B filter plates with a Packard Filtermate Universal harvester. Filter-bound radionuclide was measured on a Perkin Elmer 1450 MicroBeta. Specific binding was calculated by subtracting radioactivity that remained in the presence of 1,000-fold excess of unlabeled S1P.

To measure functional activation of the S1P receptors, [^{35}S]GTPγ *S* binding was measured. Membranes (1–4 µg of protein) were incubated in 96-well plates with test compounds diluted in DMSO in 100 µl of buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, and 2–10 µM GDP, depending on the expressed receptor. The assay was initiated with the addition of 100 µl of [^{35}S]GTPγ S (1,200 Ci/mmol or 44,400 BGq/mmol; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) for an assay concentration of 125 pM. After 60 min of incubation at room temperature, membranes were harvested onto GF/B filter plates, and bound radionuclides were measured.

Modifications of the Method

Murata et al. (2000) described a radioreceptor-binding assay for quantitative measurement of sphingosine 1-phosphate.

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Sphingosine Kinase Activation Assay

Purpose and Rationale

Sphingosine 1-phosphate produced by two sphingosine kinase isoenzymes, denoted SphK1 and SphK2, is the ligand for a family of specific G-protein-coupled receptors that regulate cytoskeletal rearrangements and cell motility. Unlike the proliferative action of SphK1, the isoenzyme SphK2 has been shown to possess antiproliferative and proapoptotic action. Both kinases have been cloned and functionally characterized (Kohama et al. 1998; Liu et al. 2000, 2003; Nava et al. 2000; Olivera et al. 2000; Igarashi et al. 2003; Paugh et al. 2003; Sanchez et al. 2003; Billich et al. 2005; Döll et al. 2005; Hait et al. 2005; Kharel et al. 2005; Okada et al. 2005; De Palma et al. 2006; Zemann et al. 2006; Gao and Smith 2011; Neubauer and Pitson 2013; Tonellli et al. 2013; Zhang et al. 2013; Ceccom et al. 2014; Plano et al. 2014; Shen et al. 2014; Tamashiro et al. 2014; Tous et al. 2014). A recent summary of drugs in clinical trials targeting the sphingosine 1-phosphate pathway illustrates the potential roles of this axis in cancer and autoimmune inflammatory disease (Kunkel et al. 2013).

Sphingosine kinase activity assays were performed in a similar way by Paugh et al. (2003) and by Huwiler et al. (2006).

Procedure

Sphingosine Kinase Activity Assay

In vitro kinase reactions were performed according to Olivera et al. (2000). In brief, 30 µg of protein lysates was incubated with 50 µmol/l of sphingosine (dissolved as 1 mmol/l stock solution in 4 mg/ml of BSA in PBS) and 10 µCi (370 kBq) of [γ-^{32}P]ATP for 15 min at 37 °C. For SK-2 activity assay, the same buffer including 1 M KCl was used to inhibit SK-1 activity (Liu et al. 2000). Reactions were terminated by addition of 20 µl of 1 N HCl followed by 800 µl of chloroform/methanol/HCl (100:200:1, v/v), 240 µl of chloroform, and 240 µl of 2 mol/l KCl. After vigorous vortexing and phase separation, 50 µl of the lower organic phase was loaded onto

TLC plates and run in 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v).

Evaluation

Spots corresponding to S1P were analyzed and quantified using an imaging system (Fuji).

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Lymphocyte Trafficking After Sphingosine 1-Phosphate Receptor Agonists

Purpose and Rationale

Adaptive immunity depends on T cell exit from the thymus and T and B cells traveling between secondary lymphoid organs to survey for antigen. After activation in lymphoid organs, T cells must again return to circulation to reach sites of infection. The immunomodulatory drug FTY720 induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing via the S1P receptor 1 (Chiba et al. 1998; Yanagawa et al. 1998a, b; Henning et al. 2001; Forrest et al. 2004; Matloubian et al. 2004; Hait et al. 2005; Kharel et al. 2005; Huwiler et al. 2006). Mandala et al. (2000) described alteration of lymphocyte trafficking by sphingosine 1-phosphate receptor agonists.

Procedure

Induction of Lymphopenia and Reduction of Thoracic Duct (TD) Lymphocytes by S1P and Analogues in Rats

Blood or thoracic duct lymph lymphocyte counts were determined by autoanalyzer (H2000, CARESIDE, Culver City, Calif., USA) and normalized to counts in vehicle controls after administration of FTY720 (2.5 mg/kg p.o.) or test compound. S1P was administered by continuous infusion beginning at 8 mg/kg/h for 20 min followed by 2 mg/kg/h for a further 220 min. The measured physiological S1P concentration in rat plasma by LC-MS was 0.5 μg/ml. This rose to a C_{max} of 2.5 μg/ml at 30 min and was maintained at 1.5 μg/ml for the remainder of the experiment. Studies on the effect on lymphocyte numbers in thoracic duct-cannulated rats were performed after the administration of FTY720 or test compound. Lymph flow remained constant

for the duration of the experiment, and numbers are shown as the average cell concentration maintained over the preceding 30 min.

FACS Measurement of Peripheral Blood Lymphocyte Depletion in Cannulated Rats

Percentage depletion by FTY720 compared to vehicle control was measured. Similar nadir lymphopenia was produced by FTY720 or non-metabolizable phosphonates. Peripheral blood samples were diluted 1:1 with phosphate-buffered saline (PBS), layered on the same volume of Lymphocyte Separation Medium (ICN Biomedicals, Aurora, Ohio, USA), and centrifuged at 400 g for 30 min. Peripheral blood mononuclear cells (PBMC) were resuspended in PBS and counted using a hemocytometer. PBMC were then stained with FITC-labeled anti-CD8, PE-labeled anti-CD45RA, and Cy-chrome-labeled anti-CD4 antibodies. Numbers of CD4-, CD8-, and CD45RA-positive cells were calculated by multiplying total PBMC count with the percentages of $CD4^+$, $CD8^+$, and $CD45RA^+$ generated from flow cytometry.

Quantitation of Lymph Node Cells

Single cell suspensions were prepared by passage of tissues through a 40-μm sieve. Peripheral blood lymphocytes were further isolated from spleens by ammonium chloride lysis of red blood cells. Cells were subsequently washed in UltraCULTURE medium (Biowhittaker, Walkersville, Md., USA), and all samples were adjusted to the same volume with PBS. An equal volume of 4 % paraformaldehyde was added while gently vortexing the samples. The total number of viable, unstained lymphocytes per sample was determined by flow cytometry (FACScan; Becton Dickinson) using CellQuest software (Becton Dickinson), based upon forward- and side-scatter characteristics. Beads (Sigma; P7458) were used as an internal standard.

Evaluation

Data were calculated as cell number per node by dividing the total number of lymphocytes quantitated by the number of nodes harvested per site (i.e., the number of Peyer's patches and mesenteric or peripheral lymph nodes collected).

Modifications of the Method

Kawa et al. (1997) reported inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate.

Fueller et al. (2003) described activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate.

Roviezzo et al. (2004) studied human eosinophil chemotaxis and selective in vivo recruitment by sphingosine 1-phosphate. Kunisawa et al. (2007) showed that sphingosine 1-phosphate may regulate peritoneal B cell trafficking and Thangada et al. (2010) using adoptive transfer experiments in wild-type mice, and mice mutated for the sphingosine 1-phosphate receptor showed that cell surface residency of the receptor determines the kinetics of lymphocyte egress. Yang et al. (2014) showed that fingolimod (FTY720) may prevent inflammation-sensitized hypoxic ischemia brain injury in newborn rats.

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In Vivo Methods for Testing Immunological Factors

Spontaneous Autoimmune Diseases in Animals

Several spontaneous autoimmune diseases have been reported in several inbred animal strains:

New Zealand black mouse (NZB mouse) (Bielschowski et al. 1959; Howie and Helyer 1968; Barthold et al. 1974; Blanchard and Bach 1980). The NZB mouse develops a spontaneous autoimmune disease with autoimmune hemolytic anemia, splenomegaly, glomerulonephritis, lymphoproliferative disorders, and peptic ulcerations.

New Zealand black/white F1 (B/W) mouse (Helyer and Howie 1963; Kessler 1968). These animals develop nephritis similar to that in human systemic lupus erythematosus and show mononuclear cell infiltration in salivary and lachrymal glands such as in human Sjögren's syndrome.

A substrain of the autoimmune-prone mouse, NZB/kl, was found to show spontaneous elevation of the auditory brainstem response threshold with age (Sone et al. 1995).

Immunodeficient alymphoplasia mice were recommended as a spontaneous model for Sjögren's syndrome (Tsubata et al. 1996). Mice homozygous for an autosomal-recessive mutation aly (alymphoplasia) lack both lymph nodes and Peyer's patches and show defects in both humoral and cellular immunity. Histopathological analyses revealed chronic inflammatory changes in exocrine organs such as the salivary gland, the lacrimal gland, and the pancreas.

The **Palmerston North autoimmune mouse strain** which exhibits both spontaneous systemic autoimmune disease and otic capsule bone formation has been proposed as a model for otic capsule osteogenesis and otosclerosis (Hertler and Trune 1990; Traynor et al. 1992).

In aging **BDF1 mice**, Hayashi et al. (1988) described spontaneous development of autoimmune sialadenitis.

Robison et al. (1994) examined the relationship between orchitis and aspermatogenesis in various strains of H_2 congenic mice and defined a genetic predisposition to spontaneous aspermatogenesis.

Motheaten mice. Mice homozygous for the autosomal-recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Su et al. 1998).

The genetic, hormonal, and behavioral influence on spontaneously developing arthritis in normal mice has been reviewed by Holmdahl et al. (1992).

Nonobese diabetic mouse (NOD mouse) (Makino et al. 1980; Miyazaki et al. 1985; Leiter et al. 1987). The inbred NOD mouse is considered a good model for type I diabetes mellitus. Mononuclear cells infiltrate the pancreatic islets of Langerhans from 6 to 8 weeks of age, followed by a progressive and selective destruction of insulin-producing β-cells and the onset of IDDM from the 12th week of age onwards.

Itoh et al. (1997) studied the requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice.

Quartey-Papafio et al. (1995) showed that aspartate at position 57 of nonobese diabetic I-A (g7) β-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus in the NOD mouse.

The NOD mouse was also recommended to study the pathogenesis of autoimmune thyroiditis (Many et al. 1996; Giarratana et al. 2007).

Inherited inflamed joints. Adipue et al. (2011) established a new spontaneous murine model of inflammatory arthritis of inherited inflamed joints (IIJ) established from AR mice that appeared in a 5B6 transgenic mouse-breeding colony.

Qi et al. (2013) developed a murine model of spontaneous liver disease resembling autoimmune hepatitis, and Yang et al. (2014) developed a murine model of spontaneous peripheral polyneuropathy.

Bio-breeding rat (BB rat) (Like et al. 1982; Field 1983; Yale and Marliss 1984). On the basis of clinical and histopathological parameters, the BB rat is considered a useful model for human IDDM. The disease in the BB rat is characterized by infiltration of lymphocytes and macrophages into the islets of Langerhans.

Allen and Thupari (1995) described spontaneous autoimmune lymphocytic thyroiditis in *BB/Wor rats.*

Obese strain chicken (OS chicken) (van Tienhoven and Cole 1962; Cole 1966; Cole et al. 1968, 1970; Wick et al. 1974). The OS chicken is perhaps the best studied model for an organ-specific, spontaneously occurring autoimmune disease, viz., spontaneous autoimmune thyroiditis, which closely resembles human Hashimoto thyroiditis. The spontaneous autoimmune thyroiditis in obese chicken was further studied by Neu et al. (1986), Kroemer et al. (1989), Cihak et al. (1995), Hala et al. (1996), and Dietrich et al. (1997).

Chickens of the University of California line 200 **(UCD-200 chickens)** develop an inherited inflammatory fibrotic disease that closely resembles human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Brezinscheck et al. 1993).

Schumm-Draeger and Fortmeyer (1996) described **autoimmune thyroiditis in the cat** as a spontaneous disease model.

Spontaneous autoimmune thyroiditis was found in **Mastomys** (*Praeomys coucha*) by Solleveld et al. (1985) and recommended as an animal model of human disease.

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Acute Systemic Anaphylaxis in Rats

Purpose and Rationale

Rats are immunized with ovalbumin and *Bordetella pertussis* suspension as adjuvant. After 11 days, the animals are challenged by intravenous injection of ovalbumin. The shock symptoms can by inhibited by corticosteroids and intravenous disodium cromoglycate.

Procedure

Female Sprague–Dawley rats weighing 120 g are immunized by i.m. injection of 10 mg/kg highly purified ovalbumin. Simultaneously 1 ml of *Bordetella pertussis* suspension (2×10^{10} organisms) is injected intraperitoneally. IgE antibodies are induced and attached to the surface of mast cells and basophilic granulocytes. Eleven days later, the animals are challenged by intravenous injection of 25 mg/kg highly purified ovalbumin. This results in the formation of antigen–antibody complexes on the surface of mast cells and basophilic granulocytes in blood and in all organs with immediate release of various mediators of anaphylaxis, such as histamine, serotonin, SRS-A,

and prostaglandins; in shock symptoms; and 80 % lethality. Corticosteroids, e.g., dexamethasone 1–10 mg/kg s.c., are given 18 h prior to challenge or 30 mg/kg disodium cromoglycate i.v. before injection of ovalbumin. Ten to 20 animals are used for each group.

Evaluation

The shock symptoms are scored and mortality counted. Results after treatment are compared with untreated controls. Pretreatment with corticosteroids or disodium cromoglycate can inhibit death and ameliorate shock symptoms. Statistical calculation is performed using the χ^2-test.

Modifications of the Method

Desensitization by repeated "microshocks" of constant strength in guinea pigs has been reported by Herxheimer (1952).

Acute systemic anaphylaxis experiments have also been performed in guinea pigs and in mice. In guinea pigs, anaphylactic bronchospasm can be measured with the Konzett and Rössler method (Davies and Evans 1973).

Moreover, anaphylactic bronchospasm can be measured in isolated guinea pig lungs according to the method of Bhattacharya and Delaunois (1955).

Anaphylaxis can be measured in the chopped guinea pig lung by assay of the supernatant in the isolated guinea pig ileum in the presence of 2×10^{-7} M atropine (Austen and Brocklehurst 1961).

Ufkes and Ottenhof (1984) sensitized Brown Norway rats with a suspension of trinitrophenyl-haptenized ovalbumin together with $AlPO_4$ as adjuvant. Bronchial and cardiovascular functions were studied after treatment with antiallergic agents and antigen challenge.

Elwood et al. (1992) studied the effect of dexamethasone and cyclosporine A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized Brown Norway rats.

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Anti-anaphylactic Activity (Schultz–Dale Reaction)

Purpose and Rationale

Guinea pigs are sensitized against egg albumin. Challenge after 3 weeks causes in isolated organs' release of mediators, e.g., histamine, which induce contraction in isolated ileum.

Procedure

Guinea pigs of either sex weighing 300–350 g are sensitized with alum-precipitated egg albumin. Alum egg albumin is prepared by dissolving egg albumin (1 mg/ml) in 6 % aluminum hydroxide gel, suspended in saline. The mixture is stirred and kept at room temperature. Each animal receives at the same time injections of 0.125 ml of this mixture in each foot pad and 0.5 ml subcutaneously. After 4 weeks, the animals are killed and the ileum is dissected out. Cleaned pieces, about 2–3 cm long, are mounted in an organ bath containing Tyrode solution at 37 °C. The strips are allowed

to equilibrate for 15 min. The contractility of the ileum strips is tested by adding 10^{-4} g/ml $BaCl_2$ solution. To one organ bath the standard (2×10^{-6} g/ml final concentration of tribenoside = 1-*O*-ethyl-3,5,6-tri-*O*-benzyl-D-glucofuranoside = Glyvenol CIBA) and to other vials the test compounds (final concentration up to 10^{-5} g/ml) are added. One organ bath serves as control. After 3 min, ovalbumin in a final concentration of 2×10^{-6} g/ml is added. The contractions are recorded with strain gauges by a polygraph.

Evaluation

The results are expressed as presence or absence of blocking activity (percentage inhibition). If anti-anaphylactic activity is observed, ED_{50} values using different doses are calculated.

Critical Assessment of the Method

Positive results can also be achieved with spasmolytics, local anesthetics, antihistaminics, and sympathicomimetics.

Modifications of the Method

The method has been modified by testing histamine release in the lung after challenging with egg albumin. Either lung strips from sensitized guinea pigs are suspended in an organ bath and their contractions are measured after addition of egg albumin or the entire lung tissue is dissected out and washed free from blood by perfusing with warm oxygenated Tyrode solution via the pulmonary artery. The lung tissue is chopped and washed with Tyrode solution in order to remove the remaining blood. The chopped lung tissue is divided into 24 samples, each of approximately 100 mg wet weight. These are incubated at 37 °C in Tyrode solution for 15 min with continuous agitation by rocking, after which 1 mg/ml of egg albumin is added to the reaction mixture. After shaking for 10 min at 37 °C, the supernatant is collected and assayed for histamine with guinea pig ileum. Atropine sulfate 2 mg/ml is added in Tyrode solution. The residual histamine is obtained by boiling the tissue in 5 ml Tyrode solution for 10 min. The tubes are then placed on ice for 1 h to allow complete diffusion. Released histamine is expressed as a percentage of total histamine content.

Koppel et al. (1981) developed a method to induce contraction of immunologically sensitized mouse trachea by antigen (Schultz–Dale reaction).

The trachea of sensitized guinea pigs was used by Omote et al. (1994). Choi et al. (2008) measured the effects of dehydroepiandrosterone on the Schultz–Dale reaction and the Th2 immune response in sensitized BALB/c mice. Guhathakurta et al. (2013) determined the effects of UNIM-352 and Naik et al. (2013) the effects of extract of Zizyphus jujuba fruits, both natural products, in a rodent model of systemic anaphylaxis.

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Passive Cutaneous Anaphylaxis

Purpose and Rationale

Passive cutaneous anaphylaxis is an immune reaction of the immediate type. By passive immunization of rats in the skin with rat anti-ovalbumin serum and a challenge 2 days later with ovalbumin at the same skin area, antigen–antibody complexes are formed in the mast cells inducing release of mediators. This results in vasodilatation, increase in permeability of the vessel walls, and leakage of plasma. To make the allergic reaction visible, Evan's blue dye is administered along with the antigen. Evan's blue dye is attached to the albumin fraction of plasma, producing a blue spot. This blue spot indicates that an anaphylactic reaction has taken place in the skin.

Procedure

For preparation of antiserum, male rats weighing 200–250 g are adrenalectomized and are allowed to recover for 3 days. Thereafter, animals are sensitized with egg albumin (1 mg/animal) using aluminum hydroxide gel (200 mg) as adjuvant. Alum egg albumin is prepared by dissolving 1 mg/ml of egg albumin in 20 % aluminum hydroxide gel, suspended in saline. Each animal simultaneously receives 0.125 ml of the above solution in each foot pad and 0.5 ml subcutaneously. After 8 days, the animals are bled and antiserum is collected.

For the test, the antiserum is diluted in such a manner as to give a wheal of 15–20 mm diameter in a preliminary titration. Aliquots of 100 µl of appropriate dilution of antiserum are injected intradermally into the shaved dorsal skin of normal male rats weighing about 100 g. After 24 h of latent period, each animal is challenged with the intravenous administration of 0.1 ml of 2.5 % Evans blue dye containing 25 mg/ml of egg albumin. In the case of intravenous administration, the test compound is administered simultaneously with the antigen and the dye. In case of oral testing, the compound is given orally 1 h prior to challenge. The animals are sacrificed 30 min after the challenge. The amount of Evans blue dye leaked at the site of passive cutaneous anaphylactic reaction is extracted and determined colorimetrically at 620 µm wavelength.

Evaluation

The amount of Evans blue extracted from passive cutaneous anaphylactic reaction is taken as 100 %. Percent inhibition of passive cutaneous anaphylactic reaction in the rats treated with the test compound is calculated. The standard disodium cromoglycate at a dose of 3 mg/kg i.v. or 30 mg/kg orally results in 80–100 % inhibition. Using different doses, ED_{50} values can be calculated.

Modifications of the Method

Goose and Blair (1969) used *Bordetella pertussis* and extracts of the worm *Nippostrongylus brasiliensis* as antigens in passive cutaneous anaphylaxis experiments in the rat.

Patterson et al. (1971) tested passive cutaneous reactivity to antihuman IgE in rhesus monkeys.

Without immunization, plasma extravasation after bradykinin injection can be tested in anesthetized Sprague–Dawley rats (Lembeck et al. 1991). Evans blue dye is injected to stain plasma proteins. After injection of bradykinin antagonists followed by bradykinin injection, the rats are perfused with physiological saline. The trachea, the urinary bladder, and the duodenum are resected, weighed, and incubated for 48 h in formamide at 50 °C (Saria et al. 1983). The

amount of Evans blue extracted is measured photometrically at 620 nm.

Vascular reactions to histamine, histamine liberator, and leukotaxine in the skin of guinea pigs using pontamine sky blue 6× as indicator were studied by Miles and Miles (1952). Babakin et al. (2008) investigated the effects of fullerene-60 in both systemic and both rat and murine passive cutaneous models of anaphylaxis, and Zhu et al. (2009) showed that the proteinase-activated receptor 2 is involved in passive cutaneous murine model of anaphylaxis and that it can be inhibited by tacrolimus.

Hitomi et al. (2010) discovered that mice deficient in the immunoglobulin-like receptor Allergin-1 developed enhanced passive systemic and cutaneous anaphylaxis, and Han et al. (2013) showed that the phytoalexin resveratrol inhibited both IgE-mediated basophilic mast cell degranulation and passive cutaneous anaphylaxis in a murine model.

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Arthus-Type Immediate Hypersensitivity

Purpose and Rationale

The immune complex-induced Arthus reaction comprises inflammatory factors that have been implicated in the acute responses in joints of rheumatic patients. Complement and polymorphonuclear neutrophils are activated via precipitating antigen–antibody complexes leading to an inflammatory focus characterized by edema,

hemorrhage, and vasculitis. Arthus reaction of the immediate type becomes maximal 2–8 h after the challenge.

Procedure

Ovalbumin Suspension

1,700 mg ovalbumin is suspended in 100 ml paraffin oil. 4.38 ml pertussis vaccine is suspended in 70 ml 0.9 % NaCl solution. Both suspensions are mixed to form an emulsion.

Wistar or Sprague–Dawley rats of either sex weighing 220–280 g can be used. Seven days prior to the start of the experiment, rats are sensitized by i.m. administration of 0.5 ml of the ovalbumin suspension. They are housed in groups of eight with standard food and water ad libitum.

Twenty-four hours and 1 h prior to induction of the Arthus reaction, test compounds are administered to groups of eight animals. The rats are challenged by injection of 0.1 ml of 0.04 % solution of highly purified ovalbumin in the left hind paw. Swelling of the paw occurs which reaches a maximum after a few hours. The footpad thickness can be measured by calipers. One group of sensitized animals treated with solvent alone serves as positive control; one group of non-sensitized animals treated with solvent alone serves as negative control. Standard doses are 30 mg/kg cortisone or 10 mg/kg prednisolone p.o.

Evaluation

The change in footpad thickness is expressed as the percent change from the vehicle control group. Comparison of experimental group to positive control is evaluated statistically using Student's *t*-test.

Modifications of the Method

Instead of ovalbumin, sheep red blood cell suspensions can be used for immunization and for challenge in mice (Omote et al. 1994).

Nagakawa et al. (1990) sensitized mice by s.c. injection of bovine serum albumin in complete Freund's adjuvant and boosted on day 21 by an intradermal injection of BSA. On day 28, the Arthus reaction was elicited by intradermal injection of BSA. Four hours later, an erythematous skin reaction over an area of more than 8 mm^2 was regarded as positive.

Kamei et al. (1991) immunized guinea pigs by injection of a mixture of egg albumin and Freund's complete adjuvant subcutaneously into the food pad or i.m. into the hind leg. The injection was repeated four times at 7-day interval. Ten days after the last immunization, 0.2 ml of 2.5 % egg albumin was injected sc. into the dorsal skin of the animals. The intensity of the Arthus reaction was evaluated by measuring the inflamed area according to scores.

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Delayed-Type Hypersensitivity (DTH)

Purpose and Rationale

Delayed-type hypersensitivity is a reaction of cell-mediated immunity and becomes visible only

after 16–24 h. The same methods as for testing immediate-type hypersensitivity can be used.

Procedure

Rats are sensitized in the same way by i.m. administration of 0.5 ml ovalbumin suspension 7 days prior to the start of the experiment as described for testing immediate-type hypersensitivity. They are challenged by injection of 0.1 ml of 0.04 % solution of highly purified ovalbumin in the left hind paw. Footpad thickness is measured immediately and 24 h after ovalbumin administration.

Modifications of the Method

Mizukoshi et al. (1994) injected female CDF1 mice intradermally with a suspension of 2×10^8 sheep red blood cells/50 μl into the left foot pad. A second booster of the same dose was given to the right foot pad on day 4. The thickness of the foot pads was measured on the following day, and the difference in the thickness between the right and the left food pads was taken as the degree of swelling.

Kamei et al. (1991) immunized mice by applying 0.15 ml of 7 % picryl chloride/ethanol solution to the skin of the shaved abdomen. The second immunization was performed 6 days later. One week after the second immunization, 1 drop of 1 % picryl chloride olive oil solution was applied to the ear, and the thickness of the ear was measured by a thickness gauge 24 h later.

Heriazon et al. (2009) investigated the induction of DTH and interferon gamma to Candida albicans and anti-hen egg white lysozyme antibody as phenotypic markers of enhance bovine immune response, and their studies suggest that this combination of test antigens could be used as phenotypic markers of immune responsiveness in cattle. Escandell et al. (2010) investigated the inhibition of DTH by the plant product cucurbitacin R which was shown to reduce human T lymphocyte proliferation.

Yang et al. (2011) used the DTH model to a three-protein cocktail with that of a purified protein derivative, and Atkinson et al. (2012) extended the model to study the similarities with collagen-induced arthritis and human rheumatoid arthritis.

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Reversed Passive Arthus Reaction

Purpose and Rationale

In the reversed passive Arthus reaction, the antigen is injected intravenously followed by a local injection – either intradermally or into the pleural space – of the respective antibody. Generation of an immune-mediated reverse passive Arthus reaction in the rat pleural cavity results in a classic acute inflammatory response. The methods are used to evaluate new anti-inflammatory agents.

Procedure

Male Lewis rats weighing 200–250 g are fasted overnight prior to use with free access to water. The animals receive 5 mg bovine serum albumin in 0.2 ml sterile saline intravenously, followed 30 min later by injection of 1 mg rabbit anti-BSA in 0.2 ml sterile saline into the right pleural cavity under light halothane anesthesia. Drugs or vehicle controls are administered by gastric gavage in 1 ml/100 g body weight at different times prior to the anti-BSA. The animals are sacrificed at various intervals after anti-BSA injections by CO_2 inhalation (after 5 min for thromboxane B_2 determination, after 10 min for leukotriene B_4 determination, and after 4 h at the peak time of neutrophil infiltration). The fluid exudate is removed from the pleural cavity by gentle vacuum aspiration and the volume is recorded. Eicosanoids in the pleural exudate are quantitated by commercial RIA kits.

Evaluation

The values after treatment with various doses of test compounds are compared with those of vehicle controls.

Modifications of the Method

The antibody can be injected intradermally into the shaved skin of rats after intravenous injection of the antigen (e. g., human albumin) together with Evans blue dye solution. Extravasated dye is determined in skin punches (Camussi et al. 1990; Burch et al. 1992; Okamoto et al. 1992).

Bailey and Sturm (1983) induced the reverse passive Arthus reaction in rats using bovine serum albumin as antigen into the tail vein and rabbit anti-bovine serum albumin into the skin site. One hour after oral dosing with vehicle or drug, animals were lightly anesthetized and their hair was shaved from the middorsal region with electric clippers. Each animal was injected intradermally with 40 µl on the left side of the middorsal line and

with 40 µl of rabbit anti-bovine serum albumin (5.0 mg/ml antibody protein), diluted 1:4 with phosphate-buffered saline on the right side of the dorsal midline. Immediately following the intradermal challenge, each rat received 0.5 ml phosphate-buffered saline containing 1.0 mg bovine serum albumin injected in the tail vein. Four hours after intradermal challenge, the animals were sacrificed. The full-thickness skin was removed from the back, and disks 8 mm in diameter were punched out with a metal punch. Wet weight of the samples from the phosphate-buffered saline- and antibody-injected site was determined, and the edema induced by the reverse passive Arthus reaction calculated as the difference between both weights.

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Adjuvant Arthritis in Rats

Purpose and Rationale

Adjuvant arthritis in rats has been described by Pearson and Wood (1959) exhibiting many similarities to human rheumatoid arthritis. Injections of complete Freund's adjuvant into the rat paw induce inflammation as primary lesion with a maximum after 3–5 days. Secondary lesions occur after a delay of approximately 11–12 days which are characterized by inflammation of non-injected sites (hindleg, forepaws, ears, nose, and tail) and a decrease of weight and immune responses. The procedure has been modified by

several authors in order to differentiate between anti-inflammatory and immunosuppressive activity (e.g., Perper et al. 1971). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by immunosuppressive agents. Two protocols, termed "preventative" (or "prophylactic") and "therapeutic" (or "established") adjuvant arthritis, have gained wide usage for assessing a drug's potential anti-arthritic activity (Schorlemmer et al. 1999).

Procedure

The choice of the animal strain has been found to be very important for the performance of this test. Wistar–Lewis rats have been proven to be very suitable in contrast to other substrains. Male rats with an initial body weight of 130–200 g are used. On day 1, they are injected into the suplantar region of the left hind paw with 0.1 ml of complete Freund's adjuvant. This consists of 6 mg mycobacterium butyricum (Difco) being suspended in heavy paraffin oil (Merck) by thoroughly grinding with mortar and pestle to give a concentration of 6 mg/ml. Dosing with the test compounds or the standard is started on the same day and continued for 12 days. Paw volumes of both sides and body weight are recorded on the day of injection, whereby paw volume is measured plethysmographically with equipment as described in the paw edema tests. On day 5, the volume of the injected paw is measured again, indicating the primary lesion and the influence of therapeutic agents on this phase. The severity of the induced adjuvant disease is followed by measurement of the non-injected paw (secondary lesions) with a plethysmometer. Purposely, from day 13–21, the animals are not dosed with the test compound or the standard. On day 21, the body weight is determined again, and the severity of the secondary lesions is evaluated visually and graded according the following scheme:

		Score
Ears	Absence of nodules and redness	0
	Presence of nodules and redness	1
Nose	No swelling of connective tissue	0
	Intensive swelling of connective tissue	1
Tail	Absence of nodules	0
	Presence of nodules	1
Forepaws	Absence of inflammation	0
	Inflammation of at least one joint	1
Hind paws	Absence of inflammation	0
	Slight inflammation	1
	Moderate inflammation	2
	Marked inflammation	3

(*continued*)

Evaluation

(a) For primary lesions: The percent inhibition of paw volume of the injected left paw over vehicle control is measured at day 5.
(b) For secondary lesions: The percentage inhibition of paw volume of the non-injected right paw over controls is measured at day 21.
(c) An arthritic index is calculated as the sum of the scores as indicated above for each animal. The average of the treated animals is compared with the control group.
(d) The total percentage change is calculated as follows by addition of:

Percent inhibition of the injected paw on day 5 + percent inhibition of the non-injected paw on day 21 + percent change of the arthritic index.

Doses of 0.3 mg/kg indomethacin p.o. and 20–50 mg/kg phenylbutazone p.o. are effective on the primary lesions when dosage is started at the day of injection of the irritant. They are not effective on the secondary lesions.

In contrast, immunosuppressants like cyclophosphamide at a dose of 7 mg/kg inhibited the secondary lesions even when started at day 9 or later.

Critical Assessment of the Method

Evidence was given that adjuvant arthritis in the rat is associated with chronic pain (Colpaert 1987). The measure of pain in this model still presents some technical problems since the evaluation is based on the somewhat biased observation of the behavioral responses.

Modifications of the Method

A review was given by Gardner (1960) on the experimental production of arthritis.

Moran et al. (1999) compared adjuvant arthritis and selected animal models of arthritis to rheumatoid arthritis with special emphasis on the mechanism of joint destruction.

Kazuna and Kawai (1975) and Rooks et al. (1982) used rats with established lesions to test analgesics in the arthritic flexion pain test. The method is claimed to be specific by detecting only central analgesics and nonsteroidal anti-inflammatory drugs but not other classes such as CNS-depressant or antihistaminic drugs.

Brackertz et al. (1977) established antigen-induced arthritis in the mouse by immunization with methylated bovine serum albumin in complete Freund's adjuvant with B pertussis vaccine.

A streptococcal cell wall-induced arthritis in rats has been described by Wilder et al. (1982, 1987) and Yocum et al. (1986).

Lewis et al. (1997) studied degradation of articular cartilage in a rat monoarthritis model induced by an intra-articular injection of *Propionibacterium acnes*.

Crossley et al. (1989) reported on a monoarticular antigen-induced arthritis in rabbits and mice.

α-2-Glycoprotein levels have been recommended as parameter for severity and inhibition of experimental immunoarthritis in the rat by Sandow et al. (1971).

Pircio et al. (1975) recommended a method for the evaluation of analgesic activity using adjuvant-induced arthritis in rats. The degree of vocalization was recorded from five rats placed together in a counting chamber.

Cruwys et al. (1994) sensitized rats on day 0 and 7 with multiple intradermal injections of methylated bovine serum albumin emulsified in Freund's complete adjuvant. On day 21, the animals were challenged by the intra-articular injection of 100 µl 0.5 % solution of methylated bovine serum albumin into the right knee. The progress of the monoarticular arthritis was monitored by daily measurement of joint diameter.

Butler et al. (1991) described a limited arthritic pain model for chronic pain and inflammation studies using injections of 0.05 ml of complete Freund adjuvant into the left tibiotarsal joint of Sprague–Dawley rats.

Issekutz et al. (1994) studied the role of tumor necrosis factor-alpha and IL-1 in polymorphonuclear leukocyte and T lymphocyte recruitment to joint inflammation in adjuvant arthritis.

Esser et al. (1995) measured radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. Digitized radiographs of the calcaneus were examined for changes in the mean and in the distribution of gray values. Periostal new bone formation was measured as an increase in image area of the calcaneus.

Mercuric chloride ($HgCl_2$) induces a syndrome of autoimmunity in Brown Norway rats characterized by a variety of IgG antibodies; very high concentrations of serum IgE, proteinuria, leukocytoclastic vasculitis which predominantly affects the cecum; and an inflammatory polyarthropathy (Kiely et al. 1995, 1996).

Kawahito et al. (2000) reported that 15-deoxy-$\Delta^{12,14}$-PGJ_2 which activates PPAR-α induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. Cuzzocrea et al. (2002) found that prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 attenuates the development of acute and chronic inflammation.

Bolon et al. (2004) described a method for rapid quantification of intralesional osteoclasts in the hind paws of Lewis rats with adjuvant-induced arthritis. A 4-µm-thick section of the decalcified hind paw was stained to demonstrate osteoclasts using an indirect immunoperoxidase method and a rabbit antihuman monoclonal antibody directed against the osteoclast marker cathepsin K, which is an osteoclast protease primarily responsible for the resorption of bone. The sections were evaluated using tiered, semiquantitative criteria to grade bone erosions and intralesional osteoclasts.

Kong et al. (1999), Campagnuolo et al. (2002), and Bolon et al. (2002a, b) used Lewis rats with adjuvant arthritis to describe the effects of osteoprotegerin, an endogenous antiosteoclast factor for protecting bone in rheumatoid arthritis.

Francischi et al. (2000) described anti-inflammatory and analgesic effects of the phosphodiesterase 4 inhibitor rolipram in the rat model of adjuvant-induced arthritis.

Boyle et al. (2001) reported anti-inflammatory effects of a non-nucleoside adenosine kinase inhibitor in rat adjuvant arthritis.

Fujisawa et al. (2002) demonstrated the effects of highly water-soluble matrix metalloproteinase inhibitors in a rat adjuvant-induced arthritis model.

Wei et al. (2004) described the effects and mechanisms of a dual inhibitor of interleukin-1 and tumor necrosis factor on adjuvant arthritis in rats.

Boe et al. (1999) reported that interleukin 6 knockout **mice** are resistant to antigen-induced experimental arthritis.

Gauldie et al. (2004) described a robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. DBA/1 and C57BL/6 male mice were injected intra-articularly into a stifle joint with FCA (5 μg in 5 μl) once per week for 4 weeks. Measurements of joint diameter and joint histopathology were used to monitor the course of arthritis. Inflammatory hyperalgesia was assessed as the pressure causing a limb withdrawal. Standard drugs, such as indomethacin or prednisolone, caused a decrease in joint inflammation and associated hyperalgesia.

Kim and Moudgil (2009) reviewed the genetic and other determinants of both susceptibility and resistance to adjuvant-induced arthritis in the rat, and Snekhalatha et al. (2013) conduced a detailed characterization of adjuvant-induced arthritis in the rat model comparing thermography, radiological imaging, and histopathology, a work extended by Vollmer et al. (2014) who used near-infrared fluorescence imaging to monitor the progress of experimental-induced arthritis in several rat models.

The adjuvant-induced arthritis model has been used to profile the activity of a number of candidate drugs which include DHOH, p38 and JAK inhibitors (Balague et al. 2012), bee venom (Darwish et al. 2013), peptides from heat shock protein 65 (Shi et al. 2014), and the saponin astragaloside IV (Wang 2014).

Consden et al. (1971), Cooke and Jasin (1972), Cooke et al. (1972), and Jasin and Cooke (1977) produced a chronic experimental monoarthritis by intra-articular injection of antigens into previously immunized **rabbits**.

Henderson et al. (1990) induced monoarticular arthritis in ovalbumin-sensitized rabbits by intra-articular injection of ovalbumin (antigen-induced arthritis) or in naive rabbits by injecting hyaluronic acid mixed with the polycation poly-D-lysine (polycation-induced arthritis).

Arner et al. (1995) compared the alterations in proteoglycan metabolism in antigen-induced arthritis and polycation-induced arthritis in rabbits and determined the involvement of interleukin-1 in the cartilage degradation that occurs in these models of rheumatoid arthritis.

Lewthwaite et al. (1995) studied the antifibrotic action of interleukin-1 receptor antagonist in antigen-induced monoarticular arthritis in New Zealand white rabbits.

Arthritis occurs in **pigs** due to infection with *Erysipelothrix rhusiopathiae* (Ajmal 1969). Experimental erysipelothrix infection in pigs can be used as a model for rheumatism research (Schulz et al. 1975a, b, 1977). Infections are established by oral or parenteral administration of standardized serotype B erysipelas strains.

Erysipelothrix arthritis could also be produced in rats and **rabbits** (White et al. 1975; Glynn 1977).

Arthritis due to infection with *Mycoplasma synoviae* occurs naturally among domestic poultry (Olson et al. 1954, 1964). Arthritis in **chickens** after mycoplasma infection has been used as experimental model (Kerr and Olson 1970; Cullen 1977).

Experimental models of arthritis due to streptococcal infections have been proposed for various species: **mice** (Cayeux et al. 1966; Hook et al. 1960; Ohanian et al. 1969), **rats** (Jasmin 1967; Koga et al. 1973), **rabbits** (Cecil et al. 1939; Cook and Fincham 1966; Ginsburg et al. 1968, 1977; Norlin 1960; Shimizu et al. 1958; Stein et al. 1973), and **pigs** (Roberts et al. 1968, 1969).

Avridine-Induced Arthritis

The injection of avridine [*N*,*N*-dioctadecyl-*N*′, *N*′-bis (2-hydroxyethyl) propanediamine/CP-20961], emulsified in Freund's adjuvant, at the base of the tail is arthritogenic in susceptible rat strains (Meacock et al. 1994; Brun et al. 1995;

Vingsbo et al. 1995; Lorentzen and Klareskog 1997; Joe and Wilder 1999; Van Bilsen et al. 2004).

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Collagen Type II-Induced Arthritis in Rats

Purpose and Rationale

As reported by Trentham et al. (1977), intradermal injection of homologous or heterologous type II collagen in incomplete Freund's adjuvant results in an inflammatory polyarthritis in rats. The demonstration of antibodies to collagen in patients with rheumatic polyarthritis suggests that autoimmunity may contribute to the pathophysiology of synovitis and joint destruction. Because of the similarities of the symptoms in rats to human disease, the test is considered to be useful to detect anti-inflammatory and immunosuppressive properties of test compounds.

Procedure

Bovine type II collagen is prepared from nasal septum cartilage, which is cut into small fragments, frozen in liquid nitrogen, and pulverized in a freezer mill. Proteoglycans are extracted

overnight by stirring 25 g of pulverized cartilage in 1 l of 0.2 N NaOH. Following centrifugation at 20,000 g for 30 min, the residue is washed with 250 ml of absolute ethanol, the supernatant aspirated, and the residue vacuum dried. Hundred mg pepsin is added to 150 ml of 0.5 M acetic acid, after which 1.0 g of cartilage is added to reach a cartilage to pepsin ratio of 10:1 (w/w). The mixture is stirred 18 h at room temperature and centrifuged at 20,000 *g* for 1 h. Acid soluble collagen present in the supernatant is precipitated by adding NaCl to reach a final concentration of 0.9 M, followed by centrifugation at 20,000 g for 1 h. The precipitate from 1.0 g cartilage is dissolved in 100 ml 1.0 N NaCl/0.005 M Tris–HCl, pH 7.5, and stirred for 3 days. Then, the solution is dialyzed against 0.02 M Na_2HPO_4, pH 9.4, and the precipitate collected by centrifugation at 30,000 *g* for 1 h. The pellet is dissolved in 0.5 M acetic acid, dialyzed against 6 l of 0.01 M acetic acid, and lyophilized. All procedures, unless otherwise stated, are performed at 4 °C.

Test procedure. Collagen is dissolved in a concentration of 2.0 mg/ml in 0.1 M acetic acid overnight at 4 °C. This solution is added dropwise to an equal volume of chilled incomplete Freund's adjuvant. Six to 12 male Wistar rats with an initial weight of about 120 g are used for each group. On day 1, each rat receives a total of 0.5 mg collagen in 0.5 ml, equally divided, in five sites. All injections are intradermal, one at the base of each appendage and one in the nape of the neck. Seven days postimmunization, the animals receive identical booster injections. Control animals receive only the incomplete Freund's adjuvant diluted with 0.1 M acetic acid.

The volume of both hind paws is measured plethysmographically on day 20. To minimize the possibility of including animals with minimal transient disease, only animals with a paw volume of 1.8 ml or greater are used for further testing. From days 20–40, the animals receive the test compounds p.o. once a day. On day 41, the paw volumes are recorded again.

Evaluation

The paw volumes of treated animals are recorded plethysmographically. The increase versus day 20 is calculated. The increase is compared with that of controls or animals treated with a standard drug. Otherwise, arthritic scores can be determined. Nonsteroidal anti-inflammatory drugs such as indomethacin in a dose of 2 mg/kg p.o. or phenylbutazone in a dose of 150 mg/kg p.o., but not acetylsalicylic acid in a dose of 50 mg/kg p.o., have been found to be active. Likewise, corticosteroids and immunosuppressives, but not D-penicillamine, were active.

Critical Assessment of the Method

Nonsteroidal and steroidal anti-inflammatory compounds are detected by this method which, however, does not allow a separation between these two groups.

Modifications of the Method

From studies with a neutrophil elastase inhibitor, Janusz and Durham (1997) concluded that the destruction of the joints in rat collagen-induced arthritis is at least partially due to neutrophil elastase.

Romas et al. (2002) reported that osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis in Dark Agouti rats.

Studies in Mice

Hom et al. (1988), Takagishi et al. (1986, 1992), Cannon et al. (1990), Nemoto et al. (1992), and Carlson et al. (1992) described the effects of immunomodulating agents in collagen-induced arthritis in mice.

Wooley et al. (1993) investigated the anti-arthritic effect of recombinant human interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice.

Joosten et al. (1994) found an accelerated onset of collagen-induced arthritis in DBA_1 lac/J mice by remote inflammation.

Miesel and Haas (1993), Miesel et al. 1994a, b) studied the effects of an active center analogue of Cu_2Zn_2-superoxide dismutase in collagen type II-induced arthritis. Furthermore, the authors described a model potassium peroxochromate-induced inflammation in rats and mice. One to

3 μmol/kg K_3CrO_8 was administered by intraplantar application into the left hind paws of anesthetized rats or mice. Arthritis index was assessed by a score system, or the inflammatory response was quantified scintigraphically under a gamma camera by intravenous injection of 500 μCi $Na^{99m}TcO_4$.

Kumar et al. (1997) compared the cellular mechanisms involved in the control of collagen II-induced arthritis and experimental autoimmune encephalomyelitis in mice.

Ruchatz et al. (1998) studied the role of IL-15 in development of antigen-induced immunopathology in collagen-induced arthritis in DBA/1 mice. A soluble fragment of IL-15 receptor profoundly suppressed the symptoms of collagen-induced arthritis.

Joosten et al. (1999) immunized male DBA-1 mice with 100 μg bovine type II collagen in CFA enriched with *Mycobacterium tuberculosis* H37Ra (4 mg/ml) at the base of the tail. The mice were boosted i.p. with 100 μg collagen dissolved in saline. After disease onset on day 28, the mice were treated either with dimerically linked PEGylated soluble p55 TNFR1 receptor or with purified rabbit anti-murine IL-1α and anti IL-1β. IL-1$\alpha\beta$ blockade prevented cartilage and bone destruction, whereas TNF-α blockade only ameliorated joint inflammation.

Using a similar protocol, Plater-Zyberg et al. (2001) found a therapeutic effect of neutralizing endogenous IL-18 activity in the collagen-induced model of arthritis and Lubberts et al. (2004) after treatment with a neutralizing anti-murine interleukin-17 antibody.

Cuzzocrea et al. (2003) found a reduction in the evolution of murine type II collagen-induced arthritis by treatment with rosiglitazone, a ligand of PPARγ.

McIntyre et al. (2003) reported that a highly selective inhibitor of IκB kinase blocked both inflammation and destruction in collagen-induced arthritis in mice.

Chen et al. (2003) tested orally active inhibitors of TNF synthesis as anti-rheumatoid arthritis drugs using collagen-induced arthritis in male DBA/1 J mice.

Nakae et al. (2002, 2003) generated IL-17-deficient mice and found a suppression of collagen-induced arthritis.

Podolin et al. (2005) described attenuation of murine collagen-induced arthritis by a selective small-molecule inhibitor of IκB kinase 2, occurring via reduction of proinflammatory cytokines and antigen-induced T cell proliferation.

Kuno et al. (2006) reported anti-inflammatory activity of a non-nucleoside adenosine deaminase inhibitor in mice.

Hegen et al. (2008), Bevaart et al. (2010), Bolon et al. (2011), and Roy and Ghosh (2013) reviewed the utility of animal models in arthritis and their suitability for therapeutic target evaluation and correlation with clinical treatment of human rheumatoid arthritis. Many compounds have been evaluated in collagen-induced arthritis including inhibitors of the Bruton's tyrosine kinase (Liu et al. 2011), inhibitors of Sphingosine-1-phosphate (Fujii et al. 2012), and agonists of the nicotinic alpha7 receptor (Hu et al. 2014). Consistent with this finding, the role of the cholinergic pathway as an anti-inflammatory mechanism has been explored in this model (Levine et al. 2014). Furthermore, technological advances for imaging inflammation and monitoring therapeutic responses have been developed (Balducci et al. 2012; Sevilla et al. 2015), which may help progress the discovery and development of new drugs, where differentiation from drugs currently in clinical practice is mandated.

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Proteoglycan-Induced Progressive Polyarthritis in Mice

Purpose and Rationale

Glant et al. (1987, 1992), Mikecz et al. (1987, 1990), and Poole (1989) described a proteoglycan-induced progressive arthritis and spondylitis in BALB/c mice as an animal model displaying similarities to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, immunological parameters, and histopathological studies of diarthrodial joints and spine.

Procedure

High buoyant density cartilage proteoglycans are prepared from fetal and adult human, canine or bovine articular cartilages, as well as 1-week-old mouse epiphyseal cartilage. Fetal human articular cartilage proteoglycan digested with chondroitinase ABC (Hascall and Heinegård 1974) is used to induce arthritis in female BALB/c mice. The mice are sensitized by intraperitoneal injection of 100 μg of chondroitinase ABC-treated proteoglycan in 100 μl of phosphate-buffered saline, pH 7.2, and in Freund's complete adjuvant in a 1:1 emulsion. They are reinjected twice more with the antigen in incomplete Freund's adjuvant after 1 and 3 weeks. All BALB/c mice immunized with human articular cartilage proteoglycan develop arthritis in diarthrodial joints after the third antigen injection. Sera from mice with progressive polyarthritis are tested for antibodies to arthritogenic proteoglycans during weeks 12–18 of immunization. The limbs of all mice are examined daily to record clinical arthritic changes. Swelling and redness, as the first symptoms of arthritis, and the thickness (diameter) of the knee, ankle (intermalleolar diameter), wrist, and the dorsovolar thickness of the paw are recorded three times a week. The most objective joint diameter is the intermalleolar one. The animals are treated with test drug or vehicle for 12 weeks and serum samples taken by retro-orbital puncture for determination of antibodies to proteoglycans. Seven weeks later, the mice are sacrificed, and limbs, tails, and lumbar spine are

fixed, decalcified, and embedded in paraffin for histological examination.

Evaluation

Mean values of intermalleolar diameter and antibody titers of treated and non-treated animals are compared by nonparametric statistics.

Modifications of the Method

Stimpson and Schwab (1989) described a chronic remittent erosive arthritis in rats induced by bacterial peptidoglycan-polysaccharide structures.

Glant et al. (2011) extended this model to generate a model based on recombinant human glycan1 containing T cell epitopes suspected of being arthritogenic. Delemarre et al. (2014) explored the efficacy of autologous bone marrow transplantation in this model showing a stabilization of arthritis scores, and Swart et al. (2014) showed that mesenchymal stem therapy provided by either the intra-articular or intraperitoneal route may suppress proteoglycan-induced arthritis in a murine model.

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Pristane-Induced Arthritis in Mice

Purpose and Rationale

The mineral oil 2,6,10,14-tetramethylpentadecane (known as **pristane**) induces a chronic inflammatory arthritis in **mice** after intraperitoneal injection (Potter and Wax 1981; Hopkins et al. 1984; Wooley et al. 1989; Chapdelaine et al. 1991;

Wooley and Whalen 1991; Levitt et al. 1992; Abe et al. 1995; Thompson et al. 1998; Wooley et al. 1998; Vigar et al. 2000). The immunological involvement in the pathogenesis of pristane-induced arthritis was studied by several authors (Bedwell et al. 1987; Thompson et al. 1990; Ghoraishian et al. 1993; Nishikaku et al. 1994; Vingsbo et al. 1996; Stasiuk et al. 1997; Morgan et al. 2004). Moreover, the genetic basis for the susceptibility to pristane-induced arthritis was studied (Lu et al. 2002; Olofsson et al. 2003; Brenner et al. 2005; Jensen et al. 2006). Not only in mice but also in **rats** arthritis could be induced by pristane injections (Vingsbo et al. 1996; Zheng et al. 2002, 2003; Webster et al. 2003; Holmberg et al. 2006).

Patten et al. (2004) characterized the model of pristine-induced arthritis (PIA) in mice by studying the response to antirheumatic agents, expression of joint cytokines, and immunopathology.

Procedure

Induction and Characterization of PIA

Male DBA/101aHsd mice were placed under isoflurane anesthesia and injected intraperitoneally with 0.5 ml of pristane (Sigma-Aldrich, Poole, UK), and an identical booster injection was given 7 weeks thereafter. The severity of arthritis was graded visually by assessing the level of swelling in each paw, including the tarsus (ankle) or carpus (wrist) joints. The following scoring system was used: 0.5 = swelling of toes only or very slight ankle/wrist swelling; 1 = slight swelling of paw; 2 = moderate swelling of paw; 3 = marked swelling of paw; and 4 = substantial swelling of paw. Thus, the maximum total score per animal was 16. All batches also contained animals that were not treated with pristane, and these served as comparators for all studies undertaken.

Mice were observed for paw or toe swelling in a time-course study lasting up to 180 days after the first pristane injection. After study termination, the initially swollen hind paws were obtained for histologic assessment and allocated to different study groups according to the duration of swelling. The remaining three paws of each animal were used in cytokine studies.

Drug Preparation and Administration Schedules

The effects of administration of established and novel antirheumatic compounds were assessed using a therapeutic dosing schedule. Separate batches of mice for each drug study were monitored weekly for the development of swollen paws from day 80 after the first injection of pristane. Mice were included in the drug studies only if they developed a score of ≥ 1 in a hind paw on two consecutive weekly observations between day 120 and day 134 after the first injection of pristane (n = 7–13 per treatment group). At study termination, paws were obtained for histologic and cytokine assessments, normally at 1 h after the final drug administration.

All orally administered treatments were undertaken by gavage. Prednisolone was suspended in 0.5 % methylcellulose and administered orally once daily at a dose of 2 mg/kg. Methotrexate was dissolved in physiologic saline and administered intraperitoneally three times per week at a dose of 9 mg/kg. Indomethacin and diclofenac were suspended in 1 % methylcellulose and given orally once daily at doses of 3 mg/kg and 2 mg/kg, respectively. Celecoxib was suspended in a solution of 66 % polyethylene glycol, 33 % water, and 1 % dimethyl sulfoxide and was administered orally twice daily at a dose of 30 mg/kg. Etanercept was dissolved in the supplied vehicle according to the instructions of the manufacturer and diluted using physiologic saline and was administered intraperitoneally three times per week at doses of 300 μg and 100 μg per mouse. Murine sTNFR, consisting of two murine p75 receptors fused to murine IgG2a, was dissolved in physiologic saline and administered intraperitoneally three times per week at doses of 300 μg and 100 μg per mouse. The selective p38 MAPK inhibitor SB242235 (synthesized at the US GSK Research Center) was suspended in 0.5 % tragacanth and 0.03 M hydrochloric acid and given orally twice daily at doses of 30 mg/kg and 15 mg/kg.

Joint Cytokine Messenger RNA (mRNA) and Protein Assays

The levels of mRNA and protein for the proinflammatory cytokines TNFα, IL-1β, and

IL-6 were measured in disaggregated joints by TaqMan real-time reverse transcription-polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISAs), respectively. At study termination and, in the drug studies, 1 h after the final drug treatment administration, the primary ankle joint was removed for histology, and the remaining paws were removed and snap-frozen in liquid nitrogen (six to eight mice per group). For cytokine assessment, the paw showing the highest score for swelling was selected with the proviso that it had also been swollen at the start of the drug study. If the remaining three paws exhibited no swelling at study termination, then the remaining ankle was selected for assay. Whole paws were frozen and pulverized using a mortar and pestle filled with liquid nitrogen.

For the mRNA studies, total RNA was isolated from homogenized paws using RNeasy Mini Kits (Qiagen, Crawley, UK). Samples were treated with 10 units of RNase-free DNase (Qiagen) for 15 min during the RNA isolation process. Reverse transcription of mRNA was carried out using TaqMan reverse transcription reagents in an MJ Research PTC-200 PCR Peltier Thermal Cycler. TaqMan probes and forward and reverse primers for the genes of interest (TNFα, IL-1β, and IL-6) and for housekeeping genes (GAPDH and cyclophilin) were designed with Primer Express TM software (PE Applied Biosystems). Cytokine mRNA expression levels were quantified by TaqMan real-time PCR using the ABI Prism 7900 Sequence Detector System (PE Applied Biosystems).

Measurement of Serum Antibody Levels

Blood was withdrawn from all mice before pristane injection and monthly thereafter. Levels of antibodies were determined by ELISA. Plates were coated with 100 µl of coating buffer (0.4 M phosphate buffer, pH 7.6) containing 5 µg of each antigen, at 4 °C overnight. The antigens assessed were bovine aggrecan, bovine biglycan, human endoplasmic reticulum molecular chaperone protein, bovine chondroitin sulfate A, bovine chondroitin sulfate B, bovine type I collagen, chick type II collagen, murine type II collagen peptide, bovine decorin, bovine double-stranded DNA, human fibronectin, lupine glucose-6-phosphate isomerase, mycobacterial 65-kDa heat shock protein, murine aggregated IgG, joint extract from normal mice, and joint extract from arthritic mice. Plates were washed three times with 0.05 % Tween 20 in PBS, and nonspecific binding was blocked by 5 % nonfat milk in PBS overnight at 4 °C. Serum samples from at least six individual mice per time point were used. Since 1:100 was the dilution determined to produce the optimal response to high-density proteoglycans, mouse serum diluted 1:100 in 5 % milk/PBS was added to each well and incubated overnight at 4 °C. Subsequently, the plates were washed six times with 0.05 % Tween 20 in PBS and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Ala., USA) at 37 °C for 1 h. Plates were again washed six times and developed for 40 min in the dark, using *p*-nitrophenyl phosphate as a chromatogen substrate. The optical density was measured at 405 nm (OD_{405nm}) using an ultraviolet max spectrophotometer (Molecular Devices, Sunnyvale, Calif., USA). To ensure uniformity of the assay, negative control sera obtained prior to blood withdrawal and a standard mouse anti-type II collagen antiserum were titered on each plate. Antibody binding was expressed as the OD_{405nm} in units, blanked against control.

Isolation of Splenocytes and Cell Proliferation Assays

Spleens were excised and immediately immersed in PBS. Tissue was mechanically disrupted to release cells, which were suspended in 10 ml of sterile PBS and centrifuged for 10 min at 1,500 rpm. Prior to resuspension in medium, red blood cells were removed from the spleen preparations by adding distilled water for 10 s and then adding PBS. Spleen cells were then counted using a hemocytometer and washed and resuspended in RPMI at a final concentration of 2.5×10^6/ml.

Next, 100 µl of spleen cell aliquots (2.5×10^6/ml) was transferred to 96-well plates with 50 µg/ml of each antigen (aggrecan, biglycan, chondroitin sulfate A, chondroitin sulfate B, type I collagen, type II collagen, type II collagen peptide,

decorin, fibronectin, and heat shock protein; all were derived from the same species as described for the serum antibody studies) in complete RPMI 1640 medium. Cells were incubated for 72 h at 37 °C in the presence of antigen. Then 20 μl of MTT solution (a mitochondrial enzyme substrate) was added to each well (5 mg/ml). After a 6-h incubation, the culture supernatant was discarded, and 200 μl of 10 % sodium dodecyl sulfate solution was added to each well. After incubation at 37 °C overnight, the OD_{590nm} was read by microplate photospectrometer (Molecular Devices). The mean OD values were recorded for each cell sample as a measure of antigen stimulation. Antigen-specific responses were calculated as follows: (OD_{590nm} [stimulated culture]) – (OD_{590nm} [spontaneous proliferation culture]).

Histopathologic Evaluation

In all studies, the primary ankle joint that was swollen at the beginning of the time-course study or drug study was excised and fixed in 10 % neutral buffered formalin. The tissues were decalcified with formic acid and embedded in paraffin blocks. Sections (4–7 μm) were cut along a longitudinal axis, mounted, and stained with hematoxylin and eosin or toluidine blue, and representative slides for each animal were assessed. The following features were scored in six to ten animals per group: inflammatory exudate, neutrophil and mononuclear cell infiltration, bone resorption, and synovial hyperplasia. For drug studies, the effects of the agents on the pristane-induced pathologic condition were scored as follows: + = mild inhibition of pathologic features, ++ = moderate inhibition of pathologic features, and +++ = marked inhibition of pathologic features.

Evaluation

Graphic and tabular data are expressed as the mean ± SEM. Statistical significance was tested by application of the Kruskal–Wallis test for clinical scores and by analysis of variance followed by Dunnett's test for the cytokine mRNA and protein time-course results. Antibody and cell proliferation studies were analyzed using the least-squares significant difference post hoc test.

Modifications of the Method

Brenner et al. (2006) published thermal signature analysis as a novel method for evaluating inflammatory arthritis activity using rats with Freund's adjuvant-induced monoarthritis and pristane-induced arthritis. The thermal imaging system employs a platinum silicide 256 × 256 pixel detector array filtered to be sensitive to infrared radiation at a wavelength of 3–5 μm.

Lange et al. (2005) investigated the mode of action of methotrexate in different models for rheumatic arthritis, such as fibroblast-induced arthritis in SCID mice, collagen-induced arthritis and anti-collagen II antibody-induced arthritis in rats, and pristane-induced arthritis in DA rats, and models of multiple sclerosis, such as experimental autoimmune encephalomyelitis in (Balb/c × B10.Q) F1 and B10.Q mice.

Pristane induces lupus-like kidney and pulmonary disease in mice (Satoh et al. 1995; Richards et al. 1998; Lin et al. 2004; Chae et al. 2006).

De Franco et al. (2014) used the pristane-induced arthritis model to dissect genetic determinants for high inflammation susceptibility and demonstrate the involvement of loci interaction with the *Slc11a1* gene.

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Streptococcal Cell Wall-Induced Arthritis

Purpose and Rationale

Streptococcal cell wall (SCW)-induced arthritis is a chronic and erosive polyarthritis which may be induced in susceptible Lewis rats by a single injection of a sterile, aqueous suspension of SCW via the intraperitoneal route of administration (Cromartie et al. 1977).

The model has been used to study the efficacy of a number of experimental drugs which include the immunosuppressant cyclosporine A (Yocum et al. 1986); antibodies to IL-4, IL-10, interferon-γ, and monocyte chemotactic protein-1 (Schrier et al. 1998; Schimmer et al. 1998); the phosphodiesterase inhibitor rolipram (Laemont et al. 1999); the bisphosphonate clodronate (Richards et al. 2001); *N*-butyryl glucosamine (Wang et al. 2007); an inhibitor of the purinoreceptor $P2X_7$ (McInnes et al. 2014); and the TNF-a inhibitor etanercept (Chakravathy et al. 2014).

Procedure

Lewis rats, typically 120–150 g at the start of the study, receive an injection into the ankle joint of SCW (Lee Laboratories, Grayson, GA, USA). Susceptible animals can be identified by intra-articular injection of SCW (5 μg) into the ankle joint up to day 21 prior to any therapeutic intervention, which may reflect an acute phase of arthritis induction. The chronic, reactivation phase of the study, during which therapeutic intervention is typically investigated, is achieved by intravenous injection of SCW (100–200 μg). Studies normally run for 6–7 days post intravenous injection of SCW but may run for up to

30 days; animals are sacrificed prior to and after intravenous challenge for blood analysis and ankle joint assessment.

Evaluation

Disease severity is typically assessed using the following criteria:

1. A direct measurement of ankle swelling and mechanical hyperalgesia by von Frey threshold using nylon filaments
2. Assessment of histopathological measures which typically include synovitis, inflammation of synovial sub-lining, chondronecrosis, and subchondral bone resorption
3. Radiographical assessment of joint structure

It is also common practice to take blood samples for analysis of biomarkers and drug pharmacokinetics. Rioja et al. (2005) conducted an extensive analysis of the gene expression profile in response to SCW-induced arthritis.

Modification of the Method

Kuiper et al. (1998) used a single intravenous injection of SCw (25 μg) and assessed the effects of TNF-α and IL-1β blockade by administration of anti-cytokine antibodies 1 h prior to arthritis induction. Wang et al. (2007) induced arthritis by a single intraperitoneal injection of SCW (15 μg/g weight of rat) and studied the disease-modifying effects of *N*-butyryl glucosamine commencing the day after SCW injection.

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Experimental Autoimmune Thyroiditis

Purpose and Rationale

Immunization of rats or mice with porcine thyroglobulin results in thyroiditis (Vladutiu and Rose 1971; Vladutiu 1983; McGregor et al. 1983; Hassman et al. 1985; Salamero et al. 1987; Fournier et al. 1990).

Procedure

Crude porcine thyroglobulin (PTg) solution is emulsified in complete Freund's adjuvant in a 1:1 ratio. Female mice (6–8 weeks old) are primed with 50 μg PTg given s.c. into four or five sites of injection and are boosted 14 days later with the same dose of PTg (s.c.) emulsified in incomplete Freund's adjutant. The test compounds are administered from day 0 (at priming) until day 21. Mice are bled on day 21 and on day 28 after priming. The sera are tested for the levels of anti-PTg antibodies using an enzyme-linked immunosorbent assay (ELISA). On day 28, the animals are sacrificed and the thyroid glands prepared. Five-micrometer-thick sections are stained with Masson-Goldner's trichrome solution.

Evaluation

The histological severity of experimental autoimmune thyroiditis is graded as a function of mononuclear cell thyroid infiltration indices:

1. Interstitial accumulation of inflammatory cells distributed between two or more follicles
2. One or two foci of inflammatory cells reaching at least the size of one follicle
3. 10–40 % of the thyroid replaced by inflammatory cells
4. More than 40 % of the thyroid replaced by inflammatory cells

Mean values of treated animals are compared with controls.

Modifications of the Method

Castagliola et al. (1994) induced autoimmune thyroid disease in BALB/c mice by immunizing with the extracellular domain of the human TSH receptor expressed as a maltose-binding protein fusion in bacteria. This type of thyroiditis could be transferred to naive BALB/c and NOD mice (Castagliola et al. 1996).

Green et al. (1995) described a spontaneous model of autoimmune thyroiditis in MRL-lpr/lpr mice.

Furthermore, Green et al. (1996) induced thyroiditis in Lewis rats by immunization with thyroid extract and thyroglobulin. A reduction of the gap junction proteins connexin 43, connexin 32, and connexin 26 was found in diseased thyroid tissue.

Wang et al. (2014) showed that overexpression of the human BH3 interacting-domain death agonist (BID) in the thyroids of transgenic mice may increase their sensitivity to iodine-induced autoimmune thyroiditis, noting that BID expression alone is not sufficient to induce thyroiditis.

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Coxsackievirus B3-Induced Myocarditis

Purpose and Rationale

The effects of immunosuppressant drugs can be studied in the murine model of coxsackievirus B3 myocarditis.

Procedure

Three-week-old male BALB/c mice are kept for 7 days before the experiment in a single, self-contained animal isolation unit to exclude pre-diseased animals. They are maintained in disposable, filter-topped cages and handled with gloves by gowned and masked personnel. The intraperitoneal route is used for injection of virus in a 0.5 ml volume.

The CVB3 virus strain is grown on either Hep-2 or VERO cells, aliquoted, and maintained at −70 °C until use. At the time of infection, seed virus is grown on either VERO or LLC-MK-2 cells with Dulbecco's modified Eagle medium, 12 % fetal calf serum, and gentamicin. Virus is harvested and adjusted to an inoculum of 1.75×10^7 plaque-forming units/0.5 ml RPM-1640. The test drugs are given subcutaneously daily for 8 days. On day 8, the animals are sacrificed, the hearts rapidly removed, and

divided into two equal cross sections. The basal portion is snap frozen for isolation of virus and determination of drug level. The apical portion is fixed in 10 % formalin, dehydrated, and embedded in paraffin. Five-mm sections are stained with hematoxylin–eosin and Masson's trichrome stains. The bases of the individual hearts are minced with a sterile scalpel, suspended in 1 ml RPMI-1640, and homogenized in a glass tissue grinder. The suspension is centrifuged at 8,000 *g* for 10 min at 4 °C. Supernatants are harvested and frozen at −70 °C until assay. Serial tenfold dilutions of heart homogenates in minimum essential medium are layered on confluent, 72-h-old VERO cells that had been grown in 96-well microtiter plates. Monolayers are checked daily for 7 days for presence or absence of virus and rate of cell destruction.

Evaluation

The slides are examined by two observers blinded to the slide code, and inflammation and necrosis are quantitated.

Modifications of the Method

Lane et al. (1991) showed that lipopolysaccharides promote CB3-induced myocarditis in otherwise resistant B10. A mice.

Beisel et al. (1991) identified a putative shared epitope between coxsackievirus B4 and mouse alpha cardiac myosin heavy chain.

Gauntt et al. (1993) found that epitopes shared between coxsackievirus B3 and normal heart tissue contribute to CVB3-induced myocarditis in mice.

Xu et al. (2004) used the murine model to deliver a chitosan–DNA vaccine and showed protection against acute CVB3 challenge. Park et al. (2009) and Yue et al. (2009) further explored approaches supportive of potential immunotherapeutics in this model using pancreatitis as an additional endpoint (Park et al. 2009). The model has also been used to investigate the innate immune response as a predictor for the progression of cardiovascular disease and heart failure in male mice (Onyimba et al. 2011) and to better understand the efficacy of further immunotherapeutic approaches where oral administration of interferon-α2b-transformed *Bifidobacterium longum* was shown to protect animals from CVB3-induced myocarditis (Yu et al. 2011).

A number of other agents have been tested in this model and include galectin-9 which ameliorated CVB3-induced myocarditis (Lv et al. 2011), IL-17 which was found to be protective (Xie et al. 2012), and the micro-RNA miR-21 which alleviated CVB3-induced myocarditis (He et al. 2013). A comparison of the effects of ivabradine and carvedilol showed an expected effect on heart rate reduction and a potential anti-inflammatory effect in the CVB3-induced myocarditis model.

Instead of coxsackievirus B3, Monrad et al. (1986) used encephalomyocarditis virus to induce experimental myocarditis in mice.

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Porcine Cardiac Myosin-Induced Autoimmune Myocarditis in Rats

Purpose and Rationale

Pummerer et al. (1991), Inomata et al. (1995), Suzuki (1995), and Dimitrijevic et al. (1998)

described autoimmune myocarditis in rats induced by porcine cardiac myosin.

Procedure

Male Sprague–Dawley or Lewis rats at the age of 8–10 weeks are immunized with porcine cardiac myosin either purchased from Sigma (St. Louis, MO, USA) or prepared from the ventricular muscle of porcine hearts according to Murakami et al. (1976). The cardiac myosin fraction is dissolved in phosphate buffer at a concentration of 10 mg/ml. The antigen solution is emulsified with equal volume of complete Freund's adjuvant supplemented with heat-killed mycobacterium tuberculosis. Rats are injected subcutaneously into the foot pad with an immunizing dose of 5 mg of antigen in complete Freund's adjuvant/kg of body weight. Rats are injected intraperitoneally with test compounds either from day 0 to 6 (early treatment group) or from day 14 to 20 (late treatment group).

Immunized rats are sacrificed on days 8, 16, 21, and 34, respectively. Disease course and severity are analyzed by macroscopic findings of the hearts and heart weight/bodyweight ratio as well as by histological and immunohistochemical analysis. Macroscopic findings are scored as follows: 0, normal finding; 1, presence of focal discolored area on the surface; and 2, presence of diffuse discolored areas (Kodama et al. 1995).

The hearts are removed and weighted immediately after the rats are sacrificed, fixed in 10 % buffered formalin, and embedded in paraffin. Serial section (5 μm in thickness) is stained with hematoxylin–eosin. The severity of myocarditis is determined according to the following scoring system: 0, no inflammation; 1, histological cross section infiltrated up to 5 %; 2, 5–10 % infiltrates/section; 3, 10–20 % infiltrates/section; greater than 20 % infiltrates/section.

For immunohistochemical staining, heart samples are embedded in OCT compound (Miles, Elkhart, IN) and rapidly frozen. Cryostat sections are cut sequentially at 7 μm in thickness, mounted on glass slides, and prepared for immunoperoxidase staining. Sections are fixed in cold acetone for 10 min and extensively washed in 0.1 M Tris buffer solution, pH 7.6. Murine monoclonal antibodies specific for different rat molecules are added at appropriate concentrations. After incubation at 4 °C overnight and further buffer washes, the sections are incubated with peroxidase-conjugated anti-mouse immunoglobulins for 60 min. Peroxidase reaction is visualized with 0.05 % diaminobenzidine in 0.01 % H_2O_2 for 7–8 min. The color development is stopped by washing slides in running water. All samples are lightly counterstained with hematoxylin, mounted in gelatin/glycerol medium, and assessed by light microscopy.

Evaluation

Macroscopic and microscopic scores are expressed as mean values. Body weights, heart weights, and heart weight/body weight ratio are expressed as mean ± SD. Student's *t*-test for paired samples is used for comparison data within groups in reference to time, while two-sample *t*-test is used for comparison data between groups.

Modifications of the Method

Koyama et al. (1995) immunized Lewis rats with human cardiac myosin suspended in complete Freund's adjuvant and induced severe active myocarditis with acute and chronic heart failure. The baseline left ventricular pressure was significantly lower in the chronic phase group, and peak dP/dt was significantly lower in both the acute phase group and the chronic phase group than in the respective controls. The animal model was recommended to study both acute heart failure related to acute myocarditis and chronic heart failure due to diffuse myocardial fibrosis.

Neu et al. (1990, 1991; Neu and Ploier 1991; Penninger et al. 1993) induced severe autoimmune myocarditis in some mouse strains by immunization with cardiac myosin in complete Freund's adjuvant.

Wahed et al. (2005) used the method of immunization with porcine cardiac myosin to test the effects of eplerenone, a selective aldosterone blocker, on the progression of left ventricular dysfunction and remodeling in rats with dilated cardiomyopathy.

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Experimental Allergic Encephalomyelitis

Purpose and Rationale

Experimental allergic encephalomyelitis was first produced in laboratory animals by Rivers et al. in 1933. This pathological model is an immunologic disease arising from a delayed hypersensitivity reaction to nervous tissue. In many respects, the model resembles autoimmune diseases, especially demyelinating diseases, in man (Constantinescu et al. 2011), and the utility of animal models as for drug discovery and development for neurological diseases especially multiple sclerosis (MS) has been extensively reviewed (Croxford et al. 2011; Denic et al. 2011; Pachner 2011; Singhal and Srivastava 2012; Tian et al. 2013). The method is used for evaluation of immunosuppressive properties of drugs (Warford and Robertson 2011; Dasgupta et al. 2011; Paris et al. 2013; Mondal and Pahan 2015).

Procedure

Preparation of the encephalitogen: 3 g of spinal cord from guinea pigs or rats is homogenized with 7.5 ml bidistilled water, 3.8 ml phenol, and 7.5 ml complete Freund's adjuvant under cooling.

Groups of 6–12 male Wistar–Lewis rats with an initial body weight of 130–200 g are used. On day 0, experimental allergic encephalomyelitis is induced by subplantar injection of 0.1 ml of the encephalitogen into the left hind paw. An equal volume of *Bordetella pertussis* vaccine concentrate (200×10^9 organisms/ml) is injected into the same foot. From days 1–2, the animals receive the test compound or vehicle only or the standard drug by oral administration once a day. Body weights of the animals are recorded every second day. The clinical signs of experimental allergic encephalomyelitis consist of ataxia or paresis, i.e., grossly irregular gait and weakness of one or both hind legs followed by flaccid paralysis of the hindquarters, urinary incontinence, fecal impaction, and abdominal wall flaccidity. Animals showing one of these clinical signs are considered positive for the purpose of evaluation.

Evaluation

Starting from day 7, the severity of clinical signs and mortality are determined daily and scored according to the following scheme:

	Score
Per 20 g loss of weight	1
Paralysis of the tail	1
Paralysis of the hind paw	3
Complete paralysis	5
Death	6

Calculation of the Results

The delay of onset of the paralytic symptoms is determined. The total score per day is recorded for treated and control groups. On the day of maximal clinical symptoms occurring among control animals, the total score of the treated groups is compared to the total score of the control group. The percentage change is evaluated.

Doses of 0.5 mg/kg p.o. methotrexate, 1 mg/kg p.o. hydrocortisone, and 2.5 mg/kg p.o. cyclophosphamide were found to be active, whereas

nonsteroidal anti-inflammatory compounds were inactive.

Critical Assessment of the Method

The model of experimental allergic encephalomyelitis in rats is suitable to distinguish between immunosuppressive and anti-inflammatory drugs. Experimental autoimmune encephalomyelitis is considered as a rodent model of the autoimmune disease multiple sclerosis (Pearson et al. 1997; Deng et al. 2002).

Modifications of the Method

The phosphodiesterase inhibitor pentoxifylline was found to prevent induction of experimental autoimmune encephalomyelitis in Lewis rats (Rott et al. 1993).

Martin and Near (1995) studied the protective effect of the interleukin-1 antagonist IL-1ra on experimental allergic encephalomyelitis in Lewis rats.

Experimental autoimmune encephalomyelitis in different strains of mice was described by Heremans et al. (1996), Glabinski et al. (1997), and Liblau et al. (1997).

Baker et al. (1990, 1991, 2000) induced experimental allergic encephalomyelitis in Biozzi AB/H mice by sensitization with 1 mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on days 0 and 7. The disease is characterized by relapsing–remitting episodes similar to multiple sclerosis in human beings. Biozzi AB/H mice also develop spasticity and tremor which can be antagonized by cannabinoids.

A chronic relapsing–remitting form of experimental autoimmune encephalomyelitis was induced in the common marmoset *Callithrix jacchus* following a single immunization with human white matter by Massacesi et al. (1995) and Genain and Hauser (1997) and recommended as a new model for multiple sclerosis. This model has been used for histopathological characterization of magnetic resonance imaging-detectable white matter lesions in a primate model of multiple sclerosis by 't Hart et al. (1998, 2004).

Experimental allergic neuritis in several animal species has been described by Waksman and Adams (1955, 1956); King et al. (1983); McCombe et al. (1990), and Nakayasu et al. (1990). This disorder has been considered to show similarities to the Guillain–Barré syndrome in man. The demyelinating process initiated by the injected antigens is a lymphocyte-mediated reaction in which activated macrophages strip myelin off the axons. Hartung et al. (1987) described the adoptive transfer experimental autoimmune neuritis in Lewis rats by injection of P2-reactive T lymphocyte cell lines.

Mix et al. (1992) studied the effect of stilbene-type anion channel blockers on the immune response during experimental allergic neuritis induced by bovine peripheral myelin.

Kojima et al. (1994) investigated the pathogenic potential of autoimmune T cell responses to nonmyelin autoantigens in the Lewis rat using the astrocyte-derived calcium-binding protein S100β as a model nonmyelin autoantigen. In contrast to the experimental autoimmune encephalomyelitis induced by the adoptive transfer of myelin basic protein-specific T line cells, S100β-specific T cell transfer induced intense inflammation not only in the spinal cord but also throughout the entire CNS and also in the uvea and retina of the eye.

Gautam et al. (1992) reported that a polyalanine peptide with only five native basic protein residues induces autoimmune encephalomyelitis in mice. This peptide, called myelin basic protein (MBP) Ac1–11, has been used by several authors for further studies on experimental autoimmune encephalomyelitis (Ratts et al. 1999; Matejuk et al. 2003).

Pearson et al. (1997) reported the induction of a heterogeneous T cell receptor repertoire in (PL/JXSJL/J) F2 mice by myelin basic protein peptide Ac1–11 and its analogue Ac1–11[4A].

Deng et al. (2002) found that expression of the tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase 1 determines the T cell activation threshold and severity of experimental autoimmune encephalomyelitis.

Maron et al. (2002) investigated the immunological properties of Cop1 (glatiramer acetate) to determine the degree to which its effects were antigen specific using myelin basic protein T cell

receptor transgenic mice. Immunization of these mice fed glatiramer acetate, myelin basic protein, or MBP Ac1–11 resulted in decreased proliferation and IL-2, IL-6, and IFN-γ production and increased secretion of IL-10 and TGF-β in glatiramer acetate-fed animals.

Gilgun-Sherki et al. (2003) reported that riluzole suppresses myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in mice.

Pollak et al. (2003) studied the experimental allergic encephalitis-associated behavioral syndrome and the modulation by anti-inflammatory treatments.

Diab et al. (2004) found that ligands for the PPAR-γ and the retinoid X receptor exert additive anti-inflammatory effects on experimental autoimmune encephalomyelitis. Duckers et al. (1997) studied the effect of a neurotropic treatment on cortical lesion development in experimental allergic encephalomyelitis in rats by longitudinal in vivo magnetic resonance imaging methods.

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Acute Graft-Versus-Host Disease (GVHD) in Rats

Purpose and Rationale

The intravenous injection of a mixture of parental splenocytes into healthy inbred F_1-rats results in graft-versus-host (GVH)-induced immune abnormalities. This is due to T lymphocytes in the donor inoculum that recognize the major histocompatibility alloantigens expressed by the F_1-animals. The host F_1 T cells are genetically unable to recognize antigens of the parental donor as foreign; thus, the response involves only donor recognition of host and not host recognition of donor. The ensuing immune abnormalities lead to clinical symptoms of an acute, lethal GVH-disease (GVHD), i.e., profound immunodeficiency, anemia, hypogammaglobulinemia, and runting.

Procedure

Three- to 4-month-old male F_1-hybrid rats of the inbred strains Lewis (Rt-1 l) and Brown Norway (BN, Rt-1n) (Zentralinstitut für Versuchstierkunde, Hannover, Germany) are used as hosts for cell grafts from the Lewis parental strain. The bone marrow cells are obtained by flushing hind femur bone shafts with culture medium. These cells are then pooled together with spleen cells (ratio 2 bones/1 spleen). The cell viability, determined by trypan exclusion, has to be more than 90 %. Each recipient is injected with about 40×10^7 cells in a 1.5 ml suspension volume. The route of injection is the penis vein, allowing an optimal control of correct intravenous application.

Prophylactic Drug Application

For this experiment, two groups of 6 F_1-hybrids each are injected with the abovementioned bone marrow/spleen cell suspension. One group receives the test drug orally and daily until the end of the experiment, homogeneously suspended in 1 % carboxymethylcellulose (CMC) solution. The other group receives CMC alone and, thus, serving as the GVHD control group. The experiment is terminated 2 weeks after disease induction, i.e., 1 week after the first appearance of GVHD symptoms. All animals are sacrificed and clinical aspects documented; spleens weighed; histology of the skin, liver, spleen, and lymph nodes performed; and organs photographed.

Therapeutic Drug Application

In this experiment, rats are separated into four groups and treatment begins with the first sign of GVHD symptoms (beginning of the second week). Because of the expected, greater therapeutic difficulty, the daily dose of the test drug has to be doubled, again for 2 weeks duration.

The experiment is terminated either by sacrificing those rats that are too sick to be able to move around the cage or at the end of the 4-week observation period, regardless of the clinical condition of the animals. The clinical-chemical parameters are determined by routine procedures conducted with a Hitachi autotechnicon.

Evaluation

The tested parameters of therapeutic success or disease, respectively, are survival rate (%), spleen weight (g), and body weight (g) as well as clinical-chemical parameters (bilirubin, alkaline phosphatase, creatinine, white cell count) after 2 and 3 weeks.

Modifications of the Method

Gelpi et al. (1994) established a chronic graft-versus-host disease in (C5BL/10 × DBA/2) F_1 mice with an injection of lymphoid cells from

the parent DBA/2 strain. Most of the animals developed antibodies against transfer RNA/protein particles.

Mosier et al. (1988) reported transplantation of human peripheral blood lymphocytes (PBL) into severe combined immunodeficient (SCID) mice to construct hu-PBL-SCID mice. Kim et al. (1997) suggested these mice for routine immunotoxicity investigations using lymph nodes of intestines as the lymphocyte sources.

Ford et al. (1970) and Schorlemmer et al. (1997, 1998) used the popliteal lymph node assay to study the local graft-versus-host reaction. The test is based on the enlargement of the draining popliteal lymph nodes as a result of injecting immunocompetent cells (1×10^8 parental Lewis spleen cells) into the hind foot pad of Lewis × Brown Norway F1 recipients. The reaction is measured at day 6 after challenge as a gain in lymph node weights.

Xu et al. (2010) explored the effects of both rapamycin and tacrolimus in the model measuring animal survival after liver transplantation and reporting a differential effect on survival between the two drugs. Xia et al. (2013) investigated the effects of Trichostatin A (TSA) in the rat model of liver transplantation and concluded that TSA did not abrogate acute graft-versus-host disease due to a downregulation of regulatory T cells.

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Influence on SLE-Like Disorder in MRL/lpr Mice

Purpose and Rationale

Systemic lupus erythematosus (SLE) is an autoimmune disease in man that affects multiple body organs and is characterized by the development of certain types of self-antigens. Primarily, the antibodies formed against double-stranded DNA (dsDNA), the most prevalent in this ailment, complex together and, with complement, deposit in the small blood vessels, leading to widespread vasculitis. MRL Mpf lpr/lpr (MRL/lpr) mice spontaneously develop a severe disease with many symptoms very similar to human SLE, i.e., hypergammaglobulinemia and glomerulonephritis (Theofilopoulos and Dixon 1981). Recent years have seen the development of numerous animal models of skin disease which have assisted the discovery of potential new drugs for clinical testing (Rottman and Willis 2010; Avci et al. 2013) which in part have allowed progression of a number of small-molecule candidate drugs (Kyttaris et al. 2013; Markopoulou and Kyttaris 2013).

Procedure

Female MRL/lpr mice (originally from Jackson Laboratories, USA), displaying distinct symptoms of SLE (between 12 and 13 weeks of age), are randomized and divided into groups of

12 animals each. At this age, the animals have already clinical manifestations of the SLE-like illness, as determined by the disease index, but have not yet developed proteinuria. Animals with early symptoms of disease are treated with various drugs, e.g., leflunomide, cyclosporine A, azathioprine, cyclophosphamide, or prednisolone, for 11 weeks, and the survival rate and disease index of these animals are followed for 24 weeks. The disease index and urine protein level are determined once weekly.

Disease Index

The subsequent clinical parameters are taken into consideration:

1. Ears: reddening of the skin, deterioration of the pinna
2. Nose: loss of hair, wasting of the skin
3. Lymph nodes: detection of swollen lymph nodes on any part of the body, especially the neck and extremities
4. Fur: general condition of fur (e.g., shabby, mangy, etc.), loss of hair
5. Skin: inflammation of the skin, scab, and/or granuloma formation
6. Eyes: exophthalmos, deterioration due to inflammation, tumor formation around the eye, swelling of the eyelid with eventual closure of the eye
7. Paws: reddening of the skin, swelling of the paw

Evaluation

A score for each of the above-described parameters is given according to the severity of the symptoms as follows:

Points for Clinical Index

Involvement	Detectable	Moderate	Severe
Ears (each)	0.5	1.0	1.5
Nose	1.0	2.0	3.0
Lymph node (each)	1.0	2.0	3.0
Fur	1.0	2.0	3.0
Skin	1.0	2.0	3.0
Eyes (each)	1.0	2.0	3.0
Paws (each)	0.5	1.0	1.5

Body weight (one point for 5 g difference from week to week)

The determination of the disease index is performed, weekly, by the same individual, but without knowledge of the group being evaluated. The points, for each animal, are registered and the total score, of each group, summarized. The average score for the group is calculated, and significance between the experimental group and the untreated diseased group is determined using the Student's *t*-test.

Proteinuria

Pooled urine is collected from each experimental group and the amount of protein in the urine is calculated.

Modifications of the Method

In addition to a lupus-like syndrome and massive T cell proliferation, MRL-1pr/1pr (MRL/1) mice develop an arthritic process very similar serologically and histologically to human rheumatoid arthritis. Boissier et al. (1989) found that in these animals, mouse type II collagen is antigenic, but not arthritogenic.

Holmdahl et al. (1991) studied the involvement of macrophages and dendritic cells in synovial inflammation of collagen-induced arthritis in DBA/1 mice and spontaneous arthritis in MRL/lpr mice.

Rordorf-Adam et al. (1985) used serum amyloid P component and autoimmune parameters in the assessment of arthritis in MRL/lpr/lpr mice.

Furukawa et al. (1996) studied the autoimmune disease-prone genetic background in relation to Fas defect in MRL/lpr mice.

Kanno et al. (1992) found spontaneous development of pancreatitis in the MRL/Mp strain of mice.

Kusakari et al. (1992) compared hearing acuity and inner ear disorders of MRL/lpr mice with those of BALB/c mice and found a significantly higher auditory brain stem response threshold. They recommended this as a model of sensorineural hearing loss.

Bundick and Eady (1992) investigated the effects of an immunosuppressive agent on the development of spontaneous lupus disease in female NZBW F1-hybrid mice.

Walker et al. (1996) reported a powerful suppressive effect of testosterone on the autoimmune disease analogous to systemic lupus erythematodes spontaneously developed by F1-hybrids of New Zealand Black (NZB) × New Zealand White (NZW) mice. A model was developed in which NZB dams carrying NZB/NZW fetuses were treated with testosterone in a dose adequate to masculinize the external genitalia in female fetuses.

Zoja et al. (1998) investigated bindarit, a compound devoid of immunosuppressive properties, in NZB/W F1 hybrid mice developing an immune complex glomerulonephritis with proteinuria and progression to renal insufficiency.

Kiberd and Stadnyk (1995) studied the role of endogenous interleukin-1 in established lupus nephritis in MRL-lpr/lpr mice by administration of the IL-1 receptor antagonist IL-1ra.

Gleichmann et al. (1982) and Schorlemmer et al. (1997) induced a systemic lupus erythematodes-like disease in mice by abnormal T and B cell cooperation. A chronic graft-versus-host reaction with the pathologic symptoms of severe glomerulonephritis is induced in B6D2 (C5Bl/6 × DBA/2) F1 hybrid mice receiving four i.v. injections (one per week) of 1×10^8 parental lymphoid spleen cells from DBA/2 donors. The inoculation of splenocytes into the BDF1 hybrid mice results in the development of a chronic GvH reaction with lymphoid hyperplasia, autoantibody production, and immune complex glomerulonephritis.

Chan et al. (1995) described ocular changes occurring in mice with experimental lupus erythematodes. The ocular disease is characterized by bilateral subacute and chronic inflammation of the eyelids (blepharitis) and hypertrophic meibomian glands. The severity of the ocular changes is strain dependent. The authors recommend this experimental eye disease as an animal model for chronic blepharitis in humans.

The changes of lacrimal and salivary glands found in MRL/lpr mice and other mouse strains with autoimmune disorders were also regarded as model of Sjögren's syndrome in human (Sullivan and Edwards 1997; Toda et al. 1999).

The MRL-lpr mouse model has been used to provide cognitive dysfunction in neuropsychiatric systemic lupus erythematosus (Jeltsch-David and Muller 2014), and peptide microarray technology has been developed which may facilitate diagnosis and early detection of CNS-SLE (Williams et al. 2014).

Several studies have investigated the effects of T cell modulation in the MRL/lpr model (Richard et al. 2013; Shinsuke and Hiroshi 2013), and the role of peptidylarginine deiminase and NET formation has been investigated in the MRL/lpr model (Knight et al. 2014).

An assessment of the value of murine lupus models for translation of findings into the clinic (Bender et al. 2014) has highlighted the individuals' strengths of the various models available.

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Prevention of Experimentally Induced Myasthenia Gravis in Rats

Purpose and Rationale

Myasthenia gravis is an organ-specific autoimmune disease in man that results in skeletal muscles' weakness. Typically, the sufferer has drooping eyelids, a blank facial expression, and weak, hesitant speech. This is due to the formation of autoantibodies against the nicotinic acetylcholine receptor (AChR). The formation of autoantibodies to acetylcholine's receptor leads to a gradual destruction of the receptors in skeletal muscles that receive nerve impulses and initiate muscle contractions. As a result, affected muscles fail to respond or react only weakly to nerve signals.

Experimental myasthenia gravis (EMG) can be induced in rats by injecting them with heterologous AChR or with recombinant α-subunits (two) of the AChR (portion of the AChR to which acetylcholine mainly binds) (Lennon et al. 1991), and the utility of clinical trials to guide the use of animal models has been recently addressed (Punga et al. 2015). The animals display symptoms of myasthenia (electrophysiological evidence of altered neuromuscular function) and detectable antireceptor antibodies. The severity of the disease can vary, but most animals display, at the very least, a weakness and fatigability of foot grip. The disease gradually leads to abnormal gait and eventually the inability of the animals to walk or even right themselves.

Procedure

Female rats of AO strain, 6–10 weeks old, are used. Three groups of rats are included in the experiment:

1. Immunized with acetylcholine receptor (AChR) protein and treated with test drug.
2. Immunized with AChR protein without drug.
3. Nonimmunized, non-treated control rats. The test drug is applied per os daily. First dose is administered on the day of immunization and the last on the day of sacrifice.

Immunization with AChR Protein

AChR protein isolated from *Torpedo marmorata* is emulsified with complete Freund's adjuvant, and 100 μg/rat is injected intradermally in the hind foot pad. As additional adjuvant, 2.6×10^{10} *Bordetella pertussis* microorganism is administered simultaneously by intramuscular injection in the hind leg.

Antibody Determination

Anti-AChR-protein antibodies are measured by enzyme-linked immunosorbent assay (ELISA) as described by Norcross et al. (1980). AChR protein is diluted to a final concentration of 2.5 μg/ml in 0.05 M carbonate buffer, pH 9.6. Two hundred ml of this solution is placed in each well of a microtitration plate (Flow Laboratories Inc.). After an overnight incubation at 4 °C, the plates are washed thoroughly with 0.01 M phosphate-buffered saline (PBS) solution containing 0.05 % Tween 20 (Sigma) subsequently referred to as PBS/T. Sera from all groups of rats are serially diluted in PBS/T, and 200 μl is added to each micron well except in the background row (control row) and incubated at 4 °C for 2 h. After washing, 200 μl of 1:1,000 diluted peroxidase-conjugated goat anti-rat immunoglobulin (Sera Lab. Sussex, England) in PBS/T is added to the micron wells and incubated for an additional 60 min at 4 °C. After plates are washed, 200 μl of substrate-citrate buffer and 0.2 μl of 10 % H_2O_2 are added and then incubated in the dark at room temperature for 30 min. The reaction is stopped by addition of 50 μl of 2 M H_2SO_4 and the OD determined by using Titert Multiscan.

Two-Color Flow Cytometry

Thymic cell suspensions are obtained by mincing tissue and passing it through 80-mm stainless mesh. After being washed three times in PBS, the cells are resuspended in PBS at a cell density of 10^7 viable cells/ml. The cell viability is determined by the trypan blue exclusion test. Erythrocytes are removed by addition of ammonium chloride. Cell staining and flow cytometric analyses are done as described by Itoyama et al. (1989). Thymocyte subsets expressing CD4 and/or CD8 molecules are defined by staining with monoclonal antibodies obtained from Serotec, Oxford, England: phycoerythrin (PE)-conjugated anti-W3/25 (CD4) and fluorescein isothiocyanate (FITC)-conjugated anti-MRC OX8 (CD8). Two $\times 10^5$–1×10^6 cells suspended in 100 ml of PBS are exposed sequentially for 30 min to FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 monoclonal antibodies. Isotype-matched control monoclonal antibodies are used to prove the specificity of binding. Cell analysis is performed using FACScan flow cytometer from Becton Dickinson. One $\times 10^4$ events per sample are analyzed by Consort 30 and Lysis software. All data are collected and displayed on a log scale of increasing green and orange fluorescence intensity. This is presented as two-dimensional contour maps and as percentage of thymocytes by integrating counts in selected areas of the contour plots.

Stereologic Analysis of Thymuses

Thymuses of animals of all groups are prepared for light microscopic analysis. For this purpose, thymus tissue is fixed in Carnoy's solution, embedded in paraffin, and 3–5-μm-thin sections are stained with hematoxylin and eosin. Cortex and medulla are analyzed stereologically using the point counting method described by Weible (1963). Volume density (*Vv*) of the examined structures is determined by the following equation: $Vv = Pi/Pt$, where *Pi* represents the number of points of the examined structure and *Pt* the total number of points. *Vv* refers to the volume fraction, i.e., volume of a feature per unit test volume (Tascaland Vaughn-Williams 1981).

Evaluation

EMG is evaluated clinically by daily examination of muscle weakness and scored as follows:

+ = weakness of grip with fatigability
++ = abnormality of gait
+++ = inability to walking and righting

Immediately after appearance of clinical signs of EMG, rats are sacrificed, and blood and thymuses are taken for determination of anti-AChR-

protein antibodies and histological analysis of thymuses and thymocyte subsets, respectively.

Statistical analysis of data is performed by Student's *t*-test (data of stereological analysis) and Mann–Whitney *U*-test (results of flow cytometric analysis of thymocyte subsets).

Modifications of the Method

McIntosh and Drachman (1987) described an in vitro suppressor assay using responder cells from the lymph nodes of Lewis rats immunized sc. with acetylcholine receptors emulsified in complete Freund's adjuvant and suppressor cells from spleens of rats immunized i.p. with acetylcholine receptors absorbed on bentonite. Antibodies were determined after stimulation with acetylcholine receptors from cocultures of responder cells and putative suppressor cells treated previously with an immunosuppressant.

Arag and Blalock (1994) developed a method of altering B cell-mediated autoimmune diseases by induction of anti-idiotypic antibodies by immunization with complementary peptides. A peptide encoded by RNA complementary to RNA for the Torpedo acetylcholine receptor main immunogenic region, AChR 67–16, was tested in the Lewis rat model of experimental autoimmune myasthenia gravis.

Russell et al. (2012) reported on the testing of CK-2017357 (Tirasemtiv) in rat model of myasthenia gravis and showed as a troponin activator it improved muscle function in this model.

Oliveira et al. (2015) describe the role of CD73 in impaired neuromuscular transmission in the EMG model and further describe the potential role of adenosine in the pathophysiology on this neuromuscular disorder.

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Glomerulonephritis Induced by Antibasement Membrane Antibody in Rats

Purpose and Rationale

Masugi nephritis and other nephritis models of immunological origin in rats have been used for evaluation of immunosuppressive activity (Heymann et al. 1959; Shibata et al. 1966; Ito et al. 1983; Thoenes et al. 1989; Ogawa et al. 1990, 1991).

Procedure

Preparation of Rabbit Antiserum Against Rat Glomerular Basement Membrane

Glomeruli are separated from the homogenate of rat renal cortex by successive use of three metal sieves (150-, 180-, and 200-mesh). The basement membrane fraction is obtained by centrifugation and ultrasonic disruption. It is then digested with trypsin, dialyzed, and lyophilized. The resultant substance is employed as antigen. An emulsion of 1 mg of the antigen in 0.2 ml saline with 0.2 ml of complete Freund's adjuvant is injected intracutaneously into white rabbits once a week for 6 weeks. One week later, production of the antibasement membrane antibody is confirmed in guinea pigs by the passive cutaneous anaphylaxis test. The blood is collected from the carotid artery, incubated at 56 °C for 30 min to inactivate components of the complement and stored at −20 °C until use.

Induction of Glomerulonephritis in Rats

Male Sprague–Dawley rats weighing about 300 g are injected with 0.5 ml of the rabbit antiserum via the tail vein. On the following day, they are further injected subcutaneously with an emulsion (0.25 ml) of physiological saline solution containing 5 mg of rabbit gamma globulin in an identical volume of complete Freund's adjuvant.

Treatment

The rat antibasement antibody is injected 5 days before the start of administration of the test compound. Before the first dose, urinary total protein is determined and rats with nephritis are so assigned as to provide almost equal distribution of severity of the disease per group. The test compounds are administered orally for 14 days. The urine is collected at 7 and 14 days of treatment. After 14 days, the animals are sacrificed, blood is collected, and the thymus and kidneys are removed. Histopathological and immunohistochemical studies are performed in kidney tissue.

Evaluation

Scores are given for **microscopic findings in the following:**

Glomeruli

- Cell proliferation in glomeruli
- PAS-positive granules in the epithelium of glomeruli
- Fibrin deposits in Bowman's space
- Adhesion to Bowman's capsule

Tubuli
- Hyaline cast
- Dilation of tubuli

Scores are also given for **immunofluorescence findings** for rat IgG, rat C3, and rabbit IgG.

Furthermore, total urinary protein, plasma total cholesterol, plasma fibrinogen, and thymus/body weight ratio are compared between drug-treated animals and controls by statistical means.

Modifications of the Method

Lan et al. (1995) investigated the pathogenic role of interleukin-1 in the progression of established rat crescentic glomerulonephritis by administration of the interleukin-1 receptor antagonist IL-1ra.

Giménez et al. (1987) and Thoenes et al. (1987) induced autoimmune tubulointerstitial nephritis in the Brown Norway rat by injection of bovine tubular basement membrane.

Development of a systemic T lymphocyte-dependent autoimmune syndrome in Brown Norway rats including glomerulonephritis with high proteinuria was induced with mercuric chloride by Baran et al. (1986), Aten et al. (1988), and Lillevang et al. (1992).

Kokui et al. (1992) induced nephrosis with proteinuria in rats by intraperitoneal injection of puromycin aminonucleoside.

Lundstrom et al. (1993) studied the Heymann nephritis antigenic complex using a rat yolk sac carcinoma cell line that expresses glycoprotein 330, the main antigen in this autoimmune disease.

Taylor et al. (2009) demonstrated a role for the purinergic P2X7 purinoreceptor in experimental glomerulonephritis showing that mice harboring a knockout for the receptor were renoprotective, further supported by a nonclinical intervention study with A-439079. Smith et al. (2010) investigated the role of spleen tyrosine kinase (SYK) in a rat model of glomerulonephritis with R788 (fostamatinib) and showed reduction of glomerular crescents and improvement in renal function establishing SYK as a target for potential future clinical investigation.

Suana et al. (2011) have shown that immunoliposomes carrying a low-dose mycophenolate mofetil cargo may prevent creatine increase and albuminuria in a model of experimental mesangial proliferative glomerulonephritis model in the rat.

D'Souza et al. (2013) developed a bicongenic rat model of experimental crescent glomerulonephritis to develop a system for investigating macrophage-dependent glomerulonephritis.

Recently Takakura et al. (2014) demonstrate an antiproliferative effect of the anti-inflammatory and antifibrotic agent pirfenidone in a rat model of basement membrane glomerulonephritis.

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Inhibition of Allogenic Transplant Rejection

Purpose and Rationale

Transplantation of allogenic organs to recipients results in rejection of the transplants (Sanchez-Fueyo and Strom 2011). This effect can be suppressed or delayed by immunosuppressive agents, and the role of B cells has been investigated in animal models suggesting a role in mechanisms of transplant tolerance (Chesneau et al. 2013). Various organs are used for allogenic transplantation in animal experiments, such as skin pieces (Schorlemmer et al. 1993), kidney (Lee 1967; Küchle et al. 1991), rat heart, rat small intestine (Xiao et al. 1994; Zhang et al. 2014), and corneal buttons (Coupland et al. 1994). The immunosuppressive activity can be evaluated either by using a major histocompatibility complex variant strain combination or a strong allogenic system, and the advances and limitations of murine models have been recently described (Schroeder and DiPersio 2011).

Procedure

For **skin transplantation** male animals of inbred strains of Fischer (F334), Lewis (LEW), Brown Norway (BN), and Dark Agouti (DA) rats are used. Rat tail skin (donor) is cut into square pieces of 0.5–1.0 cm and transplanted to the tails of recipient rats. Rejection is defined as the day when the skin graft is of red-brown color and hard consistency. As strain combination with a major histocompatibility variant, transplantation from LEW to F334 is performed. Using a strong allogenic system, the high responder DA to LEW donor-recipient combination is used. The immunosuppressive agents, e.g., cyclosporine or leflunomide, are given orally up to 20 days. Ten animals are used for each group.

Evaluation

The mean values of rejection time of treated groups are compared statistically with vehicle-treated controls using Student's *t*-test or the Mann–Whitney *U*-test.

Modifications of the Method

Schorlemmer and Kurrle (1997) used Lewis (LEW, Rtl*l) rats as receivers and Balb/c mice as donors in a xenotransplantation model of mouse-to-rat skin grafts. Rejection was defined as the day when the skin graft turned red-brown and became hard. For quantification of xenospecific IgM and IgG antibody titers, the test sera (dilution 1:10) were incubated with 1×10^6 purified T cells (by sheep anti-mouse Dynabeads, Deutsche Dynal GmbH, Hamburg, Germany) from Balb/c donor spleens for 30 min at 4 °C. The cells were washed three times with phosphate-buffered saline (pH 7.2) and then stained for IgG or IgM xenoantibodies; 50 µl of FITC-conjugated goat antibodies, specific for the Fc-portion of rat IgG or specific for the µ-chain of rat IgM, was added. After 30 min at 4 °C, the cells were washed twice and analyzed by flow cytometry.

Techniques for transplantation of several organs have been elaborated.

For **kidney transplantation**, male rats, 5–7 months of age, are used as donors and recipients for the orthotopic right kidney transplantation as described by Lee (1967) with a modification of ureter–ureter anastomosis (Thoenes et al. 1974). Because bilateral nephrectomy is performed at transplantation, animal survival is dependent upon the allograft's function. All rats that do not excrete urine on the first postoperative day are excluded from further studies. As a control concerning long survival, syngenically transplanted rats are maintained up to 300 days.

Engelbrecht et al. (1992) described a new rapid technique for renal transplantation in the rat. The method combines a special sleeve anastomotic

technique for the renal artery, conventional end-to-end anastomosis of the renal vein, and implantation of the ureter into the bladder.

A porcine renal transplant model has been used by Almond et al. (1992).

Peters et al. (1993) reviewed the therapeutic potential of tacrolimus in renal and hepatic transplantation.

For studying **heart transplantation**, heterotopic implantation of hearts from BN to LEW rats is performed (Williams et al. 1993). The diagnosis of rejection is established once the palpable cardiac allograft impulse ceases. Further studies with rat cardiac allografts have been performed by Hancock et al. (1990). The Fischer 344 rat (donor)/Long Evans rat (recipient) combination was used by Kahn et al. (1991). Walpoth et al. (1993) used magnetic resonance spectroscopy for assessing myocardial rejection in the transplanted rat heart.

Shiraishi et al. (1995) evaluated the effectiveness of the interleukin-1 receptor antagonist IL-1ra in the immune and inflammatory responses to rat heart allografts.

Cardiac transplantation between inbred rat strains that differ for weak histocompatibility antigens is associated with the development of arteriosclerosis in arteries of the donor graft myocardium (Cramer et al. 1990; Adams et al. 1992).

A heterotopic rat **heart transplant model** and the influence of infection were described by Kobayashi et al. (1993).

The **hamster to rat cardiac xenograft** model has been used by several authors (de Masi et al. 1990; Steinbrüchel et al. 1991; van den Bogaerde et al. 1991; Woo et al. 1993; Fujino et al. 1994; Schuurman et al. 1994). The hearts from Syrian hamsters were implanted heterotopically in male Lewis rats, with anastomoses between the infrarenal abdominal aorta and inferior vena cava of the recipient and the donor aorta and right pulmonary artery, respectively.

Primate cardiac xenografts were performed by McManus et al. (1993) using cynomolgus monkeys (*Macaca fascicularis*) as donors and baboons (*Papio anubis*) as recipients.

Chronic rejection of rat **aortic allograft** was studied by Mennander et al. (1991). Administration of cyclosporine induced accelerated allograft arteriosclerosis.

Heterotopic transplantation of small intestine has been performed from BN to LEW rats. The mesenteric venous drainage is reconstructed either via the vena cava or the portal vein (Xiao et al. 1994). An isolated Thiry–Vella loop was prepared by Xia and Kirkman (1990). Kellnar et al. (1990) described allogenic transplantation of fetal rat intestine with anastomosis to the normal bowel of the host. Langrehr et al. (1991) investigated under which circumstances graft-versus-host disease occurs following fully allogenic small bowel transplantation in the rat. Kirsch et al. (1991) studied the extent to which intestinal transplants in rats undergo functional and morphologic compensation.

Liver transplantation procedure has been described by Svensson et al. (1995), allowing measurement of bile secretion.

Orthotopic left **lung transplantation** was performed in inbred rats by Katayama et al. (1991).

Tracheal allografts were implanted into the abdomen of recipient rats (Davreux et al. 1993).

In vivo electrophysiology of rat **peripheral nerve transplants** was studied by Yu et al. (1990). A sciatic-tibial nerve graft was harvested from the donor rat between the sciatic notch and the ankle. In the recipient, the tibial nerve and the sural nerve were resected. The nerve graft was placed along the natural course of the native tibial nerve. Nerve repair was performed using standard end-to-end epineural microsuture technique.

A model of neurovascularized rectus femoris **muscle transplantation** in rats was established by Muramatsu et al. (1994).

The orthotopic **transplantation of vascularized skeletal allografts** (rat distal femur and surrounding muscular cuff) has been described by Lee et al. (1995).

Long-term survival of **limb allografts** in rats was studied by Kuroki et al. (1991). The donor and recipient limbs were prepared simultaneously by amputation at mid-femur. The donor limb was

fixed orthotopically by Kirschner wire. The donor and recipient femoral arteries, veins, and sciatic nerves were anastomosed using a microsurgical technique.

For **cornea transplantation**, Brown Norway rats ($RT1^{1\times n}$) serve as donors and Lewis rats ($RT1^{1}$) as recipients (Coupland et al. 1994). Both the donor and recipient rats are anesthetized with xylazine hydrochloride and ketamine hydrochloride. Twenty min prior to surgery, the recipient rats also receive 0.5 mg/kg atropine sc. and phenylephrine hydrochloride 5 % eyedrops. Under sterile conditions and using an operation microscope, two donor corneal buttons (3.5 mm) are harvested from the donor rat using a trephine and curved Castroviejo scissors. The donor animals are then sacrificed by ether inhalation. The left eyes of the recipient rats are prepared by removing a central 3.0-mm button using a trephine and curved Castroviejo scissors. A drop of sterile methylcellulose (1 %) is placed over the 3.0-mm corneal opening before the donor cornea is fixed with 10 interrupted sutures. The anterior chamber is not reestablished following surgery. Prior to closure of the eyelids with three or four interrupted sutures, Polyspectran eyelid gel is placed over the operated eye. Forty-eight hours following surgery, the eyelid sutures are removed, allowing for the first time assessment of the cornea on the slit-lamp microscope. Slit-lamp evaluations are performed every 2–3 days under i.m. anesthesia with ketamine, with assessment of the cornea by scoring graft opacity, edema, and vascularization.

Recently the role of indoleamine 2,3-dioxygenase as an immunomodulator has been reviewed in models of allogenic pancreatic islet and skin transplantation (Gill et al. 2013).

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PFC (Plaque Forming Colony) Test In Vitro

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Acute Graft Versus Host Disease (GVHD) in Rats

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Glomerulonephritis Induced by Antibasement Membrane Antibody in Rats

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Part X

Anti-Artheroslerotic Activity

Induction of Experimental Atherosclerosis

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Contents

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General Considerations

Experimental atherosclerosis was first successfully induced in rabbits by Saltykow (1908) and Ignatowski (1909). During the following years, various scientists found that dietary cholesterol was the responsible stimulus for the development of atherosclerosis. Other species are also susceptible to diet-induced atherosclerosis (Reviews by Kritchevsky 1964; Hadjiinky et al. 1991). Mouse models of atherosclerosis were reviewed by Reardon and Getz (2001), by Daugherty (2002), and by Maganto-Garcia et al. (2012). The responses of mouse models of atherosclerosis to different drugs have been summarized by Zadelaar et al. (2007). Getz and Reardon (2012) have recently compared different animal models of atherosclerosis.

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Cholesterol-Diet-Induced Atherosclerosis in Rabbits and Other Species

Purpose and Rationale

Rabbits are known to be susceptible to hypercholesterolemia and arteriosclerosis after excessive cholesterol feeding. Therefore, this approach has been chosen by many authors to study the effect of potential anti-arteriosclerotic drugs.

Procedure

Several modifications of the protocol have been described. Usually, male rabbits from an inbred strain, e.g., white New Zealand, at an age of 8–10 weeks are used. Body weight variation should be as low as possible. At the beginning of the experiment, blood is withdrawn from the marginal ear vein for the determination of total cholesterol, total glycerides, and blood sugar. Groups of 10 animals are used for treatment with drugs or as controls. The rabbits are switched from commercial food to a diet supplemented with 0.3–2 % cholesterol and kept on this regimen for a period of 10–12 weeks. One group is kept on normal diet. During and at the end of the experiment, blood is taken for analysis. Usually, cholesterol and triglyceride levels increase severalfold over the original values.

The animals are sacrificed and the thoracic aorta is removed, cleaned of surrounding tissues, and longitudinally cut and opened for fixation with formaldehyde. The tissue is stained with oil red. The percentage of the intimal surface covered by the oil red positive lesions is calculated with a computerized planimeter. In animals fed a normal diet, the aorta does not show any staining, whereas in cholesterol-fed rabbits, the aorta shows severe atherogenic lesions.

Evaluation

Data are expressed as means ± standard deviation. Statistical evaluation is performed by Dunnett's or Scheffé's test. A p-value of <0.05 is regarded as statistically significant.

Modifications of the Method

Shore and Shore (1976) studied two **different strains of rabbits** (New Zealand White and Dutch Belt) as models of hyperlipoproteinemia and atherosclerosis.

Tao et al. (2003) studied antioxidative, antinitrative, and vasculoprotective effects of a PPAR-γ agonist in New Zealand White rabbits with hypercholesterolemia induced by a high-cholesterol diet.

Studies of Kritchevsky et al. (1989) on experimental atherosclerosis in **rabbits** fed **cholesterol-free diets** revealed a greater influence of animal protein and of partially hydrogenated soybean oil on development of atherosclerosis than plant protein and unsaturated soybean oil.

Cockerels (Tennent et al. 1960) and **turkeys** (Simpson and Harms 1969) are very susceptible to cholesterol feeding and develop marked hypercholesterolemia in rather short periods.

Atherosclerosis could also be induced in cockerels by high doses of estrogen without atherogenic diet (Caldwell and Suydam 1959).

Spontaneous arteriosclerosis in **pigeons** has been described by Clarkson and Lofland (1961).

The **Japanese sea quail** (*Coturnix coturnix japonica*) is highly susceptible to the rapid development of severe experimental atherosclerosis (Day and Stafford 1975; Day et al. 1977, 1979; Day 1990; Chapman et al. 1976).

Out of 13 strains of **mice**, Roberts and Thompson (1976) selected the C57BR/cdJ and the CBA/J strain and used these strains and their hybrids as models for atherosclerosis research.

Paigen et al. (1987) described quantitative assessment of atherosclerotic lesions in mice. After 14 weeks on an atherogenic diet, C57BL/6 J female mice had aortic lesions at each of the coronary arteries, at the junction of the aorta to the heart, and in scattered areas of the aortic surface. The lesions increased after 9 months of atherogenic diet. Methods of evaluating the number and size of lesions were compared including sizing with a microscope eyepiece grid and computer-assisted planimetry.

Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice were described by Paigen et al. (1990).

Yamaguchi et al. (1993) found that addition of 10 % linoleic acid to a high-cholesterol diet enhanced cholesterol deposition in the aorta of male ICR strain mice.

In **rats** hypercholesterolemia can be induced by daily administration by gavage of 1 ml/100 g body weight of a cocktail containing in 1 l peanut oil: 100 g cholesterol, 30 g propylthiouracil, and 100 g cholic acid over a period of 7 days. The test compounds are administered simultaneously with the cocktail (Fillios et al. 1956; Lustalot et al. 1961).

Inoue et al. (1990) induced experimental atherosclerosis in the rat carotid artery by balloon de-endothelialization and atherogenic diet. A balloon catheter was introduced into the rat's carotid arteries from the iliac arteries, and the endothelium was denuded.

The **hamster** is susceptible to atherosclerosis. Nistor et al. (1987) fed male hamsters a hyperlipidemic diet consisting of standard chow supplemented with 3 % cholesterol and 15 % commercial butter for 12 months. Serum total cholesterol doubled after 3 weeks and attained a 17-fold value after 10 months. Up to 6 months, smooth muscle cells in the intima and media of the aorta as well as endothelial cells began to load with lipids. After 10 months, the affected zones looked like human atherosclerotic plaque with huge cholesterol crystal deposits, calcium deposits, and necrosis.

Especially the hybrid hamster strain Bioä F_1B (Bio Breeders Fitchburg, MA, USA) is more susceptible to dietary-induced atherosclerosis than other strains (Kowala et al. 1991). Early atherosclerotic lesions can be induced within a 3-month feeding of a cholesterol/butter-enriched diet. In these animals, simvastatin dose dependently inhibited the development of hyperlipidemia and the plaque formation by cholesterol synthesis inhibition. The histopathological examination of the aortas showed that the cholesterol/butter-fed F_1B hamster developed atherosclerotic lesions and functional changes in the aorta which are closely related to man (Schäfer et al. 1999).

Soret et al. (1976) studied the diet-induced hypercholesterolemia in the diabetic and nondiabetic Chinese hamster.

Beitz and Mest (1991) used cholesterol-fed **guinea pigs** to study the antihyperlipidemic effects of a potentially anti-atherosclerotic drug. Fernandez (2001) reviewed the advantages of the guinea pig as an animal model to study hepatic cholesterol, lipoprotein metabolism, and early atherosclerosis. Guinea pigs develop fatty streaks after 12 weeks on a cholesterol diet; the response to cholesterol is highly individualized, and animals can be a hyper-responder or a hyporesponder to dietary cholesterol.

During the last 30 years, **minipigs** have become a popular animal model for testing lipid-lowering drugs and their anti-atherosclerotic properties (Jacobsson 1987; Huff et al. 2002; Telford et al. 2003). Jacobsson and Lundholm investigated the lipid-lowering and anti-atherosclerotic effects of clofibrate, nicotinic acid, and other drugs (Lundholm 1978; Jacobsson and Lundholm 1982; Jacobsson 1987). Huff and Telford investigated the effect of atorvastatin and a bile acid absorption inhibitor on lipid metabolism and serum LDL

kinetics (Huff et al. 2002; Telford et al. 2003). The benefits of the minipig are the absence of rodent-specific peroxisomal proliferation by PPAR-α agonists and closer homology to humans in absolute and relative organ sizes. The effective doses of the tested drugs in the minipig were close to clinically effective doses in humans.

Malinow et al. (1976) recommended the **cynomolgus monkey** as a model for therapeutic intervention on established coronary atherosclerosis.

This species was used by Hollander et al. (1978) to study the development of atherosclerosis after a cholesterol- and fat-enriched diet.

Beere et al. (1992) described experimental atherosclerosis at the carotid bifurcation of the cynomolgus monkey by a cholesterol-enriched diet.

Eggen et al. (1991) studied the progression and the regression of diet-induced atherosclerotic lesions in aorta and coronary arteries on **rhesus monkeys**.

Howard (1976) recommended the **baboon** as model in atherosclerosis research because of the similarity of cholesterol metabolism and composition of the lipoproteins to man.

Kushwaha et al. (1991) determined the effect of estrogen and progesterone on plasma cholesterol concentrations and on arterial lesions in ovariectomized and hysterectomized baboons fed a high-cholesterol/high-saturated-fat diet.

Blaton an Peeters (1976) reported studies on the **chimpanzee**, the **baboon**, and the **rhesus macaque** as models for atherosclerosis.

The use of normal adult **marmosets**, a species with a lipoprotein profile similar to that of humans, may be an alternative (Crook et al. 1990; Baxter et al. 1992).

Ming-Peng et al. (1990) studied high-density lipoproteins and prevention of experimental atherosclerosis in **tree shrews** (*Tupaia belangeri yunalis*). In contrast to rabbits, no increased lipid deposition in aortic intima after cholesterol feeding was found in tree shrews.

Critical Assessment of the Method

Diet-induced hypercholesterolemia is useful for the detection of agents that interfere with the adsorption, degradation, serum clearance, and excretion of cholesterol to receive a broader effect range. It must be considered whether the effects of agents that interfere with cholesterol absorption will be enforced and therefore whether the efficacy is overestimated in those models.

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Hereditary Hypercholesterolemia in Rats

A strain of genetically hypercholesterolemic rats (RICO) was described by Müller et al. (1979). In contrast to Zucker rats, these animals are normotriglyceridemic and non-obese. The hypercholesterolemia of the RICO rat is related to a decreased rate of catabolism of chylomicrons and LDL but more specifically to an excessive production of these two types of lipoproteins. This strain has been proposed to study hypolipidemic drugs, particularly those designed to decrease the plasma concentrations of chylomicrons and LDL. Hypolipidemic effects of β-cyclodextrin were found in this model (Riottot et al. 1993).

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Hereditary Hyperlipidemia in Rabbits

Watanabe et al. (1977; Watanabe 1980) described a strain of rabbits with hereditary hyperlipidemia (WHHL rabbit) which has been used by several scientists to study development of atherosclerosis, as well as for histological and functional changes of the aorta. At the age of 10–14 months, homozygous animals exhibit an atheromatous plaque, distributed heterogeneously over the luminal surface of the aorta. Serum cholesterol is increased up to 400–600 mg/dl. The increased levels of LDL have been studied in detail (Kita et al. 1981, 1982).

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Studies in Genetically Modified Mice

Mice do not develop atherosclerotic lesions spontaneously. Paigen et al. (1985) fed ten inbred strains of mice an atherogenic diet containing 1.25 % cholesterol, 0.5 % cholic acid, and 15 % fat in order to study the variation in susceptibility to atherosclerosis among these strains. The strains were examined for plasma cholesterol and triglyceride levels and for formation of lipid-containing lesions in the aortic wall. The most susceptible strain was C57BL/6 J mice.

Several parameters which influence the development of atherosclerosis differ between mice and humans. The major lipoprotein in wild-type mice is HDL compared to LDL in humans, and mice do not have the cholesteryl ester transfer protein (CETP) which may be a target for antiatherogenic therapies in humans (Rader and deGoma 2014). These and other differences, including lack of atherosclerosis in murine coronary arteries and much faster atherosclerosis development in mice, have led to discussions, as to whether the mouse is an appropriate model to study the human disease atherosclerosis (Bentzon and Falk 2010). However, many of the processes underlying the development and progression of atherosclerotic lesions are shared by mice and humans. Since mice can be maintained and bred at relatively low costs, since atherosclerosis can be monitored within a reasonable time frame, and since mice can be genetically modified, they are currently the most frequently used species to study atherosclerosis. Several genetic mouse models of atherosclerosis have been created during the last two decades (Stoltzfus and Rubin 1993; Daugherty 2002).

The most widely used model is the ApoE knockout mouse originally created by Nubuyo Maeda, University of North Carolina, Chapel Hill, NC. ApoE-deficient mice have spontaneously elevated plasma cholesterol levels and develop atherosclerosis even on regular chow within 3–4 months (Plump et al. 1992; Zhang et al. 1992). The time-dependent progression of atherosclerosis leads to lesions of all phases of atherosclerosis similar in histopathology to those

observed in humans (Nakashima et al. 1994). This animal model is used as background for atherosclerosis research and target validation.

A disadvantage of the ApoE-deficient mice as a model for atherosclerosis is the fact that ApoE has several other functions unrelated to the metabolism of lipids, e.g., in the immune system (Getz and Reardon 2009), and transplantation of $ApoE^{-/-}$ bone marrow onto wild-type mice has been shown to lead to increased atherosclerotic lesion size independently of any effect on the plasma lipid levels (Fazio et al. 1997; Van Eck et al. 2000). This needs to be considered whenever atherosclerosis is studied in bone marrow chimeras based on the ApoE-deficient mouse model (Linton and Fazio 1999).

Fu et al. (2003) reported that the PPAR-α agonist ciprofibrate severely aggravates hypercholesterolemia and accelerates the development of atherosclerosis in mice lacking ApoE. Wang (2005) described the cardiovascular functional phenotype of ApoE knockout mice and reported that simvastatin paradoxically increased serum lipids and atherosclerosis, but decreased serum lipids and atherosclerosis in LDL-receptor knockout mice. Meier and Leitersdorf (2004) reviewed nutritional, pharmacologic, and genetic atherosclerosis intervention studies in ApoE knockout mice. Several known beneficial nutritional interventions are effective, as well as ACE inhibitors, Ang II receptor blockers, the cholesterol absorption inhibitor ezetimibe, and PPAR-γ agonists. Meier pointed out that the model helps the understanding that atherosclerosis is a form of chronic inflammation and that, despite being imperfect, the ApoE knockout mouse is currently the most popular mouse model used by researchers worldwide.

Overexpression of apolipoprotein E in transgenic mice reduced plasma cholesterol and triglyceride levels, prevented hypercholesterolemia, and inhibited the formation of fatty streak lesions (Harada et al. 1996).

An alternative to the $ApoE^{-/-}$ mouse model is LDL-receptor-deficient mice (Ishibashi et al. 1994). On normal chow, LDL-receptor-deficient mice developed hardly any lesions, and atherosclerotic lesion development requires feeding an atherogenic diet. Since the function of the LDL receptor is obviously restricted to lipoprotein metabolism, wild-type bone marrow transplanted onto LDL-receptor-deficient mice does not affect atherosclerosis development and plasma lipid levels (Linton et al. 1999).

Li et al. (2000) used LDL-receptor-deficient mice to study the inhibition of development of atherosclerosis by PPAR-γ-specific agonists. Joseph et al. (2002) investigated the inhibition of development of atherosclerosis in *LDL-receptor* knockout mice and *ApoE* knockout mice by a synthetic LXR ligand. Terasaka et al. (2003) reported inhibition of development of atherosclerosis in LDL-receptor-deficient mice by a synthetic liver X receptor ligand.

Transgenic mice overexpressing the human dysfunctional apolipoprotein E variant APOE*3 Leiden develop hyperlipidemia and are highly susceptible to diet-induced atherosclerosis (Groot et al. 1996). In some cases, APOE*3 Leiden mice have been shown to better predict effects of antiatherogenic drugs in humans compared to other mouse models of atherosclerosis (Zadelaar et al. 2007).

A general limitation of the genetic mouse models of atherosclerosis is a lack of atherosclerosis in coronary arteries. This can in part be overcome by using more complex genetic models, such as mice lacking both ApoE and the scavenger receptor SR-BI (Braun et al. 2002) or both ApoE and the LDL receptor (Caligiuri et al. 1999).

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Evaluation of Endothelial Function in Rabbits with Atherosclerosis

Purpose and Rationale

Cholesterol feeding of rabbits impairs the endothelium-dependent relaxation evoked by acetylcholine in the aorta. This phenomenon can be used to study the influence of vasodilators as well as the prevention by ACE inhibitors (Becker et al. 1991).

Procedure

Male New Zealand White rabbits weighing 3–4 kg receive a hypercholesterolemic diet containing 0.25–1 % cholesterol and 3 % coconut oil. Rabbits of the same weight receiving standard diet serve as controls. After several weeks, the serum cholesterol levels are increased from 30–40 mg/dl in the control group up to 900–1,000 mg/dl in the cholesterol-fed group. At the end of the treatment period, the animals are sacrificed by intravenous injection of sodium pentobarbital and a complete autopsy is performed.

Intact proximal parts of the thoracic aorta are sectioned into 2 mm wide rings, cut off to strips, and suspended at 2 g gauge in 25 ml organ chambers filled with a buffer solution of 37 °C comprising 113.8 mM NaCl, 20 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 5.5 mM glucose being gassed with a 95 % O_2/5 % CO_2 mixture to achieve a pH of 7.4. After 2 h, when a stable contractile tone is established, norepinephrine is added at a final concentration of 1×10^{-8} M, which produces a stable submaximum isotonic contraction. Then, acetylcholine is added in 10-fold incremental doses from 1×10^{-8} M up to final concentrations of 10^{-5} M. Relaxation of aortic strips is assessed as percentage decrease in contraction. Acetylcholine-induced concentration-dependent relaxation is greatly impaired in aorta rings from cholesterol-fed rabbits, whereas contractions to norepinephrine are only slightly diminished.

Evaluation

The data are expressed as mean ± SEM and compared by Student's *t*-test for unpaired data.

Modifications of the Method

Verbeuren et al. (1986, 1990) and Tagawa et al. (1991) used a bioassay for the determination of EDRF (Rubanyi et al. 1985). The donor thoracic aorta segments (3 cm long) are mounted horizontally in perfusion chambers filled with physiological salt solution which contains indomethacin (3×10^{-6} M) at 37 °C; the solution is gassed with a mixture of 20 % O_2, 5 % CO_2, and 75 % N_2. The aortas (control and atherosclerotic tissue) and a piece of glass tubing are perfused continuously with the same solution at 3 ml/min. The perfusate is dripping directly onto a segment (5 mm long) of either a control or an atherosclerotic abdominal aorta from which the endothelium was mechanically removed. The detector abdominal aortas are mounted vertically over two hooks and the development of isometric tension is monitored continuously. The initial tension of the detector aortas is set at 8 g. The perfusate of each donor aorta is analyzed both on a control and on an atherosclerotic detector preparation: the order of this double analysis is selected at random. A control and an atherosclerotic donor aorta are always analyzed in parallel and thus on the same detector

tissue. Drugs are added to the perfusion medium. Before the start of the experiments, the tissues are allowed to equilibrate for 45 min in the perfusion chambers. By means of a three-way system, placed at the outlet of the donor aortas, atropine (10^{-6} M) is added to the perfusate to block any muscarinic contractile effect of acetylcholine on the detector tissue. Noradrenaline (2×10^{-6} M) is infused into the perfusate via this 3-way system causing a sustained contraction in the detector tissue.

After the stabilization of the contraction to noradrenaline, the highest dose of acetylcholine (1.6×10^{-8} mol) is added to the solution for perfusion of the tubing in order to confirm no change in the contractile response to noradrenaline in the detector tissues. Moreover, the perfusate from the control and atherosclerotic donor aortas under basal conditions does not significantly affect the contraction of the detector tissues. The tissues are allowed to equilibrate, and then increasing doses of acetylcholine (0.01 to 160×10^{-10} mol) are injected into the perfusion medium close to the donor aortas. Relaxation is less pronounced in the detector tissues when atherosclerotic aortas are stimulated with acetylcholine indicating that the cholinergic agent causes a smaller release of EDRF from atherosclerotic donor aortas than from control donor aortas. The degree of the arteriosclerotic lesion can be assessed by this functional assay.

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Intimal Reactions After Endothelial Injury

Purpose and Rationale

Several attempts have been made to induce intimal injury in animals which is followed by proliferation and formation of fatty streaks similar to the alterations found in human atherosclerosis. One approach is the “balloon catheterization.”

Procedure

Male New Zealand White rabbits weighing 2.0–3.5 kg or male Sprague–Dawley rats weighing 350–400 g are used. An embolectomy catheter (Edwards Laboratories, size 4 French for

rabbits, size 2 French for rats) is introduced into the right femoral artery under surgical anesthesia and passed to the aortic arch. After inflation with room air, the catheter is withdrawn to the iliac bifurcation, deflated, and removed.

The incorporation of [^{3}H]thymidine into DNA of rabbit or rat aorta is measured 48 h after balloon catheterization. Animals are sacrificed 45 min after intravenous injection of [^{3}H]thymidine. Intima-media is prepared from whole aorta by scraping with blunt forceps. The specific activity of ^{3}H in DNA is determined after extraction of the DNA in the washed tissue homogenate using hot dilute perchloric acid. DNA is assayed by the diphenylamine method. ^{3}H incorporation is measured in a liquid scintillation counter.

For histological examination, rabbits are injected 2 weeks after balloon catheterization intravenously with heparin (500 U/kg) and then sacrificed. Fixative (2 % glutaraldehyde in 0.15 M phosphate buffer, pH 7.4) is infused at a constant pressure of 100 mmHg via a carotid cannula, and the aorta is pressure fixed in situ for 60 min. Intimal proliferation is quantified in the upper abdominal aorta, in the lower abdominal aorta, and halfway between these points.

Evaluation

[^{3}H]Thymidine incorporation and intimal proliferation is compared between drug-treated animals and controls.

Modifications of the Method

DeCampli et al. (1988) studied the effects of various drugs on accelerated myointimal proliferation in canine veno-arterial allografts by histological methods. An 8 cm length of femoral vein was removed, reversed, divided, and sewn end-to-end into carotid or femoral arteries of a recipient dog.

Berkenboom et al. (1989) induced experimental atherosclerosis in **canine** and **porcine** coronary arteries by endothelial denudation followed by a high-cholesterol diet.

Kawata et al. (1990) described the detection of regenerating cells in the aorta after ballooning by immunocytochemical demonstration of the thymidine analogue 5-bromo-2′-deoxyuridine.

Manderson et al. (1990) described changes in vascular reactivity of carotid arteries in rabbits following endothelial denudation.

Bocan et al. (1991) tested an ACAT inhibitor and selected lipid-lowering agents for antiatherosclerotic activity in iliac-femoral and thoracic aortic lesions. Atherosclerotic lesion comparable in composition to human fatty streaks were induced by chronic endothelial denudation in the iliac-femoral artery inserting a sterile, indwelling, 18 cm nylon filament with a diameter of 200 μm into the lumen of the right femoral artery in hypercholesterolemic New Zealand White rabbits. Naturally occurring fatty streaks developed in the thoracic aorta following cholesterol feeding. The effect of treatment on lesion regression was evaluated in the iliac-femoral artery, while changes in the lesion progression were evaluated in the thoracic artery.

Davies et al. (1993) performed right common carotid artery bypass grafting using the ipsilateral external jugular vein in New Zealand White rabbits and studied the response to endothelin-1 in normolipidemic and hyperlipidemic animals.

Groves et al. (1993) studied platelet adhesion and thrombus formation in a **porcine** model of balloon angioplasty.

ACE inhibitor treatment reduced the neointimal formation after endothelial denudation in the carotid artery of **rats** using a balloon catheter (Farhy et al. 1992; Linz and Schölkens 1992; Linz et al. 1993, 1994).

Lyle et al. (1995) tested the effect of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition in an atherosclerotic rabbit model of angioplastic injury. Acute ^{111}indium-labeled platelet deposition and thrombosis were assessed 4 h after balloon injury in femoral arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month).

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Influence of Lipid Metabolism

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General Considerations

Elevated lipid levels, especially hypercholesterolemia, result from increased absorption from the gut, enhanced endogenous synthesis, or inadequate clearance from serum. Therefore, there are three feasible ways to reduce hyperlipidemia: to block endogenous synthesis, decrease absorption, or enhance clearance from serum. These three factors can be evaluated in normal animals without artificial diets. Clinically used lipid-lowering compounds such as PPARα agonists (fibrates), cholesterol absorption inhibitors (ezetimibe), bile acid sequestrants, and HMG-CoA reductase inhibitors (statins) can be tested in this way, and their pharmacological activity further investigated with additional tests. For the investigation of the effects on plasma lipids, the right animal model has to be chosen. For fibrates, rats and mice are appropriate models; for LDL-lowering compounds, hamster, guinea, and rabbits. Earlier attempts to interfere with endogenous cholesterol synthesis resulted in the accumulation of sterols other than cholesterol (Holmes 1964). To date, only the inhibition of cholesterol biosynthesis with HMG-CoA reductase inhibitors has been a clinically effective approach for LDL cholesterol lowering (Ridker 2014). Inhibition of other enzymes of the cholesterol biosynthesis pathway upstream from HMG-CoA reductase, such as squalene synthetase were investigated by several pharmaceutical companies until the 1990s, but this development was discontinued for safety reasons. Inhibition of cholesterol biosynthesis upstream from HMG-CoA reductase leads to nonphysiological accumulation of metabolic intermediates. In the past, the inhibition of cholesterol absorption by ACAT-inhibitors was a widely followed approach. ACAT-inhibitors inhibit cholesterol absorption in rodents effectively, but so far all ACAT-inhibitors have been ineffective in humans (Farese 2006). The only compound that is effective in humans is ezetimibe, an azetidinone. The compound was discovered fortuitously in an ACAT-inhibitor program. During the development of that compound, it was found that ezetimibe inhibits cholesterol absorption independently from ACAT (Van Heek et al. 2000, 2001, 2003; Harris et al. 2003; Clader 2004). Ezetimibe inhibits cholesterol absorption in several animal models and is effective against plasma LDL cholesterol in humans (Couture and Lamarche 2013). Another approach to enhance hepatic LDL clearance is to interrupt bile acid recirculation. Compounds that inhibit bile acid absorption increase the conversion of cholesterol into bile acids, enhance hepatic clearance, and lower LDL cholesterol by LDL receptor upregulation in the liver.

Protective effects of calcium antagonists against experimental atherosclerosis acting mainly on other mechanisms than lipid metabolism have been claimed by various authors but the clinical relevance is still questionable (Kjeldsen and Stender 1989; Fronek 1990; Knorr and Kazda 1990; Fleckenstein-Grün et al. 1992).

New treatments emerging are systemic administration of antibodies against proprotein convertase subtilisin-kexin type 9 (PCSK9), a protein that accelerates the degradation of the low-density lipoprotein receptor (LDLR) in hepatic cells (Abifadel et al. 2003; Cohen and Hobbs 2013). This induces dramatic reductions in LDL-cholesterol levels, and the effect of this therapy on LDL-receptor activity seems to be additive to that of statin therapy. Inhibition of PCSK9 is potentially very important to the clinician, and should enable more patients to achieve their LDL-cholesterol-level goal (Robinson et al. 2015; Sabatine et al. 2015).

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Hypolipidemic Activity in Rats

Purpose and Rationale

Hyperlipoproteinemia with increased concentrations of cholesterol- and triglyceride-carrying lipoproteins is considered to be the cause of atherosclerosis with its dual sequelae of thrombosis and infarction. Lipoproteins are divided into six major classes: chylomicrons, chylomicron remnants, VLDL (very low density lipoproteins), IDL (intermediate density lipoproteins), LDL (low density lipoproteins), and HDL (high density lipoproteins). HDL promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Therefore, increased levels of HDL are desirable. On the contrary, high levels of VLDL and LDL promote atherosclerosis. LDL, especially in its oxidized form, is taken up by macrophages via a scavenger mechanism. Therefore, antiatherosclerotic drugs should reduce VLDL and LDL and/or elevate HDL.

Procedure

Groups of ten male Wistar or Sprague Dawley rats weighing 180–200 g are used. They are given once daily in the morning over a period of 5–8 days the test compounds or the standard in various doses ranging from 1 to 100 mg/kg via a stomach tube in a volume of 5 ml/kg. The control

group is given the solvent (e.g., 0.5 % HEC with 0.4 % Tween 80) only. The body weight of each animal is recorded at the beginning and at the end of the experiment. If the effect on VLDL and triglycerides is the focus of the experiment, animals should be nonfasted or postprandial, and food should be withdrawn for no longer than 4 h before blood sampling. On the morning of the first day, blood samples are taken under light isoflurane or CO_2 anesthesia by retro-orbital puncture. Then, the first dose is applied. During the whole period, the animals have free access to food and water. At the end of the experiment, blood samples are taken by retro-orbital puncture. Immediately thereafter, the animals are sacrificed and the liver removed, blotted free from blood, and weighed. Samples of liver are frozen in liquid nitrogen and stored at −25 °C for lipid analysis. The blood samples are centrifuged for 2 min at 16,000 g. Total cholesterol and triglycerides in each blood sample are determined.

To estimate the serum lipoproteins two methods are available: ultracentrifugation and liquid chromatography. For ultracentrifugation more than 1 ml serum is needed, so the serum has to be group-vice pooled. The serum lipoproteins are separated by means of a preparative ultracentrifuge.

The separation of fractions VLDL, LDL, HDL, and of the subnatant of HDL is carried out as follows:

VLDL	Native density of the serum (1.006), 16 h at 40,000 rpm
LDL	Density range from 1.006 to 1.04, 18 h at 40,000 rpm
HDL	Density range from 1.04 to 1.21, 18 h at 40,000 rpm
Subnatant of HDL	Density > 1.21

The density is adjusted by addition of a calculated amount of NaBr solution.

Another faster approach is the separation of lipoprotein by fast protein liquid chromatography (FPLC).

Distribution of cholesterol to lipoprotein fractions is performed after separation of fractions by gel permeation chromatography, using a published method (März et al. 1993). The cholesterol and triglyceride content in the isolated lipoprotein fractions after ultracentrifugation or FPLC separation is determined using standard enzymatic assays for clinical diagnostics (e.g., Roche-Diagnostics, CHOD-PAP method, Siedel et al. 1983). Triglycerides are also determined with standard enzymatic assays for clinical diagnostic (e.g., Roche Diagnostics, GPO-PAP method; Eggstein and Kreutz 1966a, b; Wahlefeld 1974). The method of Lowry et al. (1951) is applied to determine protein content.

Frozen samples of liver are thawed, homogenized and aliquots are extracted with chloroform/methanol or dichloromethane/methanol 2:1 (v/v). The extracts are purified according to Folch et al. (1957). Solvents of aliquots of the extracts are evaporated and dissolved in isopropanol for determinations of cholesterol and triglycerides, using enzymatic assays for serum diagnostics (Herling et al. 1999).

Evaluation

Average values of body weight, cholesterol and triglycerides are expressed as percentage of initial values for each group at the end of the experiment. Statistical differences between the controls and the treatment groups are evaluated by covariance analysis.

Critical Assessment of the Method

Studies with normocholesterolemic animals are faced with the difficulty that starting cholesterol levels are relatively low, and to achieve significance for lowering of cholesterol levels requires large groups of animals. LDL cholesterol lowering can be investigated in normocholesterolemic hamster, guinea pigs or minpigs, not in mice or rats. Triglyceride-lowering drugs such as fibrates are very effective against serum triglycerides and cholesterol in normolipemic male Sprague Dawley rats.

Modifications of the Method

Similar tests can be performed in various species, e.g.,:

- Male NMRI mice, weighing 25–30 g, male or female C57/bl6 mice weighing 18–24 g
- Male New Zealand obese (NZO) mice, weighing 35–40 g
- Male Syrian golden hamsters, weighing 80–120 g
- Male Pirbright guinea pigs, weighing 200–250 g
- Male miniature pigs, weighing 14–22 kg

To study long term effects, the experiments are extended for 4 weeks or 3 months.

Schurr et al. (1976) proposed high volume screening procedures for hypobetalipoproteinemic activity in rats.

März et al. (1993) described fast lipoprotein chromatography as a new method of analysis for plasma lipoproteins.

Adipokinetic actions of several hormones in slices of perirenal adipose tissue of various species were determined by Rudman et al. (1963).

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Hypolipidemic Activity in Syrian Hamsters

Purpose and Rationale

The Syrian hamster (*Mesocricetus auratus*) is a widely used animal to study the effects of drugs and diet on lipoprotein metabolism. Several in human approved lipid lowering drugs like HMG-Co A reductase inhibitors, cholestyramine, or ezetimibe lower plasma cholesterol in hamster. The lipoprotein and bile acid metabolism of the hamster is closer to human than the lipoprotein and bile acid metabolism of rats and mice (Bravo et al. 1994; Suckling et al. 1991; Kris-Etheron and Dietschy 1997). The hamster has in contrast to pig, rat, and mouse CETP (Cholesteryl Ester Transfer Protein) activity, similarly as seen in humans (Ha and Barter 1982, 1986; Ahn et al. 1994).

Increase in plasma cholesterol can be easily induced by adding small, physiological amounts of cholesterol to the diet (0.05–0.01 wt%). Additional saturated fat like coconut oil (5–10 wt%) has synergistic effects for induction of hyperlipidemia (Kowala et al. 1991). A stable hyperlipidemia with a human like lipoprotein pattern can be induced in hamster within 2–3 weeks by adding 10 wt% coconut butter and 0.2 % cholesterol into the diet. Hamster HDL cholesterol can be easily measured after precipitation of VLDL + LDL cholesterol with phosphotungstic acid/$MgCl_2$ (Weingand and Daggy 1990, 1991). LDL and HDL cholesterol can also be measured directly with Kits from Roche Diagnostics. A complete separation of all lipoprotein fractions can be done by FPLC on Superose 6 columns (Pharmacia) with a modified method of März et al. (1993).

Procedure

Male Syrian hamsters weighing 95–125 g at the start of the experiment are randomly assigned to form groups of 6–15 animals each. Test compounds can be administered by oral gavage, once or twice daily or mixed into the diet. If compounds are administered with diet, a different diet has to be prepared for each group. One control receives powdered or pellet standard hamster chow only; a second control and the treated groups, a cholesterol-enriched diet. If compounds are administered with diet a daily food consumption of 8 g should be taken in account for dose calculation. Cholestyramine, for example, is active when mixed into the diet in a concentration range between 0.3 % and 2 %. After 1–3 weeks on these diets, the animals are anesthetized with isoflurane, a blood sample is taken from the retroorbital venous plexus, the superior vena cava, or the abdominal aorta, and the liver is removed and weighed. Microsomes are prepared by ultracentrifugation from the livers.

Preparation of Plasma Lipoprotein Profiles

The blood is centrifuged at 6,000 g for 5 min and the plasma removed. The plasma is analyzed for total cholesterol and triglycerides using colorimetric enzymatic assays for clinical diagnostics, as described in the previous chapter for rats (e.g., Roche Diagnostics). The cholesterol content of HDL and LDL was determined using enzymatic assays for clinical diagnostics (Roche Diagnostics, HDL-C plus and LDL-C plus).

Quantification of High-Density Lipoprotein Cholesterol by Differential Precipitation

Weingand and Daggy (1990) compared the validity of differential precipitation with Mg^{2+} phosphotungstate for quantification of plasma high-density lipoprotein cholesterol with ultracentrifugal flotation.

As precipitating agent, 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride is added. Cholesterol is determined spectrophotometrically with an enzymatic cholesterol reagent containing microbial cholesterol esterase, in a clinical chemistry analyzer.

Fast Lipoprotein Chromatography

März et al. (1989, 1993) developed fast lipoprotein chromatography for rapid and quantitative analysis of lipoprotein fractions.

Fast lipoprotein chromatography is carried out with a chromatography system from Kontron consisting of two Model 420 pumps, a Model 432 variable-wavelength detector, and a Model 450 data system. Without using a pretreatment, 20 µl of fresh plasma are applied to a 300-mm Superose column 6 equilibrated with 100 mmol/l Na_2HPO_4, pH 7.4, and 200 mmol/l NaCl. Lipoproteins are detected on-line at 500 nm after postcolumn derivatization with CHOD-PAP cholesterol reagent (Roche Diagnostics, Mannheim, Germany). The column eluate and the cholesterol reagent are mixed in a motor-driven microchamber attached to the column outlet, and the mixture is then passed through a "knitted" capillary (20–22). The flow rate of the reagent is 70 µl/min; the run time is 80 min. Under these conditions, it takes the column eluate 2 min to pass the derivatization capillary. VLDL-C, LDL-C, and HDL-C are calculated on the basis of relative peak areas and total cholesterol, previously measured with an enzymatic assay in serum as described above.

Tissue Preparation

The liver is homogenized in a KCl (0.104 M) solution containing NaF (50 mM) using a glass Teflon homogenizer. The homogenate is centrifuged at 30,000 g, 5 °C for 25 min. The supernatant is then centrifuged at 100,000 g, 5 °C for 60 min to pellet the microsomes, which are resuspended in potassium phosphate buffer (50 mM) containing NaF (50 mM) adjusted to pH 7.4.

Determination of Enzyme Activities in Microsomal Fractions

Protein for all the following assays is determined using the method described by Bradford (1976).

HMG-CoA reductase activity is determined by quantifying the conversion of [^{14}C]HMG-CoA to

[^{14}C]mevalonic acid lactone, based on the method described by Ingebritson and Gibson (1981). ACAT activity in liver microsomes and intestinal cell homogenates is determined by the method described by Suckling et al. (1982), measuring the incorporation of [^{14}C]oleoyl-CoA into cholesteryl oleate. The cholesterol 7α-hydroxylase activity in the microsomes is quantified by determination of the percentage conversion of [^{14}C]cholesterol to 7α-hydroxy[^{14}C]cholesterol. 3 mg of microsomal protein is diluted to a volume of 5.3 ml with a potassium phosphate buffer (50 mM, pH 7.4) containing NaF (50 mM), cysteamine (31.4 mM), glucose-6-phosphate (12.7 mM) NADP (1.4 mM), and [^{14}C]cholesterol (0.22 µCi, 55 Ci/mol). After a short preincubation (5 min at 37 °C), the reaction is started by the addition of glucose-6-phosphate dehydrogenase suspension (7 µg). The reaction is stopped 1 h later by adding methanol (5 ml). The cholesterol and 7α-hydroxy-cholesterol are extracted into a mixture of chloroform and methanol (2:1, v/v, 2 × 5 ml). The product and substrate are separated by thin-layer chromatography on silica gel eluting with toluene/ethyl acetate (3:7, v/v). The radioactive regions corresponding to cholesterol and 7α-hydroxy-cholesterol are scraped off the plates and quantified by liquid scintillation counting. An alternative nonradioactive method to determine 7α-hydroxylase activity is described in detail in section "Influence on Several Steps of Cholesterol Absorption and Formation".

Evaluation

Dose–response curves for standard and test drug are established using the serum LDL cholesterol value of the standard chow fed as the maximal effect and the cholesterol control data as zero.

Critical Assessment of the Method

Potent drugs acting on cholesterol or bile acid absorption lower serum LDL cholesterol below the level measured in animals on standard chow. PPARα agonists (fibrates) increase liver triglycerides in hamsters on a normal or high-fat diet (Planke et al. 1988). HMG-CoA reductase inhibitors (statins) are not well tolerated, the therapeutic range of statins which is effective against LDL cholesterol is very narrow, and the animals have to be treated for longer than 3 weeks to see the maximal effects of statins on serum LDL cholesterol.

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Triton-Induced Hyperlipidemia

Purpose and Rationale

The systemic administration of the surfactant Triton to mice or rats results in a biphasic elevation of plasma cholesterol and triglycerides. Triton inhibits lipoprotein lipase (LPL) activity and so VLDL particle clearance (Frantz and Hinkelman 1955; Garattini et al. 1958, 1961; Holmes 1964; Tamasi et al. 1968; Millar et al. 2005).

Procedure

Male Sprague Dawley or Wistar rats weighing 200–350 g were starved for 18 h and then injected intravenously with 200 mg/kg Triton WR 1339 (isooctyl-polyoxy-ethylene phenol). Serum triglycerides increased 20-fold in a nearly linear fashion within 1 and 8 h and serum cholesterol levels increased sharply 2–3 times within 24 h (phase I). The hypercholesterolemia decreased nearly to control levels within the next 24 h (phase II). The test drugs employed or the vehicle for the controls were administered simultaneously with the Triton injection if only the acute effect of a drug was investigated or 1–3 days before. Serum triglyceride cholesterol analyses were carried out 0, 2, 4, 6, and 8 h after Triton injection in order to investigate drugs that interfere with triglyceride synthesis; and 0, 6, 24, and 48 h after Triton injection for investigation of drugs that are effective against cholesterol synthesis. In 1951, it was described by Kellner et al. that intravenous injection of nonionic detergents increased serum lipids up to 48 h. Later, it was shown by Schotz et al. (1957) that the hyperlipidemia is induced by inhibition of triglyceride hydrolysis by lipoprotein lipase. Since then, lipolysis inhibition has been used to determine hepatic VLDL secretion rates. The VLDL secretion rate can be calculated from the increase in triglyceride and cholesterol over time after the Triton injection. Drugs interfering with hepatic triglyceride and cholesterol biosynthesis were shown to be active in phase I, while drugs interfering with cholesterol clearance, excretion, and metabolism were active in phase II.

Evaluation

Mean values ± standard deviation are calculated for each group and time interval and compared statistically with the controls.

Critical Assessment of the Method

The method employing Triton hypercholesterolemia is rather simple and rapid for the detection of

compounds interfering with the synthesis of triglycerides or synthesis and excretion of cholesterol. Since the test is rather artificial, the results have to be validated by other methods, e.g., ex vivo measurement of liver cholesterol synthesis.

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Fructose-Induced Hypertriglyceridemia in Rats

Purpose and Rationale

Rats switched from a diet low in carbohydrates and high in protein to a high intake of fructose, develop an acute hypertriglyceridemia. Compounds are tested for inhibition of this phenomenon.

Procedure

Male Sprague Dawley rats weighing 200–250 g were fed over a period of 1 week a diet enriched in protein with reduced carbohydrate content, e.g., Altromin C1080 or C1009. Groups of ten animals were treated for 3 days daily with the test compound or the standard (fenofibrate 30–100 mg/kg) or the vehicle (0–5 % hydroxylethyl cellulose) by oral gavage. From the second to the third day water was withheld for a period of 24 h. Immediately afterwards, the animals were offered 20 % fructose solution ad libitum for a period of 20 h. After this time, which was also 20 h after the last application of the test compound, the animals were anesthetized with isoflurane or CO_2/O_2 (70/30 v/v) and 1.2 ml blood was withdrawn by retro-orbital puncture. The blood was centrifuged at 6,000 g for 5 min and the serum removed. The serum was analyzed for total cholesterol and triglycerides and glycerol using a colorimetric enzymatic assay for clinical diagnostics, as described in the previous chapter for rats (e.g., Roche Diagnostics).

Evaluation

The average values of total glycerol for the treated groups are compared with those for the control group using ANOVA.

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Intravenous Lipid Tolerance Test in Rats

Purpose and Rationale

Intravenous injection of a lipid emulsion results in an increase of triglycerides in serum. The lipolytic activity can be determined by measuring lipid elimination.

Procedure

Male Wistar rats weighing 200–240 g are treated daily with various doses of the test compound or the vehicle over a period of 5 days. On the fifth day, 2 h after the last administration of the test compound, the animals are anesthetized with isoflurane or 40–60 mg/kg sodium pentobarbital i.p. Then they are injected intravenously with 2 ml/kg of a 10 % lipid emulsion (Intralipid Vitrum, Hausmann AG, St. Gallen, Switzerland). Prior to the injection and 10, 20, 30, and 40 min thereafter blood is withdrawn by retro-orbital puncture for determination of triglycerides.

Evaluation

Peak levels as well as elimination constant and half-life are determined.

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Influence on Lipoprotein-Lipase Activity

Purpose and Rationale

Postheparin plasma lipolytic activity is measured by at least two lipase activities: hepatic lipase (HL) and lipoprotein lipase (LPL). Hypothyroid rats show a selective decline of postheparin plasma hepatic lipase (Murase and Uchimura 1980).

For patients with familial lipoprotein lipase deficiency a rapid diagnostic test was developed (Gotoda et al. 1991). The method was used to study the influence of drugs on lipoprotein lipase activity (Tsusumi et al. 1993).

Procedure

Male Wistar rats weighing 180–200 g receive either various single doses or one daily dose of test compounds over a period of several days. Blood samples are drawn from the tail vein into tubes containing 1 mg EDTA/ml. The animals

are then injected with 100 U/kg heparin via the tail vein and blood samples are collected 5 min later. Plasma samples are used to determine lipoprotein lipase and hepatic lipase activity.

Lipoprotein lipase activity in postheparin plasma is measured using glycerol tri[1-^{14}C]oleate as substrate and selective blocking of hepatic lipase activity with antiserum to rat hepatic lipase. Hepatic triglyceride lipase activity in postheparin plasma is obtained by subtracting lipoprotein lipase activity from total plasma lipase activity.

Evaluation

The dose-related increase of plasma lipoprotein lipase activity and the time course after drug administration are evaluated.

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Influence on Several Steps of Cholesterol Absorption and Formation

Purpose and Rationale

Cholesterol levels in the body result from two sources: absorption from the gut and endogenous *de novo* synthesis. Certain natural products and the azetidinone drug ezetimibe inhibit cholesterol absorption and reduce plasma cholesterol levels in experimental animals and are therefore of potential pharmacologic interest in the treatment of hypercholesterolemia. In rodents, cholesterol absorption can be decreased by inhibition of acyl coenzyme A: cholesterol acyltransferase (ACAT). In humans, ACAT-inhibitors are not effective. Inhibition of cholesterol resorption results in a compensatory increase of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity, which can be decreased by (HMG-CoA) reductase inhibitors (statins). The hepatic cholesterol biosynthesis can be measured with several radioactive tracers as described later in chapter "Inhibition of Cholesterol Biosynthesis," by conversion of radioactive precursors into cholesterol. In addition to serum LDL cholesterol, hepatic cholesterol biosynthesis is a biomarker for the in vivo efficacy of cholesterol absorption inhibitors. Harwood et al. (1993) and van Hek and Davis (2002) studied the pharmacologic consequences of cholesterol absorption inhibition: alteration in cholesterol metabolism and reduction in plasma cholesterol concentration induced by a synthetic saponin β-tigogenin cellobioside or ezetimibe.

Procedure

Male golden Syrian hamsters, weighing 100–120 g, are housed in a reversed light–dark cycle room (light between 3:00 p.m. and 3:00 a.m.) and receive a cholesterol-poor or cholesterol-enriched diet and water ad libitum 1 week prior to use.

The animals are assigned to groups of six animals each and are given free access to water and chow that contains the compounds to be tested in appropriate concentrations for 4 days. At 9:00 a.m. of the fourth day of the study, the animals receive a 1.0-ml oral bolus of liquid formulation of cholesterol and radiolabeled cholesterol dissolved in Intralipidcontaining 15 mg of [^{3}H]cholesterol (2.25 μCi) and 7.5 mg cholic acid for determining cholesterol absorption. At 9:00 a.m. of the fifth day (peak of the diurnal cycles of HMG-CoA reductase and cholesterol 7α-hydroxylase activities), animals are anesthetized with pentobarbital and blood samples are obtained by cardiac puncture for determining plasma cholesterol and triglyceride levels and cholesterol absorption.

Livers are removed, weighed, rinsed in 4 °C saline, and apportioned for determining hepatic cholesterol levels, hepatic HMG-CoA reductase, cholesterol 7α-hydroxylase activities, hepatic LDL receptor concentration, and cholesterol absorption.

For determination of total hepatic cholesterol, one aliquot of the liver tissue (0.5 g) is saponified in 5 ml ethanolic KOH, 3.3 % (v/v) at 65 °C for 1 h. After addition of 5 ml water and 2 mg 5-cholestene (internal standard), lipids are extracted with 15 ml petroleum ether. Samples are shaken several times to extract the sterols into the petroleum ether phase. Then 100 μl of the petroleum ether phase (upper phase) is transferred into an HPLC autosampler vial and the solvent is removed by airstream. For final HPLC analysis, the samples are mixed with an equal volume of HPLC eluent. The total cholesterol content is detected at 210 nm after isocratic HPLC separation on an RP C18 column (Merck Lichrospher 100, 250 × 4 mm, 5 μm) with 2-propanol-acetonitrile (55:45 v/v) as eluent solution (0.8 ml/min) using 5-cholestene as an internal standard.

Alternatively, hepatic cholesterol concentration can also be measured with a colorimetric enzymatic assay. Frozen samples of liver are thawed, homogenized and aliquots are extracted with chloroform/methanol and analyzed as described earlier in section "Hypolipidemic Activity in Rats" (Herling et al. 1999).

Hepatic microsomes for measurement of hepatic HMG-CoA reductase activity and cholesterol 7α-hydroxylase activity are prepared according to Harwood et al. (1984) and Junker and Story (1985). For measurement of HMG-CoA reductase activity, 0.5 g liver are immediately homogenized at 4 °C in 1 ml TEDK buffer (containing 50 mM Tris (pH 7.5), 1 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl) using 15 strikes in a Dounce homogenizer. For measurement of cholesterol 7α-hydroxylase activity, 0.5 g liver pieces are immediately homogenized at 4 °C in 1 ml PEDSKF buffer (containing 40 mM phosphate (pH 7.4), 5 mM EDTA, 5 mM DTT, 250 mM sucrose, 50 mM KCl, and 50 mM KF) using 20 strokes of a Dounce homogenizer. Homogenates are first centrifuged at 4 °C for 20 min at 10,000 g and the resultant supernatant is then centrifuged at 4 °C for 90 min at 178,000 g. The resulting microsomal pellets are resuspended in either 1.0 ml TEDK per g liver (HMG-CoA reductase determination) or 1 ml PEDSKF per g liver (cholesterol 7α-hydroxylase determination) by five strokes of a Potter-Elvehjem pestle and are stored frozen in liquid nitrogen.

For measurement of hepatic HMG-CoA reductase activity, 50 μg of microsomal protein is incubated for 30 min at 37 °C in a final volume of 75 μl of TEDK buffer containing 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 66.7 μM [^{14}C]HMG-CoA (10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6–1.2 Ci/mmol) as an internal standard, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. After incubation, 10 μl of 6 M HCl are added to terminate the enzyme reaction and to convert the newly formed mevalonate to mevalonolactone. The mevalonolactone is then separated from unreacted substrate by silica gel thin-layer chromatography. After development in toluene-acetone 1:1, the region of the chromatogram corresponding to $R_f = 0.4–1.0$ is removed, immersed in liquid scintillation fluid, and counted using a dual channel $^3H/^{14}C$ program. HMG-CoA reductase

activity is expressed as pmoles of mevalonate formed from HMG-CoA per min of incubation at 37 °C per mg of microsomal protein.

Cholesterol 7 α-hydroxylase activity was determined with a modified method according to the procedure of Hylemon (1989) with the following modifications. To measure 7α-hydroxylase activity, 2 mg microsomal protein (1 ml in assay buffer) is preincubated for 10 min at 37 °C with 20 µl cholesterol solution (1 mg/ml) in 45 % hydroxypropyl-ß-cyclodextrin (ICN) in order to saturate the enzyme with cholesterol substrate. 7α-Hydroxycholesterol is measured with isocratic HPLC analysis on a C-18 reversed-phase column (Merck 250 × 4 mm, 5 µm), at 240 nm with acetonitrile:methanol (70:30 v/v) as eluent (0.7 ml/min) using 7ß-hydroxycholesterol as internal standard. The protein content of the microsomes is measured by the BCA method (Pierce).

Cholesterol 7α-hydroxylase activity is expressed as pmoles of 7α-hydroxycholesterol formed from cholesterol per minute of incubation per milligram of microsomal protein.

Hepatic in vivo cholesterol biosynthesis is determined with a method according to Dietschy and Spady (1984). [^{14}C]Octanoic acid sodium salt (NEC 092H, concentration 0.1 mCi/ml in ethanol, NEN, Cologne, Germany) is used as tracer. The metabolism of the tracer into liver cholesterol is investigated using the following procedure. The solution is concentrated about 20-fold under a nitrogen stream and dissolved in 0.9 % NaCl to a final concentration of 50 µCi/ml. This solution is injected into the femoral vein at a dose of 10 µCi/100 g body weight under ether anesthesia.

The animals are killed 1 h after administration and the liver removed and washed in isotonic NaCl solution. Three aliquots (0.5 g) are extracted in 30 ml of 6.6 % ethanolic KOH at 65 °C for 2 h. After cooling down to room temperature, 5 ml aqua is added and the cholesterol is extracted twice with 15 ml petroleum ether into a volumetric flask. The petroleum ether is removed under an air stream. The lipid phase is resolved and transferred three times with 2 ml ethanol acetone 1:1 (v/v) into a 15-ml glass vial after heating the glass container used. The cholesterol is precipitated with digitonin after adding one drop of 1 M HCl, 2 ml 10 % aqueous aluminum chloride, 3 ml 0.5 % digitonin in ethanol water 1:1 (v/v), and incubated for 1 h at 45 °C in a water bath. The liquid phase is discarded after centrifugation. The pellet is washed with 5 ml acetone three times and once with ether. Each pellet is rolled out by hand on a table until completely dry.

To cleave the digitonin–cholesterol complex, the vials are heated to 80 °C and the cholesterol resolved with 0.6 ml pyridine. When the samples are cooled to room temperature, 5 ml ether is added twice, the samples are centrifuged, and the liquid phase is transferred into scintillation vials. The ether is removed under air stream, and the residual cholesterol resolved with 1 ml methanol and 10 ml scintillation cocktail (Ecolite ICN, Eschwege, Germany).

The radioactivity is counted in a ß-scintillation counter. The disintegrations per minute (DPM) are calculated per gram of liver tissue after background correction and correlated with the liver cholesterol concentration in a graph plotting liver cholesterol on the *x*-axis and radioactivity (dpm/g liver tissue) on the *y*-axis. Orientation of treated groups relative to cholesterol-fed and noncholesterol-fed control animals gives information about sterol turnover.

Hepatic LDL receptor levels are measured by enzyme immune blotting (Cosgrove et al. 1992). One hundred to 150 µl of the soluble extracts containing 100–600 µg protein are adjusted to 2 % SDS and 0.2 M sucrose by addition of 0.33 volumes of electrophoresis sample buffer (containing 320 mM Tris (pH 6.8), 8 % SDS, and 0.8 M sucrose). Mixtures are applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5 % (w/w) 0.1 % SDS-containing polyacrylamide slab gel of 1.5 mm thickness. Electrophoresis is conducted at room temperature with a constant current of 15 mA/gel. Prestained molecular weight markers are included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel are electrophoretically transferred to S&S BA85 nitrocellulose membranes at 18 °C with a constant voltage of 120 V for 6–8 h in 25 mM Tris, 192 mM glycine buffer (pH 8.3), containing 20 % methanol.

After transfer, the nitrocellulose paper is incubated with 100 ml of Tris buffered saline containing 3 % gelatin for 30 min at room temperature with gentle shaking. After incubation, the nitrocellulose sheet is removed from the blocking solutions, immersed without rinsing into 50 ml Tris buffer solution (TBS) containing 1 % gelatin and 250 µl of anti-LDL receptor peptide antiserum (final dilution 1:200) and incubated for 2 h at room temperature with gentle shaking. The nitrocellulose sheet is washed twice for 10 min each with TBS containing 0.05 % Tween-20 and once for 10 min with TBS. The washed nitrocellulose sheet is incubated with 50 ml TBS containing 1 % gelatin and 100 µl of goat anti-rabbit IgG-horseradish conjugate (final dilution 1:500) at room temperature for 1 h with gentle shaking. After incubation, the nitrocellulose is washed again as described above. During this final wash, 40 mg of 4-chloro-1-naphthol is dissolved in 10 ml methanol, and 50 µl cold 30 % hydrogen peroxide is added to 50 ml TBS containing 1 % gelatin. After draining the final TBS wash from the nitrocellulose, the two solutions are mixed and immediately added to the nitrocellulose. The mixture is incubated at room temperature with gentle shaking until the desired color development is observed. The nitrocellulose sheet is washed with running tap water for 15 min. The nitrocellulose is dried between pieces of filter paper and the intensity of color formation is quantitated by reflectance densitometry using a suitable instrument (e.g., Hoefer Scientific Instruments GS300 Transmittance/Reflectance Scanning Densitometer). Color intensity, which is a linear function of the number of LDL receptors in the analyzed fraction, is expressed in terms of mm peak height (arbitrary reflectance units).

Intestinal cholesterol absorption is estimated with the dual isotope feces ratio method using [^{3}H]sitostanol and [^{14}C]cholesterol. Each hamster received an intragastric dose of a mixture of [^{3}H] sitostanol and [^{14}C]cholesterol dissolved in Intralipid or plant oil, or in detergent mix (Tricaprin/Tricapril). The feces is collected for 48 h after dosing and homogenized with water; aliquots are oxidized in a sample oxidizer, which traps CO_2 and H_2O in separate vials mixed with liquid scintillation fluid, and counted using a background correction program. The absorption ratio can be calculated by changes of the $^{14}C/^{3}H$ ratio in application solution and feces with the following formula ($^{14}C/^{3}H$ application solution – $^{14}C/^{3}H$ feces)/$^{14}C/^{3}H$ application solution.

If a sample oxidizer is not available an alternative method is the recovery of radioactivity present in the liver and plasma 24 h after an oral bolus of [^{3}H]cholesterol. For assessment of hepatic radioactivity, 1.0-g liver pieces are placed in 50-ml polypropylene tubes and incubated with 2.5 ml of 2.5 M KOH for 2 h at 75 °C. After allowing the saponification mixture to cool to room temperature, 5.0 ml of 80 % ethanol, 0.1 ml [^{14}C]cholesterol (40,000 dpm, 15 dpm/nmol) as carrier and extraction standard and 10 ml of hexane are added. Tubes are capped and shaken vigorously for 1 min. Mixtures are permitted to stand to allow phase separation and are shaken vigorously for an additional minute. Mixtures are the centrifuged at 1,000 g for 5 min. Duplicate 3.0-ml aliquots of each hexane layer are removed, transferred to 20-ml liquid scintillation vials, mixed with 10 ml liquid scintillation fluid, and counted using a dual channel $^{3}H/^{14}C$ program.

After correction for recovery losses, hepatic radioactivity is calculated based on total liver weights. For assessment of total plasma radioactivity, 200-µl aliquots of plasma are added to 20-ml liquid scintillation vials and decolorized by addition of 25 µl of hydrogen peroxide. Then 10 ml aqueous scintillation fluid is added and the mixture assessed for radioactivity. Total radioactivity in the plasma is calculated based on the assumption that hamsters possess approximately 4.0 ml plasma per 100 g body weight. The degree of cholesterol absorption is expressed as a percentage of the total radioactivity administered that is present in the liver plus plasma 24 h after bolus administration.

For **determination of bile acids in feces**, pooled feces from each group of animals is collected over a period of 48 h (about 20 g). After addition of 9 ml ethanol, 1 ml water and hyodeoxycholate (internal standard) the mixture is continuously shaken at 90 °C overnight.

Samples are centrifuged and an aliquot of the liquid phase is diluted 1:5 with methanol for quantification of bile acids. Individual bile acids (deoxycholate;lithocholate; 12-oxo lithocholate) are separated and quantified by HPLC-MS (HPLC: RP C18 column Merck Purospher™, end-capped, 5 µm, 125 × 3 mm); gradient elution (20–60 % eluent B, 15 min, 0.6 ml/min; eluent A: 90 % H_2O, 10 % acetonitrile; eluent B: 10 % H_2O, 90 % acetonitrile, buffered at pH 8 with 20 mM ammonium acetate; MS detection: electrospray negative mode Hewlett Packard, 1100 MSD).

For **lipid and lipoprotein determinations**, serum or plasma can be used. Blood samples are treated either with EDTA or heparin to prevent clotting or without an anticoagulant and are then centrifuged, as described earlier in sections "Hypolipidemic Activity in Rats" and "Hypolipidemic Activity in Syrian Hamsters."

Evaluation

Inhibition of cholesterol resorption results in a decrease of hepatic cholesterol and a compensatory increase of hepatic HMG-CoA reductase activity resulting from de novo cholesterol synthesis and an increase of hepatic LDL receptor levels.

Modifications of the Method

Ogishima and Okuda (1986) described an improved method for assay of cholesterol 7α-hydroylase activity. Cholesterol 7α-hydroxylation is performed in liver microsomes utilizing cholesterol as substrate. 7α-Hydroycholesterol is converted by the action of cholesterol oxidase into 7α-hydro-4-cholesten-3-one having an intense absorption at 240 nm.

Hylemon et al. (1989) reported a modified HPLC-spectrophotometric method for measuring cholesterol 7α-hydroxylase activity by using a C-18 reverse-phase column to separate 7α-hydroxy-4-cholesten-3-one and 4-cholestene-3-one and by adding 7β-hydroxycholesterol to each reaction mixture as an internal recovery standard. This method measures simultaneously cholesterol 7α-hydroxylase activity using endogenous cholesterol and exogenous [4-^{14}C]cholesterol as substrate.

Princen and Meijer (1990) measured cholesterol 7α-hydroxylase activity in cultured rat hepatocytes.

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Inhibition of Cholesterol Biosynthesis

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General Considerations on Cholesterol Biosynthesis

The following steps are involved in cholesterol biosynthesis:

- HMG-CoA synthase (hydroxymethylglutaryl-coenzyme A synthase)
- Forming **hydroxymethylglutaryl-CoA** from acetyl-CoA and acetoacetyl-CoA
- HMG-CoA reductase (hydroxymethylglutaryl-coenzyme A reductase)
- Forming **mevalonic acid** from hydroxymethyl-glutaryl-CoA

Inhibition of cholesterol synthesis on this step is used at present successfully for therapy (see below):

- Mevalonate kinase forming **5-phosphomevalonic acid** from mevalonic acid
- Phosphomevalonate kinase forming **5-pyrophosphomevalonic acid** from 5-phosphomevalonic acid
- Pyrophosphomevalonate decarboxylase forming **3-isopentyl-pyrophosphate** from 5-pyrophosphomevalonic acid
- Isopentyl-pyrophosphate isomerase forming **3,3-dimethyl-pyrophosphate** from 3-isopentyl-pyrophosphate
- Dimethylallyltransferase forming in a two-step process **geranyl pyrophosphate** from 3,3-dimethyl-pyrophosphate
- **Farnesyl pyrophosphate** from geranyl pyrophosphate
- Squalene synthetase forming **squalene** from farnesyl pyrophosphate
- Squalene epoxidase forming **2,3-oxidosqualene** from squalene
- 2,3-Oxidosqualene cyclase forming **lanosterol** from 2,3-oxidosqualene

Following the formation of lanosterol (4,4,14α-trimethylcholesta-8(9),24-dien-3β-ol), a series of enzyme reactions is required to produce cholesterol (Bae et al. 1999):

- To 4,4,14α-trimethylcholesta-8(9)-en-3β-ol
- To 4,4-dimethylcholesta-8(9),14-dien-3β-ol
- To 4,4-dimethylcholesta-8(9)-en-3β-ol
- To cholesta-8(9)-en-3β-ol
- To cholesta-7-en-3β-ol
- To cholesta-5,7-dien-3β-ol (7-dehydrocholesterol)
- To cholesta-5-en-3β-ol (cholesterol) by 7-dehydrocholesterol reductase

Besides **inhibition of HMG-CoA reductase** (detailed description below), other approaches to inhibit biosynthesis of cholesterol are reported:

- **Inhibition of HMG-CoA synthase** (Goldstein and Brown 1990; Miller et al. 1980; Greenspan et al. 1987, 1993; Grayson and Westkaemper 1988).
- **Inhibition of squalene synthase** (Ness et al. 1994; Sliskovic and Picard 1997; Rosenberg 1998; Hiyoshi et al. 2000) Squalene synthase plays an important role in sterol biosynthesis by catalyzing the head-to-head condensation of two molecules of farnesyl pyrophosphate. The enzyme is a single 47,000 Da polypeptide that is bound to the subcellular membranes of the endoplasmic reticulum in yeast and mammalian liver (see below); severals inhibitors of squalene synthase are described (Kourounakis et al. 2010; Menys and Durrington 2003).
- **Inhibition of squalene epoxidase** (Ryder 1992) Squalene epoxidase catalyzes the conversion of squalene to (3S)-2,3-oxidosqualene, an essential step in the biosynthesis of sterols in mammals, plants, and microorganisms (see below).
- **Inhibition of 2,3-oxidosqualene cyclase** (Gerst et al. 1986; Cattel et al. 1989; Sen and Prestwich 1989; Dollis and Schuber 1994; Mark et al. 1996; Eisele et al. 1997; Morand et al. 1997; Abe et al. 1998a) The enzyme 2,3-oxidosqualene cyclase is of special interest due to its dual function: cyclization of 2,3-monoepoxysqualene to lanosterol and 2,3:22,23-diepoxysqualene to oxylanosterol. An orally active oxidosqualene:lanosterol

cyclase inhibitor (Ro48-8071) showed potent noncompetitive inhibition of bacterial squalene:hopene cyclase from *Alicyclobacillus acidocaldarius*. A tritium-labeled isotopomer of this nonterpenoid inhibitor, which possesses a benzophenone photophore, was chemically synthesized as a photoaffinity label (Abe et al. 1998b).

- **Inhibition of 7-dehydrocholesterol reductase** (Amin et al. 1996) Deficiency of this enzyme causes a severe developmental disorder with multiple congenital and morphogenic abnormalities, the Smith-Lemli-Opitz syndrome (Waterham and Wanders 2000).

Search and development of new cholesterol-lowering drugs is continued (Abe and Prestwich 1998). Whether the efficacy and safety of these agents in man will be on the long run superior to currently available therapies remain to be determined.

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Determination of HMG-CoA Reductase Inhibitory Activity

General Considerations on HMG-CoA Reductase

More than 70 % of the total production of body cholesterol in humans is derived from de novo synthesis. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme governing cholesterol biosynthesis and the synthesis of other isoprenoids in mammalian cells (Rodwell et al. 1976; Goldstein and Brown 1990). The development of HMG-CoA reductase inhibitors (called statins) offers an advance in the treatment of hypercholesterolemia by interfering with the crucial step of cholesterol biosynthesis. When inhibiting hepatic HMG-CoA reductase, the inhibitors trigger an increased production of LDL receptors in the liver. As LDL receptor activity increases, more LDL is extracted from the blood, and thus the level of circulating LDL-cholesterol is reduced. Pharmacological evaluation of HMG-CoA reductase inhibitors is based on studies on the inhibition of the isolated enzyme HMG-CoA reductase in vitro, on the inhibition of the incorporation of ^{14}C sodium acetate into cholesterol in isolated liver cells, and on the effect of HMG-CoA reductase inhibitors in vivo. HMG-CoA reductase activity has a diurnal rhythm in liver and intestine which has to be considered for in vivo studies (Shapiro and Rodwell 1969; Shefer et al. 1972).

HMG-CoA reductase inhibitors have been associated with skeletal myopathy (rhabdomyolysis) in humans and experimental animals (Flint et al. 1997; Klotz 2003; Sakamoto and Kimura 2013). The rhabdomyolysis is probably mechanism based; from the cerivastatin cases it could be concluded that the risk of rhabdomyolysis is related to active drug levels in the systemic circulation and peripheral tissues (Graham et al. 2004).

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Inhibition of the Isolated Enzyme HMG-CoA Reductase In Vitro

Purpose and Rationale

For screening purposes, studies on the inhibition of HMG-CoA reductase obtained from rat liver microsomal fraction can be used (Avigan et al. 1975; Philipp and Shapiro 1979; Parker et al. 1990).

Procedure

The inhibitory activity of the test compound on HMG-CoA reductase is estimated with soluble enzyme preparations obtained from the microsomal fraction of rat liver (Philipp and Shapiro 1979; Parker et al. 1990). The enzyme reaction is carried out with 50 µl partially purified HMG-CoA reductase in buffer containing 25 mM Tris, 10 mM EDTA, and 10 mM dithiothreitol at pH 7.5,20 µl of 910 µM HMG-CoA solution containing 100 nCi (3.7 kBq) of ^{14}C-HMG-CoA and 20 µl of NADPH regenerating system (5.2×10^{-2} M glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 5.3×10^{-3} M NADP), with the actual concentration of 50 mM NADPH. The final incubation volume is 200 µl. The main reaction is preceded by 20 min preincubation with the NADPH regenerating system at 37 °C, followed by 20 min incubation at 37 °C of the completed samples with the test compound or the standard and stopped by addition of 75 µl 2 N $HClO_4$. After 60 min at room temperature, the samples are cooled in an ice bath and neutralized by addition of 75 µl 3 N potassium acetate. Supplementing the volume with water to 500 µl, the precipitate is centrifuged, and 250 µl of the clear supernatant are applied to a column (0.6 × 8.0 cm) of BIORAD AG 1-X8 (100–200 mesh). Mevalonolactone is eluted with water discarding the first 750 µl and collecting the next 3,500 µl. Five hundred µl of the eluate are used for measurement in duplicate, mixed in vials with 10 ml Quickscint (Zinsser), and measured in a liquid scintillation counter (Beckman). The assay is generally performed in triplicate. Lovastatin sodium is used as standard.

Evaluation

The mean values with and without inhibitors are compared for the calculation of inhibition. IC_{50} values are calculated.

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Inhibition of the Incorporation of ^{14}C Sodium Acetate into Cholesterol in Isolated Liver Cells

Purpose and Rationale

De novo synthesis of cholesterol from labeled acetate can be measured in isolated liver cells. This synthesis can be inhibited by incubation with HMG-CoA reductase inhibitors.

Procedure

Monolayers of primary cell cultures from hepatocytes of female rats or of cultures of HepG2 cells (human hepatoma cells) are incubated in a lipoprotein-deficient medium for 1 h with various concentrations of the potential HMG-CoA reductase inhibitor or the standard (lovastatin). Thereafter, the labeled precursor ^{14}C sodium acetate is added to the medium, and the incubation continued for further 3 or 24 h. To one part of the cells, an internal standard of ^{3}H-cholesterol is added and the cells are saponified by alkaline. The lipids of these saponified cells are extracted with chloroform/methanol. The lipid mixture is separated by preparative thin-layer chromatography. The localization of cholesterol is identified by exposure to iodine vapor, and the amount of newly synthesized ^{14}C cholesterol is determined in a scintillation counter.

Cell protein is determined in another part of the cells. A third part of the cell culture is used to control the integrity of the cells by light microscopy and tested biologically by determination of the release of lactate dehydrogenase into the incubation medium.

Evaluation

The amount of newly synthesized cholesterol per mg of cell protein is calculated. The inhibitory capacity of the test drug is estimated against a solvent control. IC_{50} values are calculated from the results of various concentrations.

Modifications of the Method

Chen and Kandutsch (1976) studied the effects of cholesterol derivatives on sterol biosynthesis in cultures of various cell types.

Liver specificity of inhibition of sterol synthesis is studied by in vitro uptake into hepatocytes and human skin fibroblasts (Scott 1990) or in slices of various tissues after oral treatment with HMG-CoA reductase inhibitors (Tsujita 1990).

Shaw et al. (1990), however, caution against the use of HepG2 cells which may not be the cell system of choice to demonstrate liver selectivity of inhibitors of HMG-CoA reductase.

Cultures of HepG2 cells have also been used to test inhibition of cytoplasmic acetoacetyl-CoA thiolase (Greenspan et al. 1989) or inhibition of squalene epoxidase (Hidaka et al. 1991).

Pearce et al. (1992) used HepG2 cells to study the hypocholesterolemic activity of synthetic and natural tocotrienols in vitro.

Raiteri et al. (1997) investigated drugs acting at different steps of the mevalonate pathway on arterial smooth muscle cell proliferation. Competitive HMG-CoA reductase inhibitors dose-dependently decreased smooth muscle cell proliferation.

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Ex Vivo Inhibition of Cholesterol Biosynthesis in Isolated Rat Liver Slices

Purpose and Rationale

Inhibition of cholesterol biosynthesis in rat livers can be measured in an ex vivo assay after oral treatment with HMG-CoA reductase inhibitors by cholesterol synthesis from labeled sodium octanoate.

Procedure

Male Sprague Dawley rats weighing 110–130 g are kept on a reverse light cycle (lights 3:00 p.m. to 3:00 a.m.) for 14 days prior to use. Throughout the period of adaptation, the rats have free access to a low-cholesterol diet and tap water. On the day of the experiment, the test compounds are given orally between 9:00 and 11:00 a.m. as suspensions in 0.5 % methylcellulose. After 1 hour, the rats are sacrificed, the livers removed and transferred to chilled oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4). The livers are then chopped into 0.8-mm^2 pieces using a McIlwain tissue chopper (e. g., Brinkmann Instr., Westbury, USA) and are suspended in the same buffer. Aliquots of the suspension are pipetted, in triplicate, into culture tubes which contain [^{14}C]sodium octanoate (300 µM/l, 6.67 Ci/M). The assay volume is 1 ml. The tubes are gassed with 95 % O_2/5 % CO_2 for 10 s, stoppered with a serum cap, and incubated at 37 °C in a metabolic shaker at 150 oscillations/min for 90 min.

The reaction is stopped by addition of 1 ml 15 % KOH in ethanol. An aliquot of the mixture is assayed for protein concentration. An internal standard [^{3}H]cholesterol (30,000 dpm) is added to determine recovery, which ranges from 70 % to 80 %. The tubes are saponified at 75 °C for 2 h and then extracted with 10 ml of petroleum ether for 30 min. The lower aqueous phase is frozen in a dry ice/alcohol mixture, and the ether phase is removed, washed with 2 ml glass-distilled water, and then evaporated to dryness. The [^{14}C]cholesterol synthesized is separated by thin-layer chromatography on plastic silica gel plates using chloroform as eluent. After visualization

with iodine, the cholesterol spots are cut out, and radioactivity is quantitated by liquid scintillation counting.

Similar procedure was used to measure cholesterol synthesis in the ocular lens of the eye (Mosley et al. 1989).

Evaluation

Results are expressed as percentage inhibition compared to vehicle-treated control values. Using various doses, ED_{50} values of inhibition can be calculated from dose–response curves.

Modifications of the Method

Koga et al. (1990) tested the tissue-selective inhibition of cholesterol synthesis in vivo by pravastin sodium in mice. Drugs were orally administered to mice 2 h prior to an intraperitoneal injection of [^{14}C]acetate. The animals were sacrificed 1 h afterwards, and the cholesterol synthesis was determined in various organs.

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Effect of HMG-CoA Reductase Inhibitors In Vivo

Purpose and Rationale

A strain of rabbits with heritable hyperlipidemia, the WHHL strain, has been described by Watanabe (1980), Watanabe et al. (1985, 1988). These animals develop digital xanthoma and aortic and coronary atherosclerosis already at an early age. These animals are considered to be a suitable model for the evaluation of preventive or even regressive effects of drugs on hyperlipidemia and atherosclerosis.

Procedure

Male heterozygous WHHL rabbits weighing 1.8–2.5 kg at an age between 8 and 20 weeks are used. The animals are housed individually under standard conditions (standard rabbit diet and water ad libitum) and are allowed to accommodate 2 weeks prior to treatment. The test compounds are suspended in 0.5 % methylcellulose and are administered each day orally by gavage in the afternoon to insure an increased plasma level at night, since in man HMG-CoA reductase activity has been found to be higher at night than during daytime (Shapiro and Rodwell 1969; Shefer et al. 1972) similar to the enzyme in rodents. The treatment is continued for 14 days.

Blood samples are taken in the morning without previous feeding. Two ml of blood are drawn

from the outer ear vein 5 days prior to the beginning of treatment, on days 3 and 8 of treatment, and 30 days after the end of treatment for the determination of biochemical parameters. In addition, 6 ml of blood are drawn at the first and the last day of treatment and 10 days after the end of treatment for determination of biochemical parameters and lipoprotein profile. In order to obtain serum, blood is allowed to clot at room temperature and then centrifuged twice at 10,000 rpm.

The following biochemical parameters are determined in nonfrozen samples (kept at 4 °C): total cholesterol, HDL cholesterol, triacylglycerol, as well as creatinine, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), and γ-glutamyl transferase (γ-GT) using commercially available kits.

The separation of serum lipoproteins by gel permeation chromatography is performed according to Ha and Barter (1985). This method is particularly well suited for the metabolic studies of lipoproteins, because the elution profile can be obtained from the same sample under more gentle conditions than by sequential ultracentrifugation. According to their descending particle size, the elution profile of lipoproteins in the same fraction of density ($<$1.21 g/ml) shows, as the elution progresses, three major peaks which correspond to very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), respectively.

Since the amount of serum collected from one rabbit is too low to determine the lipoprotein profile from each sample, the total lipoproteins are isolated by ultracentrifugation at a density $<$1.21 g/ml, and the lipoproteins thus obtained in each group are pooled two by two prior to injection onto the cross-linked agarose HR 50 column (Superose 6B). As a result, each lipoprotein profile represents the lipoprotein size distribution of an equal volume of lipoproteins obtained from two rabbits. Cholesterol and triacylglycerol concentrations are determined in each 1 ml elution fraction.

Evaluation

The data at 5 days before beginning of treatment and of day 0 of each animal are pooled, and the mean is taken as reference value. Student's paired *t*-test is used to calculate for each group the significance of difference between mean values.

Modifications of the Method

Extension of the treatment period up to 24 weeks allows to evaluate the meanwhile apparent atherosclerotic lesions in the aorta and the coronaries by gross observation and light microscopy (Tsujita 1990).

Kasim et al. (1993) studied the effect of lovastatin on the secretion of very-low-density lipoprotein lipids and apolipoprotein B in the Zucker obese rat which is basically a model for genetic hypertriglyceridemia.

Soma et al. (1993) studied the effects of HMG-CoA reductase inhibitors on carotid intimal thickening induced by placing a nonocclusive, biologically inert, soft, hollow Silastic collar around both carotid arteries in normocholesterolemic rabbits (Booth et al. 1989).

Bocan et al. (1994) assessed atherosclerotic lesion development in the thoracic artery and chronically denuded iliac-femoral artery of hypercholesterolemic New Zealand White Rabbits using inhibitors of HMG-CoA reductase.

Krause and Newton (1995) tested the lipid-lowering activity of atorvastatin and lovostatin in several rodent species (rat models, guinea pigs, rabbits). They concluded that normal rats can be used as a preclinical tool to assess the efficacy of HMG-CoA reductase inhibitors since triglyceride lowering correlates with cholesterol lowering in LDL animal models.

Johnston et al. (2001) induced atheroma formation in the aortas of C57BL/6 mice by long-term administration of Poloxamer 407, a nonionic surfactant, and tested the potency of selected statin drugs in this model of hyperlipidemia and atherosclerosis.

Delsing et al. (2003) studied differential effects of a calcium antagonist and a statin and their combination on atherosclerosis in APOE*3-Leiden transgenic mice.

Aoki et al. (2002) induced postprandial lipemia in rats by administration of a lipid emulsion and measured the triglyceride-lowering effect of a statin.

Ugawa et al. (2002) established an experimental model of the escape phenomenon in hamsters in which plasma cholesterol, initially reduced by statins, increases again on long-term administration, and tested the effectiveness of a squalene synthase inhibitor in this model.

Yokota et al. (2003) tested the protective effect of a HMG-CoA reductase inhibitor on experimental renal ischemia-reperfusion injury in mice.

Ableshauser et al. (2002) reported a new method for biosensing of arteriosclerotic nanoplaque formation and interaction with an HMG-CoA reductase inhibitor and recommended this method for experimental and clinical studies.

Holdgate reported molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin (Holdgate et al. 2003).

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Influence of Statins on Endothelial Nitric Oxide Synthase

Purpose and Rationale

Several studies indicate that the beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) on endothelial function and cardiovascular ischemic events may be attributed not only to their lipid-lowering effect but also to direct effects on the atherosclerotic vessel wall. One such effect is the upregulation of nitric oxide synthase, which generally leads to vasorelaxation (Kano et al. 1999; Amin-Hanjani et al. 2001; Rikitake et al. 2001; Sata et al. 2001; Sumi et al. 2001; Jones et al. 2002; Laufs et al. 2002; Mitani et al. 2003a, b; Parker et al. 2003; Wassmann and Nickenig 2003; Yamamoto et al. 2003; Kumai et al. 2004, (Balakumar et al. 2012)). Statins have also proangiogenic and reendothelialization properties following vessel injury. (Walter et al. 2004).

Parker et al. (2003) compared different statins for eNOS subcellular localization, formation of pro-oxidants, and endothelial-dependent vascular function.

Procedure

Endothelium-Dependent Vascular Relaxation Ex Vivo in Statin-Treated Rats

Sprague Dawley rats (~160 g) on standard chow are treated with HMG-CoA reductase inhibitors for 8 days at doses that do not result in plasma cholesterol reductions as determined in previous experiments. To assay vascular relaxation, aortas are dissected out and perivascular tissue removed. Aortic rings (~5 mm) are incubated at 37 °C in pH 7.4 Krebs-bicarbonate buffer with 5.mM glucose and 95 % O_2/5 % CO_2 and suspended on a strain gauge transducer to measure isometric circumferential tension. After adjustment basal tension to 2.0 g over 90 min, aortic rings are contracted submaximally with a thromboxane receptor agonist (U-46619) and exposed to increasing concentrations of acetylcholine (or nitroprusside as control), which is known to induce endothelium-dependent relaxation by activating eNOS.

Data are expressed as mean (±SEM) percentage decrease in tension from the level of induced tone. Significance is determined with Student's *t*-test/two-way ANOVA.

Vascular Endothelial Cell Culture

Human arterial endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) are used at passages 3–8. Culture and statin incubations are conducted in Clonetics complete EGM medium (M-188-derived) with 2 % serum. For experiments using redox probes, cells are grown in 24-well plates or collagen-coated glass chamber slides to near confluency.

For cell-based assays using oxyhemoglobin and cytochrome c, endothelial cell cultures are pretreated with statins in flasks, then harvested by brief trypsinization, centrifuged and suspended in Krebs-Ringer buffer with 5.5 mM glucose and 95 % O_2/5 % CO_2 for assay in cuvettes.

Fluorescence Assays for Intracellular Reactive Nitrogen and Oxygen Species

As peroxynitrite indicator DHR123 is used, which is nonfluorescent and enters cells readily where it is oxidized (with limited selectivity by $^{=}$ONOO and $^{=}$OH) to the cationic, fluorescent rhodamine 123 (RH123), which is entrapped in mitochondria (Kelm et al. 1997). Statins are added to endothelial cell cultures for 4–16 h followed by addition of DHR123 (5 μM), bradykinin (1 μM), and A23187 (1 μM) to activate eNOS. At the indicated times, cells are washed with PBS, and intracellular fluorescence is assayed using a fluorescent plate reader (Cytofluor-4000, $\lambda ex = 485 \pm 20$, $\lambda em = 530 \pm 25$ nm).

DHR oxidation is also assayed ex vivo in aortic specimens isolated from statin-treated rats. Aortic rings are incubated in 300 μl of EGM medium containing DHR123 (5 μM), bradykinin (1 μM), and A23187 (1 μM) at 37 °C for 90 min. Samples are washed twice in PBS, the homogenized in PBS + 0.1 % Tween-20 + 0.1 % SDS, and duplicate aliquots are taken for fluorescence readings. Protein is determined by the Bradford method. A fluorescence standard curve for DHR123 oxidation product (HR123) is generated, and results are calculated as pmol product per mg protein.

Spectrophotometric Assay for Superoxide Anion

The assay of superoxide anion in the presence of NO in intact cells is accomplished by an adaptation of the spectrophotometric method described by Kelm et al. (1997). Endothelial cells are treated with statins in monolayer culture, then detached and resuspended in Krebs-Ringer buffer. Cells are added to cuvettes (7.5×10^5 to 1.25×10^6 cells/ml) with oxyhemoglobin (3 μM), ferricytochrome c (10 μM), and $CaCl_2$ (0.1 mM) and assayed at 37 °C with continuous stirring and 95 % O_2/5 % CO_2 flow. Agonists are added at 5-min intervals. Absorbance spectra from 400 to 600 nm are collected at 1-min intervals using a Beckman DU-7500 diode array spectrophotometer with data capture software. The superoxide anion-specific reduction of cytochrome c is measured as the change over time in absorbance difference between 465 nm and the isosbestic wavelength 525 nm.

Fluorescence Microscopy with DHR123 and eNOS Immunofluorescence

Human arterial endothelial cells are grown on glass chamber slides and incubated for several hours with the statins and 10 μM DHR123. Localization of the DHR123 oxidized product is analyzed by epifluorescence using Nikon Microphot-FXA with Chroma High-Q resorufin filter and interference contrast optics. Images are captured using a photometric image point CCD video camera. For eNOS localization, human arterial endothelial cells are fixed and processed for immunofluorescence using rabbit polyclonal anti-eNOS antibody at 2 μg/ml and LRSC-conjugated donkey anti-rabbit IgG second antibody (Jackson Immunoresearch). Images comparing control and treated cells are obtained using the same CCD exposure times and video amplifier gain.

Subcellular Fractionation and Immunoblotting of Endothelial Cells

Human umbilical vein endothelial cells are incubated with statins followed by harvesting by scraping into 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, pH 7.4, with protease inhibitors (Sigma, P8340), and lysed by brief sonification in a glass homogenizer. Cell lysates are cleared at 500 *g* and fractionated by centrifugation at 16,000 *g*. The 16,000 *g* pellet is resuspended and ultracentrifuged in a discontinuous sucrose gradient (20 mM Tris–HCl, 1 mM EDTA, pH 7.4, with steps of 0.50 and 1.12 M sucrose) to isolate plasma membranes and Golgi/mitochondria-rich intracellular membranes. Microsomes and cytosol are isolated from the 16,000 *g* supernatant. Samples are assayed for eNOS protein level by SDS-PAGE (4–20 % gradient gels) and western transfer to PVDF membranes (Novex LC-2002) and probed using rabbit polyclonal anti-eNOS antibody with peroxidase-linked

anti-rabbit IgG for chemiluminescence and densitometry. Data are given as percentage of control absorbance units from densitometry.

Sterol Synthesis in Endothelial Cells

Cholesterol synthesis is assayed in human arterial endothelial cells (Parker et al. 1990) grown to confluence and incubated for 16 h in serum-free standard medium + 1.0 % serum albumin. Statins are added in various concentrations in DMSO vehicle, and 0.5 h sterol synthesis is assayed by addition of Na-[1-^{14}C] acetate (4.0 μCi/well, 2.0 mCi/ml, 58 mCi/mmol) and incubation for 4 h at 37 °C. Radiolipids in washed and lysed cells are extracted into chloroform–methanol and cell cholesterol is resolved by silica gel thin-layer chromatography. Radioactivity is determined by phosphorimager, and the 50 % inhibitory concentration (IC_{50}) is calculated and compared to that in vehicle controls.

Evaluation

Data obtained by these methods suggest that the action of lipophilic statins in endothelium can shift eNOS localization toward intracellular domains, thereby increasing the encounter with metabolically generated superoxide anion to produce peroxynitrite and related oxidants. Under some conditions, the direct action of lipophilic HMG-CoA reductase inhibitors may unbalance NO and superoxide anion fluxes and promote oxidant stress, compromising the beneficial vascular effects of eNOS.

Modifications of the Method

Kano et al. (1999) measured upregulation of eNOS mRNA in the rabbit aorta by an HMG-CoA reductase inhibitor using competitive reverse-transcriptase polymerase chain reaction methods.

Laufs et al. (2002) determined the expression and activity of eNOS by reverse-transcriptase polymerase chain reaction, Western blotting, and arginine–citrulline assays.

Yamamoto et al. (2003) investigated the effect of a HMG-CoA reductase inhibitor on inducible nitric oxide synthase expression in rat vascular smooth muscle cells using a modification of the semiquantitative reverse transcription polymerase chain reaction.

Ikeda et al. (2003) reported the protection of ischemic-reperfused myocardium in normocholesterolemic rats by a HMG-CoA reductase inhibitor.

Baetta et al. (2002) found that in the absence of lipid lowering, tissue factor expression in infiltrated inflammatory cells and macrophage accumulation in carotid lesions of cholesterol-fed rabbits are reduced by statin treatment.

Shimizu et al. (2003) performed heterotopic murine cardiac transplants in total allogenic or histocompatibility class II-mismatched combinations. Direct anti-inflammatory mechanisms of statins may contribute to the attenuation of experimental allograft arteriosclerosis.

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Inhibition of Squalene Synthase

Purpose and Rationale

Squalene synthase (farnesyl-diphosphate:farnesyl diphosphate farnesyltransferase) is an enzyme vital for cholesterol biosynthesis. It catalyzes the dimerization of two farnesyl pyrophosphate molecules to form squalene, a key cholesterol precursor. Unlike HMG-CoA reductase inhibitors,

squalene synthase inhibitors do not lower the levels of ubiquinone and dolichol in vivo, both essential for cell growth and viability. Several natural and synthetic squalene synthase inhibitors have been described (Biller et al. 1991a, b; Oehlschlager et al. 1991; Baxter et al. 1992; Ciosek et al. 1993; Harris et al. 1995; Lindsey and Harwood 1995; Chan et al. 1996; Dufresne et al. 1996; McTaggart et al. 1996; Amin et al. 1996, 1997; Sliskovic and Picard 1997; Rosenberg 1998; Vaidya et al. 1998; Hiyoshi et al. 2000, 2003; Ugawa et al. 2000; 2002, 2003; Amano et al. 2003; Ishihara et al. 2003; Nishimoto et al. 2003; Trapani et al. 2011). Ugawa et al. (2000) determined squalene synthase activities in microsomes.

Procedure

Microsomes are prepared from the livers of rats, hamsters, guinea pigs, beagle dogs, and rhesus monkeys as well as from HepG2 cells. The tissues or harvested cells are homogenized in 50 mM HEPES buffer using a glass homogenizer. Homogenates are centrifuged at 500 *g* for 15 min at 4 °C, and the resulting supernatants are further centrifuged at 8000 *g* for 15 min at 4 °C. Microsomes are then isolated from this second supernatant by ultracentrifugation at 100,000 *g* for 60 min at 4 °C. The microsome precipitates are suspended in HEPES buffer (1–5 mg/ml) and stored in aliquots at −80 °C.

Squalene synthase activities of these microsomes are assayed using a modification of the technique of Amin et al. (1992). The test compounds are dissolved in DMSO, and the assays are carried out in 50 mM HEPES buffer, pH 7.5, containing (in mM): NaF 11, $MgCl_2$ 5.5, DTT 3, NADPH 1, farnesyl pyrophosphate (FPP) 5 μM, [^{3}H]-FPP (0.017 μM, 15 Ci/mmol), NB-598, a squalene oxidase inhibitor, 10 μM, and sodium pyrophosphate decahydrate 1 mM. After preincubation of these components at 30 °C for 5 min, the reaction is started by the addition of microsomes (10 μg protein). The reaction is carried out at 30 °C for 20 min, then terminated by addition of 100 μl of 1:1 solution of 40 % (w/v) KOH:ethanol. Synthesized [^{3}H] squalene is extracted in petroleum ether and counted in Aquasol-2 using a Beckman liquid scintillation counter.

Evaluation

Results are expressed as mean ± SEM and compared using two-way repeated analysis of variance (ANOVA).

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Inhibition of Squalene Epoxidase

Purpose and Rationale

Squalene epoxidase is a microsomal enzyme that catalyzes the oxidation of squalene to 2,3-oxidosqalene, the last reaction of non-sterol metabolites in the cholesterol biosynthesis pathway (Hidaka et al. 1990). Inhibitors of this enzyme are unlikely to block the production of biologically important non-sterol products, such as isoprenyl adenine, dolichol, coenzyme Q, or heme A, in contrast with HMG-CoA reductase inhibitors, which inhibit their production (Ghirlanda et al. 1993; Chugh et al. 2003). Several squalene epoxidase inhibitors have been described (Horie et al. 1990, 1991; Hikada et al. 1991; Moore et al. 1992; Gotteland et al. 1998; Sawada et al. 2001, 2004; Trapani et al. 2011).

Sawada et al. (2001, 2004) described a modified squalene epoxidase assay.

Procedure

HepG2 cells are cultured routinely in medium A (Eagle's modified minimum essential medium supplemented with 1 mM pyruvate and nonessential amino acids with 10 % heat-inactivated fetal calf serum) at 37 °C in a humidified 95 % O_2/5 % CO_2 air atmosphere.

For the assay, the cells are grown in 225-cm^2 culture flasks and incubated for 18 h in medium A containing 10% human lipoprotein-deficient serum and 1 μM of the squalene synthase inhibitor L-654949, to increase the squalene epoxidase activity. The HepG2 cells are washed and harvested by trypsin treatment. After centrifugation (100 *g*, 5 min at 4 °C), the supernatant fraction is removed by aspiration. The cell pellet is frozen and kept at −80 °C until use. On the day of the experiment, the cell pellet is thawed, ruptured by sonication (5 s at 4 °C) in 0.1 M Tris–HCl, pH 7.5 containing 1 mM EDTA, mixed4$:1 with 2 % Triton X-100, allowed to stand at 4 °C for 30 min and assayed for squalene epoxidase activity (Tai and Bloch 1972). Aliquots of the mixture are incubated for 90 min at 37 °C with or without test compound dissolved in 1 % DMSO in a final volume of 0.3 ml containing 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM NADPH, 1 mM FAD, 0.3 mM AMO1618 (an inhibitor of 2,3-oxidosqualene cyclase), 0.17 % Triton X-100, and 8 μM[^{3}H]-squalene (3.7 kBq) dispersed in 0.075 % Tween 80. The reaction is stopped by the addition of 0.3 ml of 10 % ethanolic KOH. After incubation for 90 min at 75 °C, nonsaponifiable materials are extracted with 2 ml of petroleum ether. The extracts are evaporated under a nitrogen stream. The residue is taken up in a small volume of diethyl ether, spotted on a silica gel thin-layer chromatography plate and developed in benzene/ethyl acetate (99.5:0.5, v/v). The radioactivity corresponding to 2,3-oxidosqualene is counted by autoradiography.

Evaluation

The results are expressed as means ± SD and evaluated by repeated measures analysis of variance, followed by Dunnett's multiple comparisons.

Modifications of the Method

A simplified squalene epoxidase assay based on HPCL separation and time-dependent UV/visible determination of squalene is published by Grieveson et al. (1997).

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Inhibition of Cholesterol Absorption

Philippe Boucher and Hans Gerhard Vogel

Contents

Inhibition of ACAT (Acyl Coenzyme A: Cholesterol Acyltransferase)

General Considerations

Acyl coenzyme A:cholesterol acyltransferase (ACAT), which catalyzes the intracellular formation of cholesteryl esters, plays an important role in the intestinal absorption of cholesterol, foam cell formation within the arterial wall, and VLDL production in the liver. Two isoforms of the enzyme exist, ACAT1 expressed in macrophages and ACAT2 expressed in intestine and liver. Cholesterol is absorbed from the gut exclusively in the unesterified form but appears in the lymph esterified with various long-chain unsaturated fatty acids. The enzyme responsible is ACAT, a microsomal enzyme that utilizes long-chain fatty acyl coenzyme A and cholesterol as substrates. ACAT inhibitors also have potential actions beyond inhibition of cholesterol absorption. Inhibition of hepatic ACAT could reduce the production of cholesteryl esters for packaging into lipoproteins, while inhibition of ACAT1 in macrophages could reduce the deposition of cholesteryl esters and prevent the formation of foam cells and atherosclerotic lesions (Nissen et al. 2006; Pal et al. 2013; Rudel and Farese 2006). Nevertheless, from investigations in ACAT2 knockout mice, it is known that ACAT2 is not the only rate-limiting pathway for cholesterol absorption. On normal chow, these mice absorb cholesterol as efficiently as wild-type mice, only if mice were fed a

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cholesterol-enriched diet. ACAT2 is important for cholesterol absorption (Lee et al. 2004; Repa et al. 2004). Under physiological conditions, alternative pathways compensate for the ACAT2 deficiency in mice (Hussain et al. 2005).

Human ACAT cDNA was cloned from a human macrophage cDNA library and expressed in an ACAT deficient line of Chinese hamster ovary cells. The cDNA, labeled K1, encoded an integral membrane protein of 550 amino acids (Chang et al. 1993). The inhibition of ACAT as treatment for hypercholesterolemia and atherosclerosis is an attractive target.

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In Vitro ACAT Inhibitory Activity

Purpose and Rationale

In vitro ACAT inhibitory activity can be determined in microsomal preparations from liver or intestine of rabbits.

Procedure

Hepatic or intestinal microsomes are prepared from rabbits. Prior to sacrifice, the animals receive chow supplemented with 2 % cholesterol and 10 % safflower oil for 6 weeks. Each assay contains 0.2 mg of microsomal protein and fatty acid-poor bovine serum albumin (3 mg/ml) in 0.04 M KH_2PO_4 buffer, pH 7.4, containing 0.05 M KCl, 0.03 M EDTA, and 0.3 M sucrose. Drug dilutions are made in DMSO (5 µl DMSO/200 µl total incubation volume). The reaction is started by the addition of [^{14}C]oleyl CoA (50 µM, 7 dpm/pmol). After 3 min, the reaction is stopped by the addition of chloroform-methanol 2:1. [^{3}H]Cholesteryl oleate is used as an internal standard. Lipid extracts are dissolved in chloroform, spotted on TLC plates (silica gel G), and developed in hexane-petroleum ether-acetic acid 80:20:1. Unlabeled, carrier cholesterol oleate is added to the internal standard to aid band visualization with iodine vapor. The band corresponding to cholesteryl esters is then scraped into scintillation vials and radioactivity determined by liquid scintillation spectroscopy. The ACAT1-specific ACAT inhibitor K-604 can be employed as a control at final concentration of 1 µM (Ikenoya et al. 2007).

Evaluation

For each compound, four concentrations are evaluated in duplicate. IC_{50} values are determined by performing a nonlinear least-squares fit of the data to a log dose–response curve.

Modifications of the Method

Rothblatt et al. (1977) studied ACAT activity in Fu5 rat hepatoma cells under the influence of hyperlipemic serum lipoproteins.

Mathur et al. (1981) studied ACAT activity in hepatic microsomes of cynomolgus monkeys during diet-induced hypercholesterolemia.

Einarsson et al. (1989) studied acyl-CoA: cholesterol acyltransferase activity in human liver microsomes.

Largis et al. (1989) found CL 277,082 to be a potent inhibitor of ACAT in microsomes from a variety of tissues and smooth muscle cells in culture.

Heffron et al. (1990) studied ACAT inhibition in microsomal fractions of the transformed mouse macrophage J774.

Bell et al. (1992) measured ACAT inhibition in cultured Fu5AH cells.

Field et al. (1991) and Krause et al. (1993) tested the inhibition of ACAT in CaCo-2 cells.

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In Vivo Tests for ACAT Inhibitory Activity

Purpose and Rationale

Most authors test the in vivo antiatherosclerotic and antihyperlipemic effect of ACAT inhibitors in cholesterol-fed hypercholesterolemic animals (Balasubramaniam et al. 1990; Harris et al. 1992; Tanaka et al. 1994). Krause et al. (1993a) used the Zilversmit dual isotope technique (Zilversmit 1972; Cayen and Dvornik 1979) for determining cholesterol absorption in rats.

Procedure

Male Sprague–Dawley rats weighing 200–225 g are fed with a diet containing 5.5 % peanut oil, 0.5 % cholic acid, and 1.5 % cholesterol with or without (control) drugs for 1 week. On the last day, food is removed at 8:00 a.m. and the isotopes administered beginning at 2:00 p.m. [^{3}H]cholesterol (13 μCi/rat) is given by oral gavage and [^{14}C] cholesterol (1.5 μCi/rat) by tail vein injection. The [^{3}H]cholesterol is prepared as an emulsion by dissolving 125 mg cholesterol in 1,625 mg olive oil. The oil phase is suspended by sonication in 25 ml of water containing 156 mg taurocholate (sodium salt). Each animal receives 1 ml. The intravenous dose is prepared by drying the labeled cholesterol (50 μCi) and then adding 300 μl warm ethanol followed by 12.5 ml of saline. Each animal receives 0.5 ml of this colloidal suspension. The rats are allowed to consume their respective diets at 3:00 p.m. and are sacrificed 48 h after the isotope administration.

Evaluation

The percentage of an oral dose of cholesterol absorbed is calculated from the plasma isotope ratio (% of the oral dose in 2 ml plasma/of the intravenous dose in 2 ml plasma × 100).

Further In Vivo Methods

Heider et al. (1983) measured cholesterol absorption via the Zilversmit dual isotope method in rabbits.

Gillies et al. (1990) studied the regulation of ACAT activity by a cholesterol substrate pool during the progression and regression phases of atherosclerosis in rabbits with dietary induced atherosclerosis.

Krause et al. (1993b) compared the activities of two ACAT inhibitors in normocholesterolemic and hypercholesterolemic rats, rabbits, guinea pigs, and dogs.

Nagata et al. (1995) tested a new ACAT inhibitor in diet-induced atherosclerosis formation in female C57BL/6 J mice.

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Lymph Fistula Model for Cholesterol Absorption

Purpose and Rationale

Direct evidence for an inhibitory effect on cholesterol absorption can be obtained by the lymph

fistula model in rats. This model also provides an indication as to the duration of inhibition and the relative selectivity of the compound on the absorption of cholesterol versus triglyceride and phospholipid.

Procedure

Rats are anesthetized by an intramuscular injection of tiletamine/zolazepam (Telazol, 40 mg/kg). Silicon rubber cannulae are placed into the main mesenteric lymph duct and into the duodenum and secured with sutures. Animals are allowed to recover from surgery overnight in restraining cages while infused intraduodenally with 2 % dextrose in saline containing 0.03 % KCl (2.5 ml/h). Drinking water is allowed at libitum during this recovery period.

At 6:00 a.m. the following day, the drinking water is removed and a 2-h basal lymph sample collected. Then, the animals are given the ACAT inhibitor at a specified dose as a single bolus into the duodenal cannula using an aqueous CMC/Tween suspension vehicle. Controls receive a bolus injection of the vehicle alone. Immediately after the drug dose, a lipid emulsion containing 0.1 % cholesterol, 0.11 % sodium taurocholate, 15 % Intralipid (20 %, Kabivitrium Inc.), 2.4 % safflower oil, and 82.6 % saline is infused into the duodenal cannula (3 ml/h). Then, four 2-h lymph collections are obtained. The lymph samples are extracted into hexane in the presence of a stigmasterol internal standard. Total and free cholesterol are quantitated by liquid gas chromatography.

Evaluation

Esterified cholesterol of lymph is determined from difference between total and free cholesterol.

Modifications of the Method

The lymph fistula model can also be used to examine the effect of ACAT inhibitors on the absorption of endogenous (i.e., biliary) cholesterol. Cannulated rats are infused intraduodenally with the saline/dextrose solution to which 2 % whole rat bile is added containing [^{14}C]cholesterol. No nonradiolabeled lipid other than that in bile is infused into the animals, and hence, lymph cholesterol is exclusively of biliary origin. Hourly collections of lymph are obtained with the use of fraction collectors. Lymph is extracted with three volumes of ethylacetate-acetone 2:1 (Slayback et al. 1977). The total ^{14}C label of an aliquot of lymph extract is determined by liquid scintillation spectroscopy. The lymph extracts are resuspended in 100 µl chloroform-methanol 2:1 and spotted on Whatman LK6D TCL plates. The plates are developed in hexane-diethylether-acetic acid 85:15:1. The distributions of ^{14}C label between the cholesteryl ester and cholesterol bands are visualized and quantified by exposing the plates to phosphor imaging plates for 16 h and then scanning the imaging screens on a Molecular Dynamics Phosphorimager.

Clark and Tercyak (1984) studied the absorption of cholesterol during inhibition of mucosal acyl-CoA: cholesterol acyltransferase in mesenteric lymph fistula rats with normal pancreatic function.

Åkerlund and Björkhem (1990) and Björkhem et al. (1993) used the lymph fistula model in rats for studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase. The thoracic lymph duct was cannulated just proximal to the cisterna magna through an abdominal approach. The proximal part of the lymph duct was ligated. The cannula exited at the back of the animal. The lymph was allowed to flow freely from the animal to the bottom of the metabolic cages. Under these conditions, the rats could move freely in the cages during the lymphatic drainage.

Critical Assessment of the Method

Gallo et al. (1987) found normal cholesterol absorption in rats in which intestinal acyl coenzyme A:cholesterol acyltransferase activity was significantly reduced by ACAT inhibitors. This challenges the value of ACAT inhibition at least in normal individuals.

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Interruption of Bile Acid Recirculation

Philippe Boucher and Hans Gerhard Vogel

Contents

Interruption of Bile Acid Recirculation

Cholestyramine Binding

Purpose and Rationale

Cholesterol is metabolized in the liver by oxidation to bile acids which undergo enterohepatic circulation. In the untreated state, approximately 95 % of the bile acids that are secreted are reabsorbed and returned to the liver, while the small loss is replaced by de novo biosynthesis from cholesterol. Increased excretion of bile acids with the feces increases the rate of oxidation of cholesterol in the liver leading to a partial depletion of the hepatic cholesterol pool. A compensatory increase in uptake via the LDL receptors results in lower serum LDL levels. This can be achieved by addition of a bile acid-binding resin, e.g., cholestyramine, to the food. The binding of unconjugated and conjugated bile-salt anions can be tested in vitro (Johns and Bates 1969).

Procedure

Rabbits weighing 2.5–3 kg are switched from standard food to a diet containing 10–20 % polymeric basic-anion exchanging resin, e.g., cholestyramine. Cholesterol levels in serum are measured at the beginning and at the end of a 4-week feeding period.

Evaluation

Cholesterol levels as means ± SD are calculated for controls and treated animals and compared by statistical analysis.

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Modification of the Method

Tennent et al. (1960) tested polymeric organic bases for action on blood cholesterol in 4-day experiments and in experiments of 7–8-week duration in cholesterol-fed White Leghorn cockerels. The birds were given a diet containing 2 % cholesterol and 5 % cotton-seed oil with or without addition of polymeric bases. The increase of cholesterol and the incidence of aortic atheromatosis were decreased by polymeric organic bases.

Day (1990) compared the hypocholesterolemic activities of the bile acid sequestrants cholestyramine and cholestipol hydrochloride in cholesterol-fed sea quail.

Quaternary ammonium conjugates of bile acid inhibited cholic acid binding and transport in everted ileal sacs of guinea pigs in vitro (Fears et al. 1990).

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Inhibition of Lipid Oxidation

Philippe Boucher and Hans Gerhard Vogel

Contents

General Considerations

Oxidative modification of the low-density lipoproteins (LDL) has been shown to cause accelerated degradation of LDL via the scavenger receptor pathway. Under conditions of high serum LDL levels, LDL particles can migrate into the subendothelial space where oxidation of LDL can occur (Heinecke 1998; Jiang et al. 2011). The actual oxidation process is believed to begin with lipid peroxidation, followed by fragmentation to result in short-chain aldehydes. These aldehydes can form adducts with the lysine residues of apo B, creating a new epitope which is recognized by the scavenger receptor of macrophages.

During the same process, lecithin is converted to lysolecithin, which is a selective chemotactic agent for monocytes. The monocytes adhere to the arterial wall and penetrate through to the subendothelium. Once there, the monocyte changes to a tissue macrophage which takes up the oxidized LDL via the scavenger receptor. The uptake of oxidized LDL continues until the macrophage is engorged with cholesteryl esters ultimately forming a foam cell. Groups of these foam cells constitute a fatty streak. By inhibiting the oxidation of LDL, it is hoped that the modification of apo B and the production of chemotactic lysolecithin can be prevented.

The family of receptors for mammalian low-density proteins has been reviewed by Hussain et al. (1999).

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Inhibition of Lipid Peroxidation of Isolated Plasma Low-Density Lipoproteins

Purpose and Rationale

Hypercholesterolemic Watanabe rabbits are considered to be a suitable model to study the effect of antioxidants as antiatherosclerotic agents (Carew et al. 1987; Kita et al. 1987; Steinberg et al. 1988; Dresel et al. 1990). Plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits is used to test the inhibition of Cu^{2+}-induced lipid peroxidation of isolated low-density lipoproteins (LDL).

Procedure

Animals of a modified Watanabe heritable hyperlipidemic rabbit strain (Gallagher et al. 1988) are used. The animals are fed over a period of 12 weeks with Purina rabbit chow diet with or without 1 % of test compound or standard (probucol). Plasma samples are collected in Na_2EDTA (0.1 % final concentration). LDLs are isolated from each rabbit plasma using a sequential ultracentrifugation technique at $d = 1.019–1.063$ g/ml (Mao et al. 1983). LDLs are then dialyzed against phosphate-buffered saline (PBS, 0.01 M sodium phosphate, 0.12 M NaCl, pH 7.4) at 4 °C for 24 h.

For determination of LDL lipid peroxidation induced by Cu^{2+}, 100 µg of each LDL sample is adjusted to a volume of 1.5 ml with distilled water. Lipid peroxidation is initiated by addition of $CuSO_4$ to a final concentration of 5 µM followed

by an incubation at 37 °C for 3 h. The reaction is stopped by adding 100 µl of 50 mM Na_2EDTA. Fifty micrograms of LDL from the reaction mixture are added to 1.5 ml of 20 % trichloroacetic acid and vortexed. Finally, 1.5 ml of 0.67 % thiobarbituric acid (TBA) in 0.05 N NaOH is added, and the mixture is incubated at 90 °C for 30 min. Samples are centrifuged at 1,500 rpm for 10 min. The absorbance of the supernatant fractions is determined at 532 nm to estimate the content of lipid peroxides (thiobarbituric acid-reactive substances). A standard curve (0–5 nmol) of malondialdehyde is generated using malondialdehyde bis(dimethyl acetal) as reference to determine the lipid peroxidation content in Cu^{2+}-treated LDL.

Evaluation

The content of lipid peroxide in LDL is plotted against the drug concentration in LDL fractions. The extent of Cu^{2+}-induced peroxidation decreases with increasing drug concentrations. The effects of test compounds are compared to the standard.

Modifications of the Method

Inhibition of iron-dependent lipid peroxidation by test compounds was measured by Braughler et al. (1987) and Yoshioka et al. 1989).

Yamamoto et al. (1986) studied the effects of probucol on lipid storage in macrophages in vitro in the presence of acetylated low-density lipoprotein using macrophage-like cells (UE-12) established from a human histiocytic lymphoma cell line.

Barnhart et al. (1989) used LDL from human plasma to study the concentration-dependent antioxidant activity of probucol.

Parthasarathy et al. (1986) incubated LDL from human plasma samples with rabbit aortic endothelial cells and measured the increase in electrophoretic mobility, the increase in peroxides, and the increase in subsequent susceptibility to macrophage degradation.

Mansuy et al. (1986) studied the inhibition of lipid peroxidation induced in liver microsomes either chemically by $FeSO_4$ and reducing agents (cysteine or ascorbate) or enzymatically by NADPH and CCl_4.

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Internalization of Labeled LDL into HepG2 Cells

Philippe Boucher and Hans Gerhard Vogel

Contents

Internalization of Labeled LDL into HepG2 Cells

Purpose and Rationale

Enhanced uptake of low-density lipoproteins (LDL) via the LDL receptor into liver cells results in reduced plasma cholesterol levels. This can be tested in the cultured hepatoma cell line HepG2.

Procedure

Heterozygous WHHL rabbits are treated with the test compound over a period of 4 weeks. Blood is withdrawn twice weekly before, during, and after treatment for determination of total plasma cholesterol, low-density lipoprotein cholesterol, total triglycerides, and high-density lipoprotein cholesterol. Lipoprotein-deficient serum is prepared by ultracentrifugation (Goldstein et al. 1983). Serum of treated and untreated animals is used for the uptake assay being prepared by centrifugation to remove the clots, followed by heat inactivation of the complement system and sterilization through a 0.45 μm filter. In each preparation, 2 mg LDL (*d*, 1.019 to 1.050 g/ml) at 10 mg/ml is iodinated by the monochloride method to a specific activity of 300 cpm/ng (Huettinger et al. 1984).

HepG2 cells are grown in 5 cm dishes in Eagle's minimum essential medium to 60 % confluence. Serum prepared from treated animals and

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from the same animals in the pretreatment period is added from 1 % to 50 % to the medium used to grow HepG2 cells. After incubation of 18 h, cells are washed and incubated in Eagle's minimum essential medium plus 2 % bovine serum albumin and 8 µg labeled LDL for 3 h. The cells are then washed and solubilized in sodium hydroxide, and the content of each dish is counted for radioactivity. An aliquot is used to determine protein content.

Similar experiments were done in HEK 293 cells using fluorescent 3,3′-dioctadecylindocarbocyanine (DiI) particles (Fisher et al. 2007).

Evaluation

Values are given for specific uptake, which is calculated from the difference of total uptake minus uptake measured when a 40-fold excess of unlabeled LDL is present in the incubation medium of duplicate or triplicate incubations. The effect of the test drug is demonstrated by an increase of uptake with increasing percentage of serum of treated animals containing the active drug.

Modifications of the Method

Sprague et al. (1993) measured the inhibition of scavenger receptor-mediated modified low-density lipoprotein endocytosis in cultured bovine aortic endothelial cells.

Takano and Mowri (1990) produced a monoclonal antibody which recognizes peroxidized lipoproteins.

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Influence of Peroxisome Proliferator-Activated Receptors (PPARs) and Liver X Receptors (LXRs) on Development of Artherosclerosis

Philippe Boucher and Hans Gerhard Vogel

Contents

General Considerations

Peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) are nuclear receptors that regulate systemic glucose and lipid metabolism. They are expressed by macrophages in which they modulate cholesterol homeostasis and inflammation. These receptors heterodimerize with retinoid X receptors (RXRs) to modulate transcription at the promoters of target genes. They act as lipid sensors and bind with micromolar affinities to ligands derived from either intracellular metabolism or dietary lipids. Endogenous ligands of PPARs include fatty acids and eicosanoids, whereas metabolites of oxidized cholesterol activate the LXRs (Rigamonti et al. 2008).

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Influence of PPAR Activation

Purpose and Rationale

Peroxisome proliferator-activated receptors (PPARs) play a major role in atherosclerosis (Brun et al. 1996; Pineda Torra et al. 1999; Duval et al. 2002; Tailleux et al. 2003, Gizard and Bruemmer 2008; Huang and Glass 2010; Takano and Komuro 2009; Tontonoz and Spiegelman 2008). The PPAR family consists of three proteins: α, β/δ, and γ. Experimental data suggest that PPARα and PPARγ activation decreases atherosclerosis progression not only by correcting metabolic disorders but also through direct effects on the vascular wall. PPARs modulate the recruitment of leukocytes to endothelial cells, control the inflammatory response and lipid homeostasis of monocytes/macrophages, and regulate inflammatory cytokine production by smooth muscle cells. PPAR agonists, such as fibrates and thiazolidinediones, are recommended for the treatment of atherosclerosis, diabetes, and obesity (Willson et al. 2000; Etgen and Mantio 2003; Francis et al. 2003; Wang and Tafuri 2003; Ferré 2004; Kota et al. 2005). Scavenger receptors are expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors (Chinetti et al. 2000). PPARγ is a regulator of monocyte/macrophage function (Ricote et al. 1999, Glass and Saijo 2010).

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PPARα

Purpose and Rationale

Several studies indicate the lipid-lowering role of PPARα in rodents (Chinetti et al. 2001; Vosper et al. 2002; Fruchart 2009). PPARα is highly expressed in the rodent liver where activation of these receptors with fibrates induces a massive increase in peroxisomal fatty acid oxidation in hepatocytes. This provides a powerful action for the clearance of fat from the serum. Fibrates also increase the expression of the liver fatty acid-binding protein (Lawrence et al. 2000) and the hepatocyte-secreted apolipoproteins apoA-I and apoA-II (Vu-Dac et al. 1995). Lee et al. (1995) demonstrated that PPARα is responsible for clofibrate-induced fatty acid oxidation by disrupting the PPARα gene in mice. Mice lacking apolipoprotein E are characterized by severe hypercholesterolemia. Tordjman et al. (2001) reported that PPARα deficiency reduces insulin resistance and atherosclerosis in apoE-null mice (Lalloyer et al. 2011). Fu et al. (2003) found that the PPARα agonist ciprofibrate severely aggravates hypercholesterolemia and accelerates the development of atherosclerosis in mice lacking apolipoprotein E. Species differences have to be taken in account. Lawrence et al. (2001) reported that PPARα fails to induce peroxisome proliferation-associated genes in human cells independent of the level of receptor expression.

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Effect of PPARα Agonists in Mice

Purpose and Rationale

Duez et al. (2002) and (Cao et al. 2014) described reduction of atherosclerosis by the peroxisome proliferator-activated receptor α agonist fenofibrate in mice.

Procedure

Animals

Homozygous human apoA-I transgenic (Rubin et al. 1991) and apoE-deficient C57Bl/6 mouse

strains were used. Human apoA-I transgenic mice were crossed with apoE-deficient mice to generate heterozygous human apoA-I transgenic mice in a heterozygous apoE-deficient background. These mice were further mated with homozygous apoE-deficient mice to generate human apoA-I transgenic mice in a homozygous apoE-deficient background (hapoA-I Tg × apoE-deficient mice). Human apoA-I expression was analyzed in plasma by enzyme-linked immunosorbent assay using specific antibodies (Berthou et al. 1996). Homozygous apoE-deficient mice were identified after determination of total cholesterol levels. After weaning, apoE-deficient and hapoA-I Tg × apoE-deficient male mice were maintained on a standard pellet rodent chow.

Ten-week-old apoE-deficient male mice were given a Western diet containing 20 % fat and 0.2 % cholesterol before the treatment period. Then one group was treated for 8 weeks with fenofibrate (0.05 % added to the diet, corresponding to 100 mg/kg per day); the other group served as control. At the end of the study, blood was collected under anesthesia by retro-orbital puncture and tissues collected for further analysis.

ApoE-deficient male mice on a C57Bl/6 background were treated at an age of 20 weeks with 100 mg/kg per day fenofibrate by gavage. Another group served as control. After 6 weeks, the animals were sacrificed and their aortic cholesterol and cholesteryl ester content were determined.

Ten-week-old hapoA-I Tg × apoE-deficient male mice were given a Western diet for 2 weeks before treatment with either control or fenofibrate (0.05 % added to the diet, corresponding to 100 mg/kg per day) for another 8 weeks. At the end of the study, blood and tissue samples were collected for further analysis.

Lipids, Apolipoprotein, and Lipoprotein Measurements

Plasma cholesterol and triglyceride concentrations were determined by an enzymatic assay adapted to microtiter plates using commercially available reagents. Plasma human apoA-I levels were measured by an immunoelectrophoretic assay using species-specific polyclonal antibodies (Berthou et al. 1996). Lipoprotein cholesterol profiles were obtained by fast-protein liquid chromatography. This system allows the separation of three major lipoprotein classes, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) + LDL, and HDL, according to their size.

Cholesterol Measurement in the Descending Aorta

After collection of the blood, the vasculature was gently perfused through the left ventricle with cold PBS and 5 mM EDTA. For collection of aorta for biochemical analysis, all branches and adipose tissue connected to the aorta were removed, and each aorta was carefully excised from the aortic root to the right renal artery. The aortas were kept briefly on ice in PBS and then blotted dry, weighed, minced, and extracted with chloroform/methanol (2:1) according to Folch et al. (1957). The lipid extracts were dried down, resuspended quantitatively in chloroform/ methanol (2:1), and stored at −20 °C until the time of assay. Total and free cholesterol levels in the aortic extracts were determined with an enzymatic fluorometric assay (Sparrow et al. 2001). The solvent was evaporated and the lipid residue was redissolved in 100 µl of reagent-grade ethanol. Aliquots of cholesterol and cholesteryl oleate standard solutions in chloroform/ethanol (1:1) were treated similarly. To determine free cholesterol, samples and standard were incubated for 1 h at 39 °C in a total volume of 1.01 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.03 % Triton X-100 and 0.9 mM sodium cholate. Cholesterol oxidase (Roche Molecular Biochemicals), peroxidase (Roche Molecular Biochemicals), and p-hydroxyphenylacetic acid (Aldrich) were added for an additional 1-h incubation at 37 °C. The fluorescent product was measured (excitation 325 nm, emission 425 nm). For total cholesterol determinations, cholesterol esterase (Calbiochem) was included in the first incubation step, and cholesteryl oleate was used as standard. The esterified cholesterol content in each sample was calculated by subtracting the value of free cholesterol from that of total cholesterol. All values were expressed as nmol/aorta.

Analysis of Atherosclerotic Lesions in the Aorta

Hearts and ascending aortas were fixed in formaldehyde, and serial 10-mm-thick cryosections were cut from the aortic arch to the ventricles for quantitative analysis of atherosclerosis. The distance between each section was 50 mM and the final magnification 2.5-fold. Sections were stained with Oil Red O and counterstained with hematoxylin. The atherosclerotic lesion and the Oil Red O-stained areas of each section were quantified using a computer-assisted video imaging system. To avoid morphological heterogeneity between mice, the expression of average atherosclerotic lesion areas in the region of the aortic sinus, from the appearance to the disappearance of the aortic valves, was normalized at ten equal sections.

Evaluation

A nonparametric Mann–Whitney test was used to analyze for significant differences between the experimental groups. Analysis of variance (ANOVA) and Tukey post-hoc tests were used for analysis of the lesion area data.

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Effect of PPARα and PPARγ Agonists in Human Macrophages

Purpose and Rationale

Lee and Evans (2002) reviewed the role of the peroxisome proliferator-activated receptor γ in macrophage lipid homeostasis.

Chinetti et al. (1998) reported that PPARα and PPARγ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway.

Millar (2013) reported how peroxisome proliferator-activated receptors (PPARs) (α, γ, and δ) regulate several factors associated with cardiovascular risk (Millar 2013).

Procedure

Transactivation Assays of the RPR-5 Compound

Transactivation assays are performed with the RPR-5 compound in A10 cells (a rat smooth muscle cell line) using full-length human PPARα and PPARγ and the PGL3-J_3-TK vector (Vu-Dac et al. 1995). After 24 h, luciferase activity is measured with LucLite (Packard, Meridian, CT, USA) according to the manufacturer's instructions.

Cell Culture

Mononuclear cells are isolated from the blood of healthy normolipidemic donors by Ficoll gradient centrifugation and cultured (Chinetti et al. 1998). Mature monocytes are used for experiments after 10 days of culture. For treatment with the different activators, medium is changed to medium without human serum but supplemented with 1 % Nutridoma HU

(Boehringer). Efflux studies are performed in the absence of any serum. Human monocytic THP-1 cells (ATCC, Rockville, MD, USA) are maintained in RPMI 1640 medium containing 10 % of FCS and differentiated for 48 h with 100 nM PMA.

RNA Extraction and Analysis

After incubation with the PPAR activators, cells are washed with PBS and used for RNA extraction. Total RNA is extracted after 10 days from differentiated macrophages and THP-1 cells treated for 24 h with the compounds using Trizol (Life Technologies, France). For Northern blot analysis, membranes are hydrolyzed containing 10 μg of total RNA with radiolabeled ABCA1 or 36B4 control cDNA probes. An EcoRI LXRβ and a HindIII–HincII LXRα cDNA fragment are used as probes. For the human ABCA1 probe, a 1.1-kb cDNA fragment is cloned into a pCR4–TOPO vector (Invitrogen).

Transient Transfection Assay

At 24 h before transduction, COS1 cells are plated in 24-well plates at a density of 5×10^4 cells/well and cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum. Cells are transfected by lipofection using ExGen 500 (Euromedex) in Opti-MEM 1 medium, with 50 ng of reporter plasmid and increasing quantities of LXRα and RXR expression plasmids (10, 25, and 50 ng) in the presence of 50 ng of the internal control β-gal expression vector, for 3 h in serum-free medium. Fresh medium containing 0.2 % fetal calf serum and 10 μM 22(R)-hydroxycholesterol (Sigma) or its solvents are subsequently added, and cells are further incubated for 48 h.

Cholesterol Loading and Efflux

Ten-day-old human macrophages were pretreated for 24 h and thereafter every 24 h with PPAR activators (e.g., rosiglitazone 100 nM, Wy14643 50 μM) and cholesterol loaded by incubation with AcLDL (50 μg/ml, with or without [^{3}H]cholesterol) in RPMI 1640 medium supplemented with 1 % Nutridoma (Boehringer) for 48 h. After this incubation period, cells are washed twice in PBS and apoA-I-mediated cholesterol efflux studies are immediately performed by adding fresh RMPI medium without Nutridoma with or without 100 μg/ml of apoA-I for 24 h. Because, in macrophages, the equilibrium between esterified and free cholesterol is not obtained even after a 24-h additional incubation period, the experiments are performed in the absence of equilibrium.

Evaluation

For Transactivation Assays

ED_{50} values are calculated after curve fitting with XLFit (human PPARαEC_{50} = 0.13 μM, PPARγ EC_{50} = 5 μM).

For Transient Transfection Assay

Cell extracts are prepared and assayed for luciferase activity (Delerive et al. 2000). Results are normalized to internal control β-gal activity.

For Cholesterol Binding and Efflux

At the end of the incubation, intracellular lipids are extracted in hexane/isopropanol and dried under nitrogen, and free cholesterol, total cholesterol, and phospholipids are measured by enzymatic assays (Boehringer). Esterified cholesterol is measured as the difference between total and free cholesterol. Cellular proteins are collected by digestion in NaOH and measured by Bradford assay (BioRad). The percent change of intracellular cholesterol amounts in the presence of apoA-I relative to apoA-I-free medium is expressed according to the following equation:

$$\begin{aligned}&\text{Percent decrease in cellular cholesterol}\\&= \left\{\left[(\text{cellular cholesterol})_{\text{RPMI}}\right.\right.\\&\left.-(\text{cellular cholesterol})_{\text{apoAI}}\right]\\&\left.+(\text{cellular cholesterol})_{\text{RPMI}}\right\} \times 100.\end{aligned}$$

In the experiments with [^{3}H]cholesterol, radioactivity is measured, by scintillation counting, in centrifuged medium and in cellular lipids extracted with hexane/isopropanol. ApoA-I-induced [^{3}H]cholesterol is measured as the fraction of total labeled cholesterol appearing in

the medium in the presence of apoA-I after subtraction of values for apoA-I-free medium. For the phospholipid efflux study, lipids are isolated from culture medium by chloroform/methanol extraction. Extracted lipids are subsequently dried under nitrogen pressure and phospholipid mass determined by an enzymatic assay (Boehringer).

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PPARγ

Purpose and Rationale

PPARγ is a transcription factor required for the activation of many adipose-specific genes (Vosper et al. 2002). PPARγ essentially controls the accumulation of lipids in adipocytes, and many experiments have demonstrated the ability of the receptor to direct adipocyte stimulation (Rosen et al. 2000; Tontonoz and Spiegelman 2008). Reginato et al. (1998) reported that prostaglandins can both promote and block adipogenesis through opposing effects on PPARγ. PPARγ is a molecular target for the thiazolidine group of insulin-sensitizing drugs (Rieusset et al. 1999). PPARγ promotes lipid clearance in the liver of mice (Memon et al. 2000).

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Effect of PPARγ Agonists in Mice

Purpose and Rationale

Collins et al. (2001) reported the inhibition of the formation of early atherosclerotic lesions in diabetic and nondiabetic LDL receptor-deficient mice by a PPARγ agonist. However, the clinical use of TZDs is limited by the occurrence of several adverse events, including body-weight gain, congestive heart failure, bone fractures, and possibly bladder cancer (Cariou et al. 2012).

Procedure

Transendothelial Monocyte Migration

THP-1 cells, a human monocytic leukemia cell line, were added to a human aortic EC monolayer covering a gelatin-coated 8-mm porous

membrane and incubated for 30 min at 37 °C to facilitate their attachment. Cells were then pretreated with the ligands or vehicle (dimethyl sulfoxide) for 30 min at 37 °C. Migration was induced by the addition of monocyte chemoattractant protein-1 (MCP-1, 50 ng/ml) to the lower compartment. After 90 min, non-migrating THP-1 cells and human aortic ECs were removed with a cotton tip, and the membranes were fixed and stained with the Diff-Quik stain set (Dade, Miami, FL, USA) to identify migrated cells. The number of migrated cells was determined per 3,320 high-power field. Experiments were performed in duplicate and were repeated at least 3 times.

Western Blots

Western immunoblots were performed (Goetze et al. 1999). Membranes were incubated with rabbit polyclonal antibodies (1:1,000 dilution, New England Biolabs) that recognize either: (1) total extracellular signal-regulated kinase (ERK) or (2) ERK phosphorylated on threonine 202 and tyrosine 204.

Animals and Diets

Male LDLR2/2 mice were obtained (C57BL/6 J-Ldlrtm1Her, stock No. 002207, Jackson Laboratory) and were group-housed under a 12-h light and 12-h dark regimen. At 3 months of age, the mice were randomly assigned to one of five dietary regimens: (1) chow (Harlan Teklad 8604), (2) high-fat complex carbohydrate (Research Diets), (3) high-fat complex carbohydrate with 4 g troglitazone/kg of food, (4) high fructose (Research Diets), or (5) high fructose with 4 g troglitazone/kg of food. The high-fat diet consisted of 21 % fat, 20 % protein, 50 % carbohydrate, and 0.15 % cholesterol. The high-fructose diet contained 4 % fat, 16 % protein, 71 % fructose, and 0.15 % cholesterol. Mice and feed were weighed weekly, and the rate of consumption of drug was computed. The mice were fed for a period of 12 weeks.

Metabolic Measurements

Blood samples from the retro-orbital sinus were obtained from the mice before the beginning of treatment and every month thereafter and from the abdominal vena cava at euthanasia. Mice were fasted overnight before the collection of the blood samples. Plasma glucose was measured by glucose oxidase reaction (Beckman Glucose Analyzer 2, Beckman Instruments). Plasma lipids and plasma insulin were measured. Blood pressures were obtained by using an indirect tail-cuff method with a controlled-temperature chamber.

Vessel Preparation and Image Analysis

Mice were euthanized and perfused with 7.5 % sucrose in 4 % paraformaldehyde. Aortas were dissected out, split longitudinally, pinned flat in a dissection pan, and stained with Sudan IV to detect lipids and determine lesion area. Images were captured by use of a Sony 3-CCD video camera and analyzed using ImagePro image analysis software. The extent of lesion formation is expressed as the percentage of the total aortic surface area covered by lesions.

Cross Sections: Determination of Intimal Macrophage Content

The largest lesions from the aortic arch were excised and embedded in paraffin. The avidin–biotin–peroxidase complex technique for immunostaining was used. Macrophages were stained by using monoclonal antibody to CD68 (titer 1:100, KP1 clone, M0814, Dako). Nonimmune serum was used as a control. Primary antibody incubations were performed in 1 % BSA/2 % goat serum containing PBS for 60 min. Biotinylated rabbit anti-mouse (Dako) was applied; incubation with a streptavidin–peroxidase complex followed. Peroxidase activity was detected with the use of diaminobenzidine tetrahydrochloride as a chromogen. Slides were then counterstained with hematoxylin. Images of the stained sections were analyzed by using the software described above. After tracing the intimal area to be measured with a cursor, five pixels of color, which defined the anti-CD68 stain, were sampled by the operator. The area encompassed by the pixels, which was

not contiguous, in the color range for anti-CD68 was then computed automatically by the software.

Evaluation

Statistical analysis was performed by using 2-factorial ANOVA with Student–Newman–Keuls to determine the differences between individual group means.

Modifications of the Method

Li et al. (2000) used LDL-receptor-deficient mice to study the inhibition of atherosclerosis by PPARγ ligands.

Chen et al. (2001) used apolipoprotein E knockout mice to study the inhibition of atherosclerosis by a thiazolidinedione.

Gavrilova et al. (2003) used lipoatrophic A-ZIP/F-1 mice to study the contribution of peroxisome proliferator-activated receptor γ to hepatic steatosis, triglyceride clearance, and regulation of body fat mass.

Tao et al. (2003) found antioxidative, antinitrative, and vasoprotective effects of a peroxisome proliferator-activated receptor-γ agonist in hypercholesterolemic New Zealand white rabbits.

Iwaki et al. (2003) used C57BL/KsJ (db+/db+) mice to study the induction of adiponectin, a fat-derived antidiabetic and antiatherogenetic factor, by nuclear receptors.

Corti et al. (2004) performed an in vivo study by high-resolution magnetic resonance imaging of male New Zealand white rabbits. A selective PPARγ agonist showed an additive effect on plaque regression in combination with a statin in experimental atherosclerosis.

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Effect of PPARγ Agonists on Gene Expression in Macrophages

Purpose and Rationale

Hodgekinson and Ye (2003) used a combination of expression microarray and Northern blot analyses to identify target genes for peroxisome proliferator-activated receptor (PPAR)γ in RAW264.7 macrophages. PPARγ natural ligand 15-deoxy-Δ12–14 prostaglandin and synthetic ligands ciglitazone and rosiglitazone increased the expression of scavenger receptor CD36 and ATP-binding cassette transporter A1, as well as adipophilin (a lipid droplet-coating protein involved in intracellular lipid storage and transport), calpain (a protease implicated in ABCA1 protein degradation), and ADAM8 (a disintegrin and metalloproteinase protein involved in cell adhesion).

Procedure

Cell Culture

Macrophage RAW264.3 cells were purchased from ATCC and cultured in DMEM (Sigma, Poole, UK) at 37 °C in a humidified atmosphere with 95 % air–5 % CO_2. When cells reached ~80 % confluence, WY14,643, 15d-PGJ2, ciglitazone, rosiglitazone, and BADGE were added to the medium as described below to give final concentrations of 100, 5, 50, 1, and 500 μM, respectively.

RNA Extraction

Total RNA from RAW264.7 macrophages was extracted using the BD Clontech Nucleobond RNA purification system (BD Clontech, Basingstoke, UK). Cells were lysed by a lysis buffer containing guanidine thiocyanate. After removing cellular proteins from the lysate, RNA was precipitated, then immobilized in a column containing silica glass fibers and subjected to RNase-free DNase I digestion to remove genomic DNA. The RNA sample was further purified by washing and finally eluted from the column into nuclease-free water. The integrity of the RNA sample was verified by spectrophoretic analysis and formaldehyde agarose gel electrophoresis.

Microarray Filters and Hybridization

cDNA microarray nylon filters were purchased from Research Genetics. Radiolabeled cDNA probes were generated by reverse transcription of RNA extracted from rosiglitazone- or ciglitazone-treated and untreated (control) RAW264.7 macrophages using a Research Genetics GeneFilters Probe Labeling and Purification Kit (Invitrogen, Paisley, UK). Briefly, 4–10 μg of total RNA was mixed with 0.4 mg of oligo (dT) and incubated at 70 °C for 10 min. Then, 10 nmol each of dATP, dGTP, and dTTP, 37.5 U of AMV reverse transcriptase, and 100 μCi of [α-^{33}P]dCTP (3,000 Ci/mmol; Amersham, Bucks, UK) were added, and the solution (30 μl of final volume) incubated at 42 °C for 2 h. The labeled cDNA probe was then purified using spin columns supplied with the kit to remove unincorporated radioactive nucleotide. Prehybridization was carried out in 5 ml of MicroHyb buffer (Research Genetics) supplemented with poly(dA) (1 μg/ml) and denatured human Cot-1 DNA(1 μg/ml) at 42 °C for 2 h. Radiolabeled cDNA probe was denatured at 100 °C for 3 min and then added to the above solution, followed by hybridization at 42 °C overnight. The filters were then washed twice in a solution containing 2 × SSC and 1 % SDS at 50 °C for 20 min and a brief wash (5 min) in a solution containing 0.5 × SSC and 1 % SDS at room temperature. The filters were exposed to a low-energy phosphor storage screen, which was then scanned using a Storm Phosphorimager (Molecular Dynamics), and the resultant TIFF images analyzed using Pathways 4 software (Research Genetics). The intensities of the spots were normalized to mean intensities of all spots on each filter. Normalized intensities of corresponding spots were compared between two filters. In order to check the data, the filters were stripped and re-probed with the opposite radiolabeled cDNA probe, thus a filter probed with control cDNA was stripped and probed with treated cDNA and vice versa.

Northern Blot and Hybridization

RNA was denatured in 50 % (v/v) formamide and 6 % (w/v) formaldehyde at 65 °C for 10 min and

subjected to electrophoresis on 1.5 % (w/v) agarose gel in MOPS buffer [20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0)] containing 1.9 % (w/v) formaldehyde. Integrity and equal loading of the RNA was confirmed by observing ethidium bromide-stained 28S and 18S mRNA bands under UV illumination. The RNA was transferred overnight to Hybond-N nylon membrane (Amersham) by capillary action and the RNA linked to the membrane by baking at 80 °C for 2 h. cDNA probes for ABCA1, ADAM8, adipophilin, calpain-L3, CD36, protein phosphatase 2A non-catalytic subunit, and thymosin were generated from the IMAGE clone which had been isolated, sequenced, and verified by Research Genetics. Purified plasmid DNA was cut with the appropriate restriction enzymes, and the digested insert was purified by electrophoresis on 0.8 % agarose in TAE buffer [4 mM Tris, 1 mM EDTA, and 0.0114 % (v/v) acetic acid (pH 7.6)] containing 1 μg/ml ethidium bromide. The purified insert was purified from the gel using a QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) and 25 ng of cDNA was denatured at 95 °C for 2 min, and the cDNA was radiolabeled with [α-32P]dCTP by random priming using the Klenow labeling kit as supplied by Promega. Unincorporated label from cDNA labeling was removed using a QIAquick Nucleotide Removal Kit (Qiagen). Hybridizations with cDNA probes were performed with PerfectHyb (Sigma, Poole, UK) at 65 °C. Prehybridization was carried in 5 ml PerfectHyb supplemented with 150 μg of denatured salmon sperm DNA (Sigma) for 2 h. Hybridization with the radiolabeled cDNA probe was carried out in the same mixture overnight. Membranes were washed twice at room temperature with 2 × SSC–0.1 % SDS for 10 min followed by a 10 min wash in 0.1 × SSC–0.1 % SDS. A high-stringency wash (5–10 min) was performed in 0.1 × SSC–0.1 % SDS at 65 °C. The membranes were then subjected to autoradiography at −70 °C for between 12 h and 7 days.

Evaluation

Autoradiographs of Northern blots were scanned and analyzed using Phoretix software.

Modifications of the Method

Tontonoz et al. (1988) reported that PPARγ promotes the differentiation of monocytes/macrophages and the uptake of oxidized LDL.

Moore et al. (2001) reviewed the role of PPARγ in macrophage differentiation and cholesterol uptake.

Arimura et al. (2004) found that the peroxisome proliferator-activated receptor γ regulates expression of the perilipin gene in adipocytes. Perilipins are a family of proteins coating the surface of lipid droplets in adipocytes.

In macrophages, PPARγ activation suppresses inflammation by regulating gene expression and increases cholesterol uptake and efflux (Duan et al. 2008).

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PPARδ

Purpose and Rationale

Vosper et al. (2001) reported that PPARδ promotes lipid accumulation in human macrophages. PPARδ in macrophages also improve fatty acid metabolism and insulin sensitivity (Desvergne 2008).

Procedure

Isolation of Human Monocytes and In Vitro Monocyte/Macrophage Differentiation

Human monocytes were isolated from buffy coat preparations of whole blood taken from healthy volunteers. The buffy coat was mixed with OptiprepTM (Robbins Scientific) in a ratio of 2.5:1 and then overlaid with a discontinuous OptiprepTM gradient. Following centrifugation for 25 min at 600 g, the monocyte layer formed within the top 5–10 ml of the gradient was removed, washed with phosphate-buffered saline, and resuspended in RPMI 1640, supplemented with 2 mM glutamine and 10 % human serum (Sigma). Cell viability was assessed by the ability to exclude trypan blue and was typically 95 %. Monocyte purity was determined by differential counts of Diff-Quik-stained (Porvair Sciences) cell preparations and was typically >95 %. For monocyte–macrophage differentiation, monocytes isolated as above were resuspended in culture medium at a density of 2.5×10^6/ml and seeded into 12-well tissue culture plates; medium was changed every 48 h.

Transient Transfection

COS-1 cells were transfected by the DEAE-dextran method. The reporter construct pFABPLUC contained four copies of the peroxisome proliferator response element from the human liver FABP gene in front of the herpes simplex virus thymidine kinase promoter, cloned immediately upstream of the cDNA encoding firefly luciferase in pGLBAS (Promega). The PPAR expression vectors contained the coding sequence for human PPARα, −δ, and −γ under the control of the enhancer/promoter of the human cytomegalovirus. PSVGal was cotransfected with each sample to act as an internal control for transfection. Cell lysates were prepared, and luciferase and β-galactosidase activities were assayed using kits as described by the manufacturer (Promega).

Data are presented as the relative light units obtained with the luciferase assay divided by the absorbance obtained at 405 nm in the β-galactosidase assays (Luc/β-galactosidase).

Culture and Differentiation of THP-1 Cells

THP-1 cells were obtained from ATCC. Cultures were grown in RPMI supplemented with 10 % heat-inactivated fetal calf serum. Differentiation was initiated by the addition of 5 ng/ml PMA in the above medium. All drugs were added in Me_2SO and were replaced at intervals of 48 h. After 4 days, the cells were fixed with 0.66 % paraformaldehyde then stained with Oil Red O and hematoxylin. Quantification of lipid accumulation was achieved by extracting Oil Red O from stained cells with isopropyl alcohol and measuring the optical density of the extract at 510 nm. The value obtained using a control culture was subtracted from the resulting values. The Oil Red O absorbance was corrected by co-staining DNA with SYBR Green dye (Molecular Probes) and quantified on a Labsystems FluoroSkan Ascent FL microplate fluorometer. Cell number was determined from a standard curve.

Isolation of Stable Cell Lines

The entire coding sequence of human PPAR was subcloned, in both sense and antisense orientations, into the eukaryotic expression vector pCLDN. The resulting plasmids were transfected into THP-1 cells using a modified DEAE-dextran procedure. The cells were maintained in medium containing 1 mg/ml G418 and 10 % THP-1 conditioned medium, with rigorous washing procedures to remove dead cells. This was continued until cell killing stopped and robust growth was observed.

Western Blotting

Cultures were lysed in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and analyzed by Western blotting using standard procedures. PPAR antiserum was used at a 1:2,000 dilution, and the anti-AFAR antiserum was used at a 1:3,000 dilution. A peroxidase-conjugated mouse anti-rabbit IgG antiserum (Sigma) was used as a secondary detection reagent at 1:3,000, and the results were visualized using enhanced chemiluminescence (ECL+) as described by the manufacturer (Amersham Pharmacia Biotech).

Lipid Extraction and Measurement

Cultures were treated as above and then extracted with methanol/chloroform/phosphate-buffered saline (1:1:1, v/v) containing stigmasterol as an internal standard for cholesterol. The organic phase was analyzed for cholesterol with and without saponification by GC–MS using a Finnigan ThermoQuest Trace 2000. Triglycerides were quantified colorimetrically using a kit from Sigma and triolein as a standard. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent.

Cholesterol Efflux Assays

THP-1 cells were differentiated with PMA in the absence or presence of 100 nM compound F for 3 days. PPAR SENSE cells were differentiated with PMA for 3 days. The cells were labeled with 2 μCi (40 nmol) of [4-^{14}C]cholesterol in 2 ml of medium containing 10 % fetal calf serum for 3 h. ApoA-1-specific efflux was determined (Oliver et al. 2001). For apoA-1-independent efflux assays, the cells were incubated for a further 120 h in medium containing 10 % fetal calf serum.

Radioactivity release into the medium was determined by scintillation counting at 1, 4, 18, 24, 48, 72, and 120 h. All cultures incorporated similar levels (between 80 and 95 %) of the labeled cholesterol.

RNA Extraction and Analysis

RNA was extracted from THP-1 cells using TRIZOL reagent as recommended by the manufacturer. cDNA was synthesized from such RNA using "Ready-To-Go You-Prime" beads from Amersham Pharmacia Biotech. TaqManTM real-time PCR analysis was applied using prepared reagents from PerkinElmer Life Sciences. The primers and probes are described elsewhere (Kliewer et al. 1992). Relative levels of mRNA were calculated using the values obtained with each target gene compared with the values obtained with the 18S ribosomal RNA probes.

Evaluation

All graphs and statistics were prepared using Graphpad Prism for the Macintosh v3.0 (Graphpad Software Inc., San Diego, CA, USA). Nonlinear regression was applied using a sigmoidal response model for the dose response to compound F in transient transfection experiments. P-values were calculated using a standard Student's t-test.

Modifications of the Method

Wang et al. (2004) described the engineering of a mouse capable of continuous running up to twice the distance of a littermate. This was achieved by targeted expression of an activated form of PPARδ in skeletal muscle, which induces a switch to form increased numbers of type I muscle fibers. Treatment of wild-type mice with PPARδ agonist elicits a similar type I fiber gene expression profile in muscle.

Forman et al. (1997) developed a conformation-based assay that screens activators for their ability to bind to PPARα/δ and induce DNA binding. Specific fatty acids, eicosanoids, and hypolipidemic drugs are ligands for PPARα or PPARδ. Because altered fatty levels are associated with obesity, atherosclerosis, hypertension, and diabetes, PPARs serve as molecular sensors that are central to the development and treatment of these metabolic disorders.

Procedure

Cell Culture and Transfection

CV-1 cells were grown and transfected (Forman et al. 1995a). The reporter construct, PPREx3-TK-LUC, contained three copies of the acyl-CoA oxidase PPRE upstream of the herpesvirus thymidine kinase promoter (Kliewer et al. 1992). Expression vectors contained the cytomegalovirus IE promoter/enhancer (pCMX) upstream of wild-type mouse PPARα, mouse PPARγ 1-ΔN (Met-105-Tyr-475), mouse PPARδ-ΔN (Leu-69-Tyr-440), mouse PPARα-G (Glu-282 Gly) (Hsu et al. 1995), or Escherichia coli β-galactosidase as an internal control. Cells were exposed to the compounds for 24 h and then harvested and assayed for luciferase and β-galactosidase activity. All points were performed in triplicate and varied by less than 10 %. Normalized luciferase activity was determined and plotted as n-fold activation relative to untreated cells. Each experiment was repeated three or more times with similar results.

Electrophoretic Mobility-Shift Assays

In vitro-translated mouse PPARα (0.2 μl) and human RXRα (0.1 μl) were incubated for 30 min at room temperature with 100,000 cpm of Klenow-labeled acyl-CoA oxidase PPRE as described (Forman et al. 1995b) but with 150 mM KCl.

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Influence of Liver X Receptor Agonists

Purpose and Rationale

Liver X receptors (LXR) are nuclear receptors that are intracellular sensors, which regulate the expression of genes controlling cholesterol absorption, excretion, catabolism, and cellular efflux in target organs including small intestine, liver, and macrophages (Gabbi et al. 2014; Hong and Tontonoz 2014). The LXRs belong to a subgroup of nuclear receptors that are considered "metabolic receptors." Also included in this group are the PPARs and FXR (farnesol-activated receptor). The endogenous ligands for these receptors are intermediates or end products of metabolic pathways. The two LRXs, LRXα and LRXβ, are activated by physiological concentrations of oxidized cholesterol, such as 22(R)-hydroxycholesterol and 24(S),25-epoxycholesterol (Huang 2014). LRXα is expressed at high levels in liver, intestine adipose tissue, and macrophages, whereas LRXβ is expressed ubiquitously (Kovanen and Pentikäinen 2003; Jaye 2003; Joseph and Totonoz 2003). Macrophage liver XC receptors are identified as inhibitors of atherosclerosis (Tangirala et al. 2002, Parikh et al. 2014). Liver X receptors are regarded as potential therapeutic agents for dyslipidemia and atherosclerosis (Lund et al. 2003, Hong and Tontonoz 2014).

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Stimulation of Cholesterol Efflux

Purpose and Rationale

LXRs induce ATP-binding cassette (ABC) transporters, e.g., ABCA1 (Costet et al. 2000) and ABCG1 (Venkateswaran et al. 2000), which are involved in the transport of cholesterol and phospholipids from cells to extracellular receptors (Westerterp et al. 2014; Westerterp et al. 2013).

Sparrow et al. (2002) described a potent synthetic LXR agonist that is more effective than 22 (R)-hydroxycholesterol in inducing ABCA1 mRNA and stimulating cholesterol efflux.

Procedure

Cells

Cells were cultured at 37 °C in a humidified atmosphere consisting of 95 % air and 5 % carbon dioxide. Primary human fibroblasts were obtained from the Camden human cell repository. The cells were grown, handled, and cholesterol-loaded as described by Francis et al. (1995). Human primary hepatocytes were maintained in phenol red-free Dulbecco's modified Eagle's medium (high glucose) containing 10 % charcoal-stripped FCS, 1 % nonessential amino acids, 1 % glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. Caco-2 cells were obtained from ATCC and grown in Opti-MEM containing 10 % FCS, nonessential amino acids, and vitamins. THP-1 cells were obtained from ATCC and were grown in RPMI medium containing 10 % FCS, 0.05 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotic–antimycotic solution (100 units/ml penicillin, 0.1 μg/ml streptomycin, 0.25 μg/ml amphotericin B). THP-1 cells were differentiated into macrophages in six-well tissue culture dishes at a density of 1 million cells/well by incubation in the same medium plus 100 nM tetradecanoyl phorbol acetate for 3 days. After differentiation into macrophages, cells were used for mRNA measurements or for cholesterol efflux assays as described below. Human primary monocytes were prepared as described by Wright and Silverstein (1982) and differentiated to macrophages by culturing for 7 days in Teflon jars in RPMI 1640 medium supplemented with 12 % human serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. They were then plated in the same medium to initiate experiments.

Assays of Cholesterol Efflux Using [^{3}H]Cholesterol

Cells were labeled by incubation for 24 h in fresh growth medium containing [^{3}H]cholesterol (10 μCi/ml). In some experiments, cells were simultaneously labeled with [^{3}H]cholesterol and cholesterol-loaded using acetylated LDL. Following labeling with [^{3}H]cholesterol, cells were washed and incubated for an additional 24 h in serum-free media containing 1 mg/ml bovine serum albumin to allow for equilibration of [^{3}H]cholesterol with intracellular cholesterol. Cholesterol efflux was initiated by adding the indicated amount of apoA-I, usually 10 μg/ml, with or without APD, in serum-free medium. APD was added to cell culture medium from Me_2SO solutions, and control cells received an equivalent amount of Me_2SO, never exceeding 0.1 %. After 24 h, media were harvested, and cells were dissolved in 1 mM HEPES, pH 7.5 containing 0.5 % Triton X-100. Media were briefly centrifuged to remove non-adherent cells, and then aliquots of both the supernatants and the dissolved cells were subjected to liquid scintillation spectrometry to determine radioactivity.

Assays of Cholesterol Efflux Using Gas Chromatography–Mass Spectrometry

At the end of the 24-h efflux assay, cells and media were extracted. Cells were extracted twice (10 min with shaking each time) with 1 ml of hexane/isopropyl alcohol/water (3:2:0.1, v/v/v)/5 cm^2 of cell surface area. Media were extracted with 2.5 vols of chloroform/methanol (2:1, v/v). Internal standard [26,27-^{2}H_{6}]cholesterol was added to the extracts, and the samples were taken to dryness under a stream of argon. Half of the cellular lipid extract was saponified for 1 h at 60 °C in a solution of 3 % KOH in 90 % ethanol. One volume of water was added, and the mixture was extracted with 2 vols of hexane. The extracts of media and cellular lipids with or without saponification were taken to dryness under argon, derivatized to trimethylsilyl ethers by treatment with Sigma-Sil-A for 1 h at 60 °C, redissolved in hexane, and analyzed by gas chromatography/mass spectrometry. The amount of cholesterol in the original sample was determined using a standard curve.

Gas Chromatography/Mass Spectrometry

Gas chromatography/mass spectrometry was performed using a ThermoQuest GCQ instrument equipped with an RTX-5MS column (30 m × 0.25 mm inner diameter, 0.25-μm phase thickness; Restek, Bellefonte, PA, USA). The gas chromatography program was 180 °C for 1 min, followed by a temperature gradient of 20 °C/min to 290 °C and a final elution at 290 °C for 20 min. The injector was operated in the split mode (1:10 split), and the temperature was kept at 275 °C. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The instrument was operated in the electron ionization mode with the electron energy set to 70 eV. The ion trap was used for the selected ion monitoring of m/z 458 and 464 for determination of cholesterol and [26,27-$^2H_6$]cholesterol, respectively.

Transactivation Assays

Expression constructs were prepared by inserting the ligand-binding domain of human LXRα and LXRβ cDNAs adjacent to the yeast GAL4 transcription factor DNA-binding domain in the mammalian expression vector pcDNA3 to create pcDNA3-LXRα/ GAL4 and pcDNA3-LXRβ/ GAL4, respectively. The GAL4-responsive reporter construct, pUAS(5x)-tk-luciferase, contained five copies of the GAL4 response element placed adjacent to the thymidine kinase minimal promoter and the luciferase reporter gene. The transfection control vector, pEGFP-N1, contained the green fluorescence protein gene under the regulation of the cytomegalovirus promoter. For transient transfections, HEK-293 cells were seeded at 4×10^4 cells/well in 96-well plates in Dulbecco's modified Eagle's medium (high glucose) containing 10 % FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 5 % CO_2. After 24 h, transfections were performed with Lipofectamine (Invitrogen) according to the instructions of the manufacturer. Transfection mixes contained 0.002 μg of LXRα/ GAL4 or LXRβ/GAL4 chimeric expression vectors, 0.02 μg of reporter vector pUAS(5x)-tk-luc, and 0.034 μg of pEGFP-N1 vector as an internal control of transfection efficiency. Compounds were characterized by incubation with transfected cells for 48 h across a range of concentrations in phenol red-free Dulbecco's modified Eagle's medium (high glucose) containing 10 % charcoal-stripped FCS, 1 % nonessential amino acids, 1 % glutamine, and 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Cell lysates were prepared from washed cells using cell lysis buffer (Promega, Madison, WI, USA).

Measurement of mRNA Levels by Real-Time Quantitative Reverse Transcription-PCR (TaqMan)

Real-time quantitative PCR analysis was used to determine the relative levels of ABCA1 and ABCG1 mRNA. Reverse transcription and PCRs were performed according to the manufacturer's instructions. Sequence-specific amplification was detected with an increasing fluorescent signal of FAM (reporter dye) during the amplification cycle.

Amplification of the mRNA for the human 23-kDa highly basic protein, also called ribosomal

protein L13a, was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Levels of the different mRNAs were subsequently normalized to highly basic protein mRNA levels. Oligonucleotide primers and TaqMan probes were designed using Primer Express Software (Applied Biosystems) and were synthesized by Applied Biosystems. Sequences of forward primers, reverse primers, and probes (respectively) were as follows:

ABCA1, TGTCCAGTCCAGTAATGGTTCTGT, AAACGAGATATGGTCCGGATT, 6FAM-ACACCT GGAGAGAAGCTTTCAACGAGACTAACCTAMR A; ABCG1, TGCAATCTTGTGCCATATTTGA, CC AGCCGACTGTTCTGATCA, 6FAM-TACCACAACCCAGCAGATTTTGTCATGGA-TAMRA; SREBP-1c, GGTAGGGCCAACGGCCT, CTGTCTTGGTT GTTGATAAGCTGAA. 6FAM – ATCGCGGAGCCA TGGATTGCACT – TAMRA; HBP, GCTGGAAGT ACCAGGCAGTGA, ACCGGTAGTGGATCTTGG CTTT, VIC-TCTTTCCTCTTCTCCTCCAGGGTGG CT-TAMRA.

Evaluation

For Cholesterol Efflux

Cholesterol efflux is expressed as a percentage, calculated as ([^{3}H]cholesterol in medium)/([^{3}H]cholesterol in medium + [^{3}H]cholesterol in cells) × 100.

For Transactivation Assay

Luciferase activity in cell extracts was determined using luciferase assay buffer in a ML3000 luminometer (Dynatech Laboratories). Green fluorescence protein expression was determined using the Tecan Spectrafluor Plus at an excitation wavelength of 485 nm and emission at 535 nm. Luciferase activity was normalized to green fluorescence protein expression to account for any variation in efficiency of transfection.

Modifications of the Method

Chawla et al. (2003) used null stem cell of mice to demonstrate that PPARδ is a very-low-density lipoprotein sensor in macrophages.

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Liver X Receptor Binding

Purpose and Rationale

Several authors have studied the structure and function of the liver X receptors (Spencer et al. 2001; Yoshikawa et al. 2002; Hoerer et al. 2003; Yu et al. 2003; Williams et al. 2003).

Janowski et al. (1999) studied the structural requirement of ligands for the oxysterol liver X receptors LXRα and LXRβ and developed a ligand-binding assay.

Procedure

Ligands

Purchased ligands include the following: 9-cis-[20-methyl-^{3}H]retinoic acid (72 Ci/mmol; 1 Ci =37 GBq) and [26,27-^{3}H(N)]-24(S),25-EC (76 Ci/mmol); 22(R,S)-HC, 25-HC, 7α-HC, 24,25-dehydrocholesterol, cholic acid, pregnenolone, 9-cis-retinoic acid (9cRA). Stereocontrolled syntheses for the following ligands were performed: 24(S),25-EC; 22(R)-hydroxy-24(S),25-EC; 24(R),25-EC; 22(S)-hydroxy-24(R),25-EC; 7-keto-, 7β-hydroxy-, and 7α-hydroxy-24(S),25-ECs; 24(S),25-iminocholesterol; and 22(R),24(S)-dihydroxycholesterol. The methyl ester and dimethylamide of cholenic acid were prepared from cholesterol trisnorcarboxylic acid via acid chloride by reaction with methanol and dimethylamine, respectively.

Cell Cotransfection Assays

CV-1 cell conditions and transient transfections were performed (Willy et al. 1995). Receptor expression plasmids encoding full-length human LXRβ (CMX-hLXRβ) or LXRα (CMX-hLXRα) (Willy et al. 1995) were cotransfected with a luciferase reporter plasmid [TK-CYP7a-LXRE(X3)-LUC] (Peet et al. 1998) containing three tandem copies of the sequence (gcttTGGTCActcaAGTTCAagtta) from the rat Cyp7a gene (Chiang and Stroup 1994). Increasing concentrations of ligand (0.1–40 mM) were added to cells in media containing 5 % lipid-depleted calf bovine serum. Transfection data were normalized to a β-galactosidase internal standard.

Data are presented as mean relative light units (RLU) from triplicate assays ± SEM. EC_{50} values generated from duplicate assays were determined by fitting the data to a sigmoidal dose–response curve (GraphPad Prism, GraphPad Software, San Diego, CA, USA). Efficacy values represent the fraction of maximal-fold activation of each compound relative to 24(S),25-EC. Fold activation was determined by dividing the maximal activation of each compound by the activation observed in the absence of compound.

Receptor Protein Purification

Polyhistidine human RXRα (His10-hRXRα) (Petty 1995), LXRα LBD, or LXRβ LBD fusion proteins (His_6-hLXRα-LBD, His_6-hLXRβ-LBD) were expressed in Escherichia coli strain BL21 (DE3) (Novagen). Cultures grown in Luria-Bertani medium were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at 25 °C. Pellets were suspended in lysis buffer (250 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1 % Triton X-100, 10 mM imidazole, 200 mg of lysozyme per ml). The supernatant was incubated with Ni^{2+}-NTA agarose (Qiagen). The resin was washed twice with 20 vols of 50 mM HEPES, pH 7.5, 250 mM NaCl supplemented first with 50 mM then 75 mM imidazole. Protein was eluted with a linear 75–500 mM imidazole gradient. Peak fractions were tested for purity by SDS-PAGE, pooled, and cleared of imidazole over a PD-10 column (Pharmacia) equilibrated with 20 mM HEPES, pH 7.5, 200 mM NaCl, 2.5 mM EDTA, 5 mM DTT. Concentrations were determined by UV spectral analysis.

Ligand-Binding Assay

Scintillant-filled beads precoated with polylysine to permit protein binding (Amersham) were diluted in scintillation proximity assay (SPA) buffer [10 mM K_2HPO_4, 10 mM KH_2PO_4, 2 mM EDTA, 50 mM NaCl, 1 mM DTT, 2 mMCHAPS, 10 % (vol/vol) glycerol, pH 7.1; CHAPS is 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate] to a final concentration of 10 mg/ml. Binding assays were performed in 96-well plates (Packard) in a total volume of 100 ml containing beads (0.2 mg per well) and His_6-hLXRα-LBD (600 ng per well), His_6-hLXRβ-LBD (250 ng per well), or His_6-hRXRα (250 ng per well). The amount of protein used did not deplete ligand concentrations. [^{3}H]-24(S),25-EC or [^{3}H]-9cRA was diluted in SPA buffer and added to wells for a final concentration of 25 nM or 5 nM, respectively. Competition-binding assays using a single concentration of unlabeled competitor contained 25 mM 24(S),25-EC or 5 μmM9cRA. In other competition-binding assays, unlabeled ligands were serially diluted in SPA buffer and then

added at final concentrations ranging from 3 nM to 50 μM. Plates were shaken at 25 °C for 3 h, and then radioactivity was measured with a Packard Topcount at 1 min per well. All concentrations were assayed in triplicate and the results were averaged. Values from wells void of competitor represented 100 % binding.

Evaluation

Generation of K_i Value

Competition curves were generated by nonlinear regression analysis with GraphPad Prism, and apparent equilibrium dissociation constants (K_i values) were determined by using a method described by DeBlasi et al. (1989) based on the Cheng–Prusoff equation. K_i values for compounds that served as weak competitors in the assay (competition <70 % at the highest concentrations tested) are described as poor competitors. All K_i and SEM values reported are averages generated from duplicate or triplicate assays.

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Inhibition of Atherosclerosis by LXR Ligands

Purpose and Rationale

Several authors studied the potential antiatherosclerotic effects of LXR ligands (Hong and Tontonoz 2014; Parikh et al. 2014). Alberti et al. (2001) used LXRβ-deficient mice to study hepatic cholesterol metabolism and resistance to dietary cholesterol.

Chawla et al. (2001) reported that the nuclear receptor LXRα mediates a transcriptional cascade via PPARγ, which induces ABCA1 expression and cholesterol removal from macrophages. Ligand activation of PPARγ leads to primary induction of LXRα and to coupled induction of ABCA1 (Tall 2008). Transplantation of PPARγ null bone marrow into LDLR$^{-/-}$ mice results in a significant increase in atherosclerosis, consistent with the hypothesis that regulation of LXRα and ABCA1 expression is protective in vivo.

Procedure

Reagents

Oxysterols were dissolved in ethanol prior to addition to cells (<1 µl/ml).

Cell Culture, Stable Cell Lines, and RNA Analysis

THP-1 cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum or 10 % lipoprotein-deficient fetal bovine serum (LPDS). ES cells were cultured in the presence of rLIF (Gibco) as described (Keller et al. 1993). Macrophage differentiation was induced in ES cells in a two-stage process. Initially, ES cells were differentiated into embryoid bodies. Day-6 embryoid bodies were isolated, disaggregated, and replated in the presence of interleukin-3 and macrophage colony-stimulating factor-1 to induce differentiation of monocytes and macrophages, respectively. Total RNA was isolated using TRIzol reagent (Gibco), and Northern blot analyses were performed.

Transfection Assays

Murine LXRα and β promoters were amplified by PCR using the published genomic structure and sequence. Primers for PCR of LXR promoters were LXRα: 5′-ATCCTGTCCCTTCTGTCC-3′ and 5′-CCTCCAGAGTCAGCGTTC-3′ and LXRβ: 5′-CAGTGAGCGCATACAGGT-3′ and 5′-TCTCCGACTCTGTTGCCC-3′. To determine the transcription initiation site for mABCA1, 5′ RACE was performed using the 5′/3′ RACE kit (Roche) with RNA from the RAW 274 cell line and mouse liver and the gene-specific 3′ primer (5′-CTGAGGCCAACAAGCCAT-3′). The extended RACE products were used to screen a mouse genomic library (Stratagene). Sequence analysis of the isolated clones by the BLAST program revealed that the identified initiation site and 5′ regulatory region of ABCA1 were identical to the sequences deposited in GenBank (accession number AJ017356). The 2.5-kb genomic fragment of the mABCA1 promoter was cloned into a pGL3-basic vector. CV-1 cells were transfected by lipofection and assayed for reporter activity as described by Forman et al. (1995). Transfections were performed in triplicate and normalized to an internal CMX-βgal control. pCMX expression vectors for the PPARs and LXRs have been described (Kliewer et al. 1994; Willy et al. 1995).

Gel Mobility Shift Assays

Gel mobility shift assays were performed using in vitro translated receptors and 32P end-labeled oligonucleotides in a buffer containing 20 mM HEPES (pH 7.4), 100 mM KCl, 1 mM β-mercaptoethanol, 10 % glycerol, 100 mg/ml polydI-dC, and 5 mg/ml BSA. For competition studies, an excess of unlabeled oligonucleotide was added.

Cholesterol Efflux

Cholesterol efflux assays were performed as described by Venkateswaran et al. (2000), with minor modifications. Cells were plated at 50 % confluence. On day 2, cells were washed and incubated for 24 h in RPMI 1640 supplemented with 10 % LPDS. Cells were labeled with [^{3}H] cholesterol (1.0 mCi/ml), either in the presence of the ACAT inhibitor (58–035; 2 µg/ml) or with acLDL (50 µg/ml) in the absence of the ACAT inhibitor. Ligands for PPARβ (BRL; 5 µM), RXR (LG268; 50 nM), or LXR (22[R]-OHC; 2.0 µg/ml) were added to the cells. To equilibrate cholesterol pools, cells were washed twice with PBS and incubated for 8 h in RPMI containing 0.2 % BSA plus the ligands, but lacking radiolabeled cholesterol or acLDL. Cells were again washed with PBS and incubated in RPMI containing 0.2 % BSA in the absence or presence of HDL

(50 μg/ml) or apoA-I (15 μg/ml), for 4 h. An aliquot of the medium was removed and centrifuged at 14,000 *g* for 2 min, and the radioactivity was determined by liquid scintillation counting. Total cell-associated radioactivity was determined by dissolving the cells in isopropanol.

The data are presented as percent HDL-specific efflux or apoA-I-specific efflux, which is efflux in the presence of acceptor minus efflux in the absence of acceptor. Each assay was performed in triplicate.

Bone Marrow Transplantation

LDL-R$^{-/-}$ mice backcrossed onto the C57BL/6 background were initially obtained from Jackson Laboratories. Mice were fed a chow diet ad libitum until they were enrolled into the study. PPARg2/2ES cells were injected into C57/Bl6 blastocysts to generate PPAR$\gamma^{-/-}$ chimeric mice. The majority of the chimeric mice obtained were high-percentage chimeras. Peripheral blood, spleen, and bone marrow of PPAR$\gamma^{-/-}$ chimeras were extensively analyzed by FACS analysis with antibodies directed against Ly 9.1, CD11b/CD18, F4/80, CD3, B220, and Ly 6.1 to quantify the extent of chimerism. Chimeras, which were greater than 90 % null in their contribution to the monocytic/macrophage lineage, were used for bone marrow transplantation, along with wild-type control mice. In all, 24 6-week-old male LDL-R$^{-/-}$ mice were γ-irradiated with 1000 rads to eliminate the majority of their bone marrow cells. Each of the γ-irradiated mice were reconstituted via a tail vein injection with 2,000,000 bone marrow cells isolated from either PPARγ +/+ mice (designated PPARγ +/+ BMT) or PPARg 2/2 mice (designated PPARg 2/2 BMT). The mice were placed on a chow diet for 4 weeks while their marrow repopulated with the donor cells. For induction of atherosclerosis, the transplanted mice were placed on an atherogenic diet containing 15.8 % (wt/wt) fat and 1.25 % cholesterol (no cholate; diet 94059, Harlan Teklad) for 8 weeks. At weeks 0 (before BMT), 4, and 12, blood was drawn via the retro-orbital plexus following an 8-h fast, and total plasma cholesterol was measured via an enzymatic assay.

Lesion Analysis and Immunohistochemistry

Atherosclerotic lesions were quantified in the aortic valve of each mouse as described by Boisvert et al. (1998). The OCT-embedded, frozen aortic valves were sectioned serially at 10-μm thickness for a total of 300 μm beginning at the base of the aortic valve, where all three leaflets are first visible. Every fourth section for a total of five sections from each animal was stained with Oil Red O to identify the lipid-rich lesions. The stained areas were quantified using a computer-assisted video imaging system, and the mean area of the five sections from each animal was used for comparison analysis. The mouse aortic valve lesions were analyzed immunohistochemically with the following antibodies: anti-MOMA-2 (Serotec) for detection of intimal macrophages, anti-β-galactosidase (Cappel) for detection of PPAR$\gamma^{-/-}$ cells, and anti-α-actin (Dako) for detection of smooth muscle cells in the lesion. The frozen tissue sections were blocked with 5 % normal sera and incubated for 2 h at room temperature with the primary antibody (1–10 μg/ml). The sections were blocked for endogenous peroxidase activity with Peroxo-Block (Zymed, South San Francisco, CA, USA), followed by incubation with the appropriate secondary antibody (5 μg/ml) for 1 h. The washed sections were finally incubated for 30 min with Vectastain ABC Elite solution (Vector Laboratories, Burlingame, Calif., USA), developed with 9-amino-3-ethylene-carbazole (AEC; Vector Laboratories), and counterstained with hematoxylin.

Evaluation

Statistical analysis was performed using the Mann–Whitney *U* test, and data are expressed as mean ± SD.

Modifications of the Method

Dressel et al. (2003) used mouse myogenic C2C12 cells to study the influence of a peroxisome proliferator-activated receptor β/δ agonist on the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells.

Tanaka et al. (2003) used skeletal muscle cells of mice to study the influence of peroxisome

proliferator-activated receptor δ on fatty acid β-oxidation in skeletal muscle and the metabolic syndrome.

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See Herling (2006).

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Part XI

Activity on the Gastrointestinal Tract

Test on Salivary Glands

Andreas W. Herling

Contents

Measurement of Salivation

Purpose and Rationale

Symptoms of several human diseases are manifested as increased salivation (e.g., Parkinson's disease) or decreased salivation (e.g., xerosis). Studies to find and to evaluate sialagogues, such as substance P and its synthetic derivatives, as well as to search for salivation inhibitors are necessary. Saliva excretion is greatly influenced by anesthetics. Wagner et al. (1991) proposed a simple method to study saliva secretion in conscious rats and to evaluate sialagogues and sialagogue antagonists.

Procedure

Fed, male Sprague–Dawley rats (200–300 g) are weighed and distributed randomly into groups of six animals. Conscious rats are injected i.v., via the lateral tail vein, with either the vehicle or the sialagogue, e.g., substance P (0.3–3 μg/kg in 1 ml saline/kg). The rat's oral cavity is swabbed immediately after i.v. injection by placing and holding a pre-weighed, absorbent foam cube (e.g., 5/16″, Texwipe Company, Upper Saddle River, NJ) sublingually for 10 s using a triceps foam pencil (Texwipe Company, Upper Saddle River, NJ). Conscious rats are restrained during the 10 s collection period by gently holding the animal and opening the mouth using a plastic-coated snare,

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which is looped around the maxillary incisors and drawn back over the animal's head and the hand holding the rat, drawn around in front of the rat, and looped around the mandibular incisors. Gentle pressure on the snare opens the rat's mouth allowing the placement of the absorbent cube. Foam cubes are reweighed immediately after use. The difference between the initial weight of the cube and the weight of the cube after use represents saliva secreted.

Evaluation

Data are analyzed with Dunnett's *t*-test that compares several treated groups with a control group. Regression analysis is used to determine dose response and relative potency.

Modifications of the Method

Martinez et al. (1978, 1981) inserted appropriate plastic cannulae into the main excretory ducts of the two submandibular glands in **rats**.

Giuliani et al. (1988) studied the relative contributions of various neurokinin receptors (NK-1, NK-2, NK-3) to the sialogogic response after i.v. application in urethane-anesthetized rats.

Direct cannulation of the glandular duct with polyethylene tubing was performed by Bodner et al. (1983) and Kohn et al. (1992).

Bianciotti et al. (1994) cannulated the ducts of both the submaxillary and parotid glands in male Wistar rats anesthetized with 10 % ethyl urethane. No basal flow of saliva was observed from either gland; however, dose–response curves could be established after intravenous injection of sialogogic agents, such as methacholine (0.3–10.0 μg/kg), norepinephrine (3–60 μg/kg), isoproterenol (1–30 μg/kg), methoxamine (30–300 μg/kg), and substance P (0.3–10.0 μg/kg). Atrial natriuretic factor enhanced the salivary response to methacholine, methoxamine, and substance P.

Lohinai et al. (1997) determined salivary amylase secretion in conscious rats. Under ether anesthesia a catheter was introduced into the esophagus for salivary juice collection, and a cannula was inserted into the jugular vein for infusions. After postanesthesia recovery, submaximal carbachol infusion was given as a background to obtain steady secretion because of the low basal secretory rate. After application of drugs, volume and amylase were determined in saliva samples collected for 30 min.

Iwabuchi et al. (1994) studied salivary secretion after administration of a muscarinic agonist in MRL/lpr **mice**. Saliva was collected from the floor of the mouth of anesthetized rats with a capillary micropipette every 5 min for 60 min.

A method for the quantitative comparison of atropine substitutes on the salivary secretion of the **cat** has been published by Bülbring and Dawes (1945). Cats anesthetized with pentobarbitone were used. A cannula is tied into Wharton's duct and attached to a bottle containing tap water. The tap water, displaced by the saliva, passes out of the bottle through a tube which actuates a drop timer.

Ekström et al. (1994) used morphometric analyses to study the parotid acinar degranulation in cats after stimulation of the parasympathetic auriculotemporal nerve.

Izumi and Karita (1994, 1995a, b) investigated the secretory and vasodilator effects of nerve stimulation in the submandibular gland of cats. Cats of either sex were anesthetized with ketamine and a mixture of chloralose and urethane, paralyzed by intravenous injection of pancuronium bromide, and artificially ventilated. Blood flow changes in the submaxillary glands and lips of the cats were measured using a laser Doppler flowmeter. The duct of the submandibular gland was cannulated with a polyethylene cannula inserted distal to the intersection between the chorda lingual nerve and the duct. The amount of saliva secreted in response to nerve stimulation was determined gravimetrically by collecting the saliva in pre-weighed tubes.

Boldyreff (1925) described the preparation of salivary fistulae in the **dog**.

For preparing a **parotid fistula**, a fine sound is introduced through the orifice of the parotid duct, which is found opposite to the largest upper molar tooth, to the depth of 6–8 cm. Around the orifice

and at a distance of about 0.5 cm from it, four sutures are laid on the mucosa at equal distances one from the other. After this, a round piece of mucosae, about 1 cm in diameter around the orifice, with the sutures at the edge of this piece, is cut out with small sharp scissors. The duct is then separated from surrounding tissues about 2 cm from the orifice in the direction of its length. Then an opening is made through the cheek into the mouth (from the point half way on the vertical line from the front or the back corner of the eye to the mouth) to the base of the prepared duct. The orifice of the duct is now led outside by pulling out with the forceps. Four sutures on the piece of mucosa are made around it. The piece of mucosa is sutured carefully to the skin with knot sutures. The wound inside the mouth is closed with a continuous suture. The piece of mucosa must be covered daily with Vaseline to prevent drying. Sutures must be taken out slowly, beginning 3 days after operation. For the first 10 days after operation, it is necessary to produce on the dog an intensive salivary secretion, twice a day, by introducing into the mouth of the animal dry bread or meat powder or 0.5 % solution of hydrochloric acid. Saliva is collected into graduated test tubes.

In a similar way, one can produce a **fistula of the submaxillary or sublingual glands**, usually a common fistula for both glands, because their ducts have a common orifice.

Ogawa et al. (2003) developed a model of chronic parotitis in rats by a direct injection of complete Freund's adjuvant into the unilateral parotid gland via the parotid duct without skin incision.

Lambert et al. (1994) **cultured acinar cells** from lacrimal and submandibular glands as well as epithelial cells from rat small intestine in supplemented, serum-free media and measured the secretory components after treatment with various agents by radioimmunoassay.

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Tests on Esophagus

Andreas W. Herling

Contents

Tunica Muscularis Mucosae of Esophagus In Vitro

Purpose and Rationale

The tunica muscularis mucosa preparation of the rat esophagus (Bieger and Triggle 1985; Ohia et al. 1992) has been recommended for the evaluation of 5-HT_4 receptor ligands since it possesses a homogeneous population of 5-HT_4 receptors which mediates a well-defined relaxant response to 5-HT (Baxter et al. 1991; Baxter and Clarke 1992; Reeves et al. 1991; Waikar et al. 1992; Eglen et al. 1993, 1995; Yang et al. 1993; Gale et al. 1994; Monge et al. 1994; Sagrada et al. 1994; Elz and Keller 1995; Hegde et al. 1995; Cohen et al. 1996, 1998; Wong and Eglen 1996; Nagakura et al. 1999; Takeda et al. 1999).

Procedure

Male Sprague-Dawley rats (200–300 g) are sacrificed by asphyxiation with CO_2, and 2-cm segments of intrathoracic esophagus, proximal to the diaphragm, are excised and placed in Tyrode solution of the following composition (mM): NaCl 136, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.0, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, pH 7.4. The external muscularis propria, containing the outer longitudinal and circular muscle layers of the esophagus, is carefully removed in order to isolate the inner smooth muscle tube of the tunica

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muscularis mucosae. The tunica muscularis mucosae are suspended in a 10-ml tissue bath containing Tyrode solution at 37 °C and aerated continuously with 95 % O_2/5 % CO_2. Tissues are placed under 2.5-mN tension and are left to equilibrate with Tyrode solution for 60 min (washing every 15 min) prior to starting the experiment. Responses are recorded isometrically using a Hugo-Sachs Elecktronik (Biegestab K30) transducer coupled to a Graphtec (Linearcorder WR3310) four-channel chart recorder.

The preparation of tunica muscularis mucosae is contracted with carbachol (3 μM, approximate EC_{50}). Concentration-effect curves (relaxation) to 5-HT (or other agonists) are constructed in a cumulative fashion, followed by washout, with a 60-min interval between the first and second curve.

In antagonist studies, the antagonist is incubated with the tissue for 60 min following washout, and the second concentration-effect curve is constructed in the presence of the antagonist. Responses are measured as decreases in isometric tension and are expressed as percentage relaxation of the carbachol-induced tone.

Evaluation

Concentration-Effect Curves and Agonist Potencies

All agonist concentration-effect curves are fitted using a nonlinear, iterative curve-fitting program according to the following relationship (Parker and Waud 1971):

$$E = E_{\max}[A]^n / \left([A]^n + EC_{50}^n\right).$$

This relationship describes curves with a maximal response $E_{\max}$, half-maximal response EC_{50} (both in terms of molar concentration), and a slope factor determined by the power n. $[A]$ represents agonist concentration and E is response.

Antagonist Potencies

pA_2 estimates of test compounds versus 5-HT in the rat tunica muscularis mucosae are determined by the method of Arunlakshana and Schild (1959) and computed using Statview II (Brain Power Inc., Calabasas, CA). Concentration ratios are determined using the iterated EC_{50} values in the absence and the presence of the test compounds.

All remaining pA_2 estimates are determined by the method of Furchgott (1972) using a single concentration of agonist. The method assumes a competitive interaction and is calculated as follows:

$$pA_2 = -\log([\text{antagonist}]/[\text{concentration ratio} - 1]).$$

Statistics

CL (95 %) and statistical significance of the difference between samples (single comparisons, unpaired student's t-test) are determined using Statview II.

Modifications of the Method

De Boer et al. (1993) divided the **rat** esophagus into two parts, cervical and thoracic, each of a length of 10–15 mm. Both parts were cut longitudinally and pinned on a silicon mat with the outer, striated muscle coat up. After dissection of the striated muscle, the remaining muscularis mucosae was divided into 4 (5 × 2 mm, thoracic part) and 6 (5 × 1.5 mm, cervical part) strips. Strips from different parts showed no differences in pharmacological behavior.

Cohen et al. (1994) found 5-HT_4 receptors in rat but not guinea pig, rabbit, or dog esophageal smooth muscle.

Several authors (de Boer et al. 1993, 1995; Kelly and Houston 1996; Lezama et al. 1996; Oriowo 1997, 1998) showed that β_3-adrenoceptors mediate the relaxation of the rat esophageal muscularis mucosae.

Eglen et al. (1996) studied the functional interactions between muscarinic M_2 receptors and 5-hydroxytryptamine $(5\text{-HT})_4$ receptors and β_3-adrenoceptors in the isolated esophageal muscularis mucosae of the rat.

Goldhill et al. (1997) investigated the 5-HT_4 receptor modulation of tachykinergic excitation of rat esophageal tunica muscularis mucosae.

The tunica muscularis mucosae of **guinea pigs** was used by various authors:

Yoshida et al. (1993) studied the effect of a gastroprokinetic agent on electrically evoked contractions in tunica muscularis from isolated guinea pig esophagus.

Watson et al. (1995) investigated the interactions between muscarinic M_2 receptors and β-adrenoceptors in guinea pig esophageal muscularis mucosae.

Uchida et al. (1998a, b) examined the effect of Ba^{2+} on acetylcholine- and KCl-induced contractions and characterized the endothelin-induced contraction of the guinea pig esophageal muscularis mucosae.

Malmberg et al. (1991) studied muscle activity of isolated muscle strips from the middle pharyngeal constrictor, the inferior pharyngeal constrictor, the cricopharyngeal muscle, and the cervical esophagus from **rabbits** in organ baths in response to drugs and electrical field stimulation.

Kohjitani et al. (1993, 1996) divided the lower esophagus of rabbits into three regions (lower esophagus, transitional zone, lower esophageal sphincter) and studied the influence of anesthetics and peptides on contractions induced by acetylcholine or electrical field stimulation.

Percy et al. (1997) studied the pharmacological characteristics of rabbit esophageal muscularis mucosae in vitro.

Based on studies by Bitar and Mahklouf (1982) and Biancani et al. (1987), isolated smooth muscle cells of the lower esophageal sphincter from **cats** were used by Hillemeier et al. (1996) to investigate the influence of protein kinase C on spontaneous muscle tone. Esophagus and stomach from sacrificed cats were removed and opened along the lesser curvature. The location of the squamocolumnar junction was identified, and the mucosa was peeled and removed by sharp dissection under a microscope. The underlying circular muscle layer was cut into slices 0.5 mm thick with a tissue slicer. The last slices containing the myenteric plexus, longitudinal muscle, and serosa were discarded. The slices of circular muscle were placed flat on a wax surface, and tissue squares were made by cutting twice with a 2-mm blade block, the second cut at right angle of the first. Isolated smooth muscle cells were obtained by enzymatic digestion with collagenase. Agonist-induced contraction of isolated muscle cells was achieved by exposing them to IP_3 and a protein kinase C agonist. The cells were fixed in acrolein at a final concentration of 1 %. The length of 30 consecutive intact cells encountered at random was measured with a phase contrast microscope and a closed-circuit video camera.

Muscle strips from *cat* lower esophageal sphincter were used by Dobreva et al. (1994), Kortezova et al. (1994), and Preiksaitis and Laurier (1998).

Uy Dong Sohn et al. (1995) investigated muscle-type-specific signal transduction pathways in esophageal and lower esophageal sphincter circular smooth muscle of cats.

Tokuhara et al. (1993) studied the influence of adrenoceptor agonists on the striated muscle portion of the esophagus by use of isolated strips from **dogs**.

Saha et al. (1993) examined the effects of nitric oxide-containing compounds on **opossum** esophageal longitudinal smooth muscle in vitro.

Da Rocca et al. (1992) examined the effects of metoclopramide in transverse muscular strips from **pigeon** esophagus.

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Esophageal Sphincter In Vivo

Purpose and Rationale

Salapatek et al. (1992) and Xue et al. (1996) studied the control of esophageal peristalsis and function of the lower esophageal sphincter in anesthetized cats.

Procedure

Adult cats of either sex are fasted overnight. Anesthesia is induced with 15 mg/kg ketamine i.m. and maintained with i.v. infusion of 15 mg/kg/h. The cats can tolerate intubation while continuing to swallow spontaneously or with pharyngeal stimulation. Esophageal motility is continuously monitored by a multi-lumen catheter passed orally. The catheter has a sleeve (Dent 1976) which is positioned within the lower esophageal sphincter, with recording ports 2, 4, 6, and 8 cm above the lower esophageal sphincter and one port just below the sleeve. The recording catheter channels are continuously perfused with distilled water at 0.3 ml/min by means of a pressurized infusion pump. At the recording ports within the esophageal body, the system is able to record a pressure rise of 300 mm Hg/s. Pressures are recorded using transducers. Respiration is continuously monitored with a belt pneumograph placed around the animal's chest. All pressures are recorded on a Beckman eight-channel direct writing chart recorder with input couplers, preamplifiers, and amplifiers while simultaneously taped on an eight-channel FM tape recorder. Drugs are administered intravenously.

For each esophageal contraction, amplitude is measured at each esophageal level, using mean intraesophageal pressure as baseline. Onset of contraction is determined as the point of rapid upstroke at each level, and progression of the wave along the esophagus is expressed as the lag of time or delay (in seconds) between two adjacent recording sites. Basal lower esophageal sphincter pressure is measured using intragastric pressure as reference. Maximum lower esophageal sphincter relaxation and lower esophageal sphincter after-contraction are also assessed.

Evaluation

Statistical analysis is performed by using one-way analysis of variance and a student's *t*-test where appropriate.

Modifications of the Method

Greenwood et al. (1992) used a similar multilumen catheter assembly system in **cats** and registered additionally the electromyogram of the mylohyoid muscle.

Further studies in cats were performed by Preiksaitis et al. (1994) and Lichtenstein et al. (1994).

Using a miniature perfused sleeve/side-hole catheter, Kawahara et al. (1994) measured gastric, lower esophageal sphincter, and esophageal pressure in urethane-anesthetized **rats**.

Rouzade et al. (1996) monitored manometrically esophageal, lower esophageal sphincter, and fundus pressure in conscious **dogs**.

Blackshaw et al. (1995) and Blackshaw and Dent (1997) measured responses of the lower esophageal sphincter in urethane-anesthetized **ferrets**.

Smid et al. (1998) studied lower esophageal sphincter function in a model of esophagitis in ferrets. Esophagitis was induced by acid (0.15 M HCl) and 1 % pepsin infusions in anesthetized ferrets. Lower esophageal sphincter strip responses were measured in vitro after various agents and after electrical field stimulation.

Knudsen et al. (1994) measured esophageal pressured with a four-channel, perfused catheter assembly in lightly anesthetized **opossums**.

Further studies in opossums were performed by Harrington et al. (1991) and de Arruda-Henry and Uchida-Athanasio (1994).

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Permanent Fistula of the Esophagus in the Dog

Purpose and Rationale

Esophagostomy was made for the first time by Pavlov (1902) for the purpose of obtaining pure gastric juice from a dog. Pure gastric juice can be obtained in great quantities only after shame feeding from a gastric fistula, so it is necessary that the dog shall have two fistulae: esophageal and gastric fistula. The technique to prepare a permanent gastric fistula in the dog was described by Boldyreff (1925).

Procedure

It is necessary to obtain beforehand a well-nourished dog with a gastric fistula. The operation must be done aseptically with the usual anesthesia. The incision on the neck is made on a median line 2–3 cm below the larynx and is 12–15 cm long. The esophagus is found under the trachea and a little to the left. It is separated as little as possible from the surrounding tissue with a knife handle and pulled outside. The esophagus is now divided, cross section, and the ends of the dissected esophagus are carefully sutured into the respective corners of the wound. In suturing the mucosa of the esophagus with the skin of the wound, it is necessary to take into the suture the muscle layer of the esophagus also. After the ends of the esophagus are sutured into the corners of the wound, the part of the wound between them is closed. The wound should be covered with thick antiseptic ointment. After the operation it is necessary to examine the wound daily. The sutures must be taken out gradually beginning 5 or 6 days after the operation.

Esophagotomy can be performed more simply if the esophagus is not divided but a longitudinal incision, 12–15 cm long, is made through its wall. The edges of this incision are sutured together with the edges of the wound in the neck. When the wound is healed, this fistula can be temporarily closed during feeding by means of a bandage or with a special device. The dog can then eat and drink normally and the saliva is not lost through the fistula.

Modifications of the Methods

Boldyreff (1925) described also the preparation of crop fistulas in the **rooster**. A longitudinal incision 2–3 cm long is made through the skin in the middle part of the crop. Then, with the aid of a pair of surgical forceps, the front wall of the crop is lifted up, and an opening about 2 cm long is made in it with scissors. After this a fistular tube is introduced into the crop with the aid of a large hook, and if necessary, the opening of the crop is sutured with one suture tightly around the tube.

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Pharmacological Effects on Gastric Function

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Contents

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Acid Secretion

Acid Secretion in Perfused Rat Stomach (Gosh and Schild Rat)

Purpose and Rationale

Originally Gosh and Schild (1958) introduced a method for the continuous recording of gastric acid secretion in the stomach lumen-perfused anesthetized rat. In this model gastric acid secretion can be stimulated by histamine, carbachol, or gastrin. Stimulated gastric acid secretion is inhibited by test drugs with antisecretory potential.

Procedure

(*Modified After* Gosh and Schild 1958)

The animals are fasted for 18 h prior to the experiment with free access to water. Anesthetized rats with a body weight of 300–350 g are used. Anesthesia is induced by pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h). Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The jugular veins are then exposed and cannulated with polyethylene tubes beveled at the tip. The abdomen is opened through a midline incision. The esophagus and pylorus are ligated, and a double-lumen perfusion cannula is inserted and fixed in the forestomach. The stomach is perfused continuously with warm (37 °C) saline at a rate of 1 ml/min. The perfusate is collected at 15-min periods and its acid concentration measured. Histamine (10 mg/kg/h), desglugastrin (100 μg/kg/h), or carbachol (30 μg/kg/h) are administered by i.v. infusion into the jugular vein after a basal period of 45 min. Ninety minutes after the onset of the secretagogue infusion, acid output had reached a stable plateau. As soon as acid secretion has reached a plateau, test drug or a standard is injected intravenously.

Evaluation

The perfusate is collected at 15-min periods, and its acid concentration measured by titration against 100 mM NaOH to an endpoint of pH 7 and acid output (μmol H^+/15 min) is calculated. Using various doses of the test drug and of a standard, dose–response curve can be established, and activity ratios with confidence limits can be calculated.

The maximal inhibition of acid secretion (AI_{max}) and the inhibition of acid secretion during 1 h (AI_t) are calculated.

$$AI_{\mathbf{max}} = (pH_i - pH_s) \times (pH_b - pH_s)^{-1} \times 100$$
$$AI_{\mathbf{t}} = 100 - F_i \times F_s{}^{-1} \times 100$$

whereby:

$\mathbf{pH_b}$ = pH value at basal H^+ secretion.

$\mathbf{pH_s}$ = pH value at stimulated H^+ secretion.

$\mathbf{pH_i}$ = highest pH value after administration of test compound.

$\boldsymbol{F_s}$ = integral of pH curve over 60 min before administration of test compound.

$\boldsymbol{F_i}$ = integral of pH curve over 60 min after administration of test compound.

Critical Assessment of the Method

The method of Gosh and Schild being modified by several authors can be used for standardization of secretagogues, like gastrin, and for evaluation of acid secretion inhibiting antiulcer drugs.

For the specific pharmacological assessment of inhibitors of gastric acid secretion, like H_2 blockers, anticholinergics, and H^+/K^+ − ATPase inhibitors, this method reveals valid results with respect to the antisecretory potential of the test compound. However, only parenteral administration of the test drug, preferentially i.v., is feasible and should be preferred.

As different pathways of stimulation are exclusively initiated by histamine, gastrin, or carbachol, it is possible to estimate the potential interaction of the test drug with the secretory pathways of acid secretion. Test drugs affecting:

1. The H_2 receptor inhibit histamine- and gastrin-stimulated gastric acid secretion
2. The gastrin receptor inhibit only gastrin-stimulated gastric acid secretion
3. The muscarinic receptor inhibit only carbachol-stimulated gastric acid secretion,

4. Carboanhydrase activity inhibit gastric acid secretion, irrespective of the kind of stimulation,
5. H^+/K^+-ATPase (gastric proton pump) inhibit gastric acid secretion, irrespective of the kind of stimulation

Modifications of the Method

Burn et al. (1952) described the evaluation of substances which affect gastric secretion using perfusion of the stomach in anesthetized **cats**.

Lawrence and Smith (1974) described the measurement of gastric acid secretion in the rat by conductivity. The stomach of an anesthetized rat is continuously perfused with 2 ml/min of an isotonic (0.308 M) glucose solution at 37 °C. The conductance of a solution depends on the total ion concentration and is therefore not specific for hydrogen ions. Since hydrogen ions have an equivalent conductance nearly five times greater than any other ion found in gastric juice and since they are secreted in a far greater concentration than other ions, conductivity measurements can be regarded as a relatively specific measure of hydrogen ions. Using Mullard conductivity cells (type E 791/B) and a commercially available meter (Phillips PW 9501), simultaneous measurements in six rats were performed.

Gallo-Torres et al. (1979) described in detail a method for the bioassay of antisecretory activity in the conscious rat with acute gastric fistula with additional collection of the biliary and pancreatic secretion by means of a catheter in the common bile duct. The gastric secretions are collected by gravity via a cannula in the most gravity dependent site of the glandular stomach.

Larsson et al. (1983) described studies in the acutely vagotomized rat. Truncal vagotomy is performed under ether anesthesia by cutting the dorsal and ventral branches of nervus vagus just below the diaphragm. The pylorus is then ligated, and a polyethylene catheter (PP 200) is inserted into the duodenum, close to the pylorus. Each animal is placed in a modified Bollman cage and is allowed to recover at least 1 h before the experiment. Gastric juice is collected by free drainage in 30-min samples.

Herling and Bickel (1986) showed that gastric acid secretion in stomach lumen-perfused rats can be stimulated in vivo on the subreceptor level by IBMX (phosphodiesterase inhibitor) and forskolin (non-receptor activation of the adenylate cyclase). H^+/K^+-ATPase inhibitors and H_2 antagonists show, according to their different modes of action, also a different inhibitory profile in this assay.

Hammer et al. (1992) used anesthetized female Sprague–Dawley rats weighing 200–320 g. After insertion of a tracheal cannula, a 3-mm silicon tubing is placed through the mouth and advanced to the stomach. The tubing is tied to the esophagus at the neck. A 4-mm drainage tube is inserted into the stomach through a laparotomy incision and an incision in the duodenum and ligated in place at the pylorus. Gastric perfusate (0.9 % saline at 37 °C) is collected on ice every 5 min for titration to pH 7.0.

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Isolated Rat Stomach

Purpose and Rationale

The isolated whole stomach of the rat was recommended for evaluation of H_2 receptor antagonists (Bunce and Parsons 1976).

Procedure

Fed immature rats of either sex weighing 38–42 g are anesthetized with sodium pentobarbitone i.p. The abdomen is opened and the esophagus ligated close to the stomach. An incision is made in the rumen of the stomach, and the contents washed out with warm Krebs–Henseleit solution. A second incision is then made at the pyloric sphincter, and polyethylene cannulae are inserted and tied into the stomach via these incisions. The stomach is rapidly dissected out and placed immediately into a 10-ml organ bath containing Krebs–Henseleit solution at 37 °C. The lumen of the stomach is perfused at a rate of 1 ml/min with a modified Krebs–Henseleit solution (without Na_2CO_3 and KH_2PO_4) at 37 °C. Both solutions are gassed with 95 % O_2/5 % CO_2. The effluent perfusate from the stomach is passed over a micro dual electrode. The changes in pH are converted to a function of hydrogen ion activity by an antilog function generator and continuously recorded on a potentiometric pen recorder. The drugs are added, in a volume not exceeding 0.5 ml, to the complete Krebs–Henseleit solution bathing the serosal surface of the stomach. After setting up the stomach preparation, the basal H^+ output is allowed to stabilize, both under control conditions and in the presence of the H_2 antagonist, before the secretory responses to histamine are investigated. The response to a dose of histamine is assessed by measuring the amount of acid secreted at peak response above the preceding basal level.

Evaluation

The rate of acid secretion is expressed as $[H^+]$ moles $\times$ 10^{-8}/min. The effect of the antagonist is assessed by measuring the potency of the agonist. An estimate of potency is firstly obtained using the agonist alone, and then a second estimate is obtained in the presence of the agonist–antagonist combination. The potency ratio is calculated according to Finney (1964).

Modifications of the Method

A similar method was described by Szelenyi (1981) and Stanovnik et al. (1988) using the isolated stomach of the **mouse**.

Weigert et al. (1995) evaluated the effect of the opiate receptor antagonist naloxone on vagally stimulated secretion of bombesin-like immunoreactivity, somatostatin, and gastrin from the isolated rat stomach which was perfused via the celiac artery with Krebs–Ringer buffer. Vagal stimulation was performed with 1 ms, 10 V and 2, 5, or 20 Hz, respectively.

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Chronic Gastric Fistula in Rats

Purpose and Rationale

A permanent gastric fistula using an especially designed Pavlov's type of cannula in the rat has been described by Lane et al. (1957) and Komarow et al. (1963), Komarow and Brawlow (1960). Moreover, the preparation of chronically denervated gastric pouches in the rat has been reported by Alphin and Lin (1959).

Procedure

For a **permanent gastric fistula** in rats, Komarow and Brawlow (1960) and Komarow et al. (1963) developed a stainless steel cannula of the type extensively used in Pavlov's laboratories. The cannula is 10–12 mm in length with an inner bore of 3 mm diameter and a beveled flange at each end. The inner bore at one flange is threaded to permit insertion of a removable screw which acts as a stopper between experiments. The lumen of the cannula permits a snug fit with a No. 8 French catheter.

Male Wistar rats weighing 280–300 g are used. Under appropriate anesthesia, the left upper quadrant of the abdomen is shaved and the animal placed on its right side. An incision of 5–10 mm in length is made about 5 mm below and parallel to the lower left costal margin between the parasternal and mammary lines, and the deep abdominal muscles are divided. The area of the rumenal wall is then drawn up through the incision. This area should be close to the greater curvature but far enough away from the glandular portion of the stomach to avoid sacrificing secretory mucosa. A small incision is made in the exposed portion of the rumen, and a purse-string suture is loosely placed around the margin of the opening. The unthreaded flange of the cannula is inserted into the rumen, and the suture is pulled tight around the shaft of the cannula and tied. The incision is then closed by two layers of interrupted sutures. The deep abdominal muscle is closed first by a suture at each side of the cannula, using a bit of rumen to ensure a tight fit. The incision is closed with skin sutures.

During the postoperative period of 2–3 weeks, the animals are conditioned to the experimental procedure by keeping them in a restraining cage for several hours each day. Food is withdrawn 18 h prior to the experiment. The animal is placed in a restraining cage, the stopper screw removed from the cannula, and the stomach gently washed twice with 2–3 ml warm saline, and 6 cm length of rubber catheter is inserted through the lumen of the cannula. Hourly collections of gastric fluid in a graduated centrifuge tube are then started. The animal model can be used for physiological studies as well as for standardization of secretagogues and for measuring the inhibition of gastric secretion by antisecretory drugs.

For preparation of **chronic denervated gastric pouches** according to Alphin and Lin (1959), male Wistar or Sprague–Dawley rats weighing 250–350 g are used. Under anesthesia, a midline incision is made along the abdominal wall, and the stomach is gently pulled to the outside by means of a stainless steel hook. Two small clamps are then gently applied along the stomach between the smaller and greater curvature, care being taken not to injure the blood vessels supplying the glandular and the nonglandular portions of the stomach.

An incision is made between the clamps from one end to the other end of the greater curvature with as little involvement of the mesenteries as possible. The mucosa and the muscular wall are sewed up separately. A new opening is made

midway along the greater curvature of the denervated pouch into which one end of a stainless steel cannula is secured by double purse-string sutures. The stainless steel cannula is 3 cm in length with an inside diameter of 3 mm. The other end of the cannula is led through a small puncture 1 cm on the left of the midline of the abdomen. The gastric end of the cannula is firmly anchored to the body wall by silk sutures. After closing the midline incision of the body wall by silk sutures, the animal receives 150 MU procaine penicillin. The animals receive a pasted diet for some days and are kept constantly in an air-conditioned room with constant temperature during the recovery period of 2–3 weeks.

For experiments, the animals are deprived of food, but not of water prior to the test, placed in restraining cages and the gastric juice is collected in graduated centrifugation tubes. The amount of secretion into the pouch and the acidity of the fluid are measured after secretagogues, e.g., 20 mg/kg histamine s.c., alone and then two hours later after administration of potential antisecretory drugs.

Evaluation

The effect on volume and HCl secretion at 30 min intervals after administration of the test compound is compared with the control values. The decrease is expressed as percentage of control.

Modifications of the Method

Larsson et al. (1983) described a modified technique for the chronic fistula rat. Under methohexital anesthesia, a plastic cannula is implanted in the rumen of rats close to the glandular part of the stomach wall. At the same time, a polyethylene tube (PE 50) for compound administration is inserted into the upper part of duodenum and fixed to the cannula. The duodenal tube is subcutaneously guided to the neck, where the free end is guided through the skin. One week recovery is allowed before experiments. For the experiment, the rats are placed in modified Bollman cages, and six consecutive 1-h samples of gastric juice are collected from the gastric cannulae. After the first two collection periods, the test compounds are given intraduodenally.

Altar (1980) described the design and method of implantation of a chronic gastric cannula in adult rats.

Johnson et al. (1990) described a chronic gastric cannula for feeding ethanol liquid diet to rats.

Tsukamoto et al. (1984) reported an improved method for intragastric infusion in conscious rats with simultaneous implantation of a cannula into the jugular vein for blood sampling and drug administration.

Rossowski et al. (1997) measured the inhibition of gastric secretion by adrenomedullin, amylin, calcitonin gene-related peptide, and their fragments in rats equipped with chronic gastric fistulas.

Bickel et al. (2004) measured sham-feeding in rats with chronically implanted gastric fistula after treatment with an anorectic compound.

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Chronic Gastric Fistula in Dogs

Purpose and Rationale

A simple technique for preparing chronic gastric fistulas in dogs which does not require placement of cannulae or tubes has been described by Foschi et al. (1984).

Procedure

Male dogs weighing 12–18 kg are used. During appropriate anesthesia (e.g., inhalation anesthesia with isoflurane), a round incision (2 cm) is made on the left side of the abdomen below the costal arch. The muscular fascial layers are cut across to either the peritoneum or the abdominal cavity. The stomach is grasped on the greater curvature, as high as possible, and is gently pulled through the abdominal wall. A round excision of the serous–muscular layer (0.5 cm diameter) is made, and then the mucosa is cut and sutured to the serous layer with catgut. A purse string of 3–0 silk is placed 1.5 cm around the gastrostomy hole to invert the opening and to form an antireflux flap. The stomach is also sutured to the peritoneum, the fascial layer, and the skin with catgut. After 5 days, a silver cannula has to be inserted once a week to avoid closure of the gastrostomy.

After 10–14 days, the animals are trained to lie in a cage to their left sides, supported by a 30° angle with the caudal part of the body raised to avoid the passage of gastric juice into the duodenum. For secretion studies, a short plastic tube is inserted through the fistula, and gastric juice is collected for periods of 15 min. After 30 min, secretagogues (e.g., pentagastrin 6 μg/kg/min) are given as continuous infusion and samples collected every 15 min.

Evaluation

The samples are titrated with 0.1 N NaOH to pH 7.0 by a pH meter. The results are expressed as volume (ml), pH, acid concentration (μE/ml), and output (mE/h).

Critical Assessment of the Method

The technique described by Foschi et al. (1984) has the advantage of simplicity.

Modifications of the Methods

Boldyreff (1925) reported improvements and details of the technique described by Pavlov (1902).

Thomas (1941) described an improved cannula for gastric and intestinal fistulas using a removable flange being inserted separately into the stomach or intestine.

Emås (1960) reported on gastric secretory responses to repeated intravenous infusions of histamine and gastrin in non-anesthetized and anesthetized cats with gastric fistula. A gastric cannula as well as a duodenal cannula is inserted.

Daly et al. (1980) described an apparatus for intragastric titration in the conscious dog. A titration display unit provides a record of the secretory response both as a digital printout and a bar chart display.

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Heidenhain Pouch in Dogs

Purpose and Rationale

The preparation of a chronic gastric pouch, as described by Heidenhain in 1878, is one of the classic techniques in experimental surgery. This model has much contributed to the understanding of the physiology and pathology of the stomach and to modern techniques of abdominal surgery in man. The surgical technique has been described again in detail by deVito and Harkins (1959). A preparation of chronic denervated pouches in the rat has been described by Alphin and Lin (1959). Both preparations can be used as pharmacological models for testing antisecretory drugs.

Procedure

Dogs weighing 15–20 kg are fasted 24 h preoperatively. The abdominal surgery is performed during appropriate anesthesia (in former times by e.g., 30 mg/kg pentobarbital sodium; nowadays more appropriate by inhalation with isoflurane). The abdominal part is shaved with electric clippers and then with a razor. The skin is disinfected with Zephiran 70 % alcohol. Sterile drapes are applied to cover the whole surgical field. A midline linea alba incision from xiphoid to umbilicus provides excellent exposure and ease for closure. As the posterior sheath is divided, the large ventral fat pad present in dogs should be excised completely. A self-retaining retractor is applied, and the stomach is palpated for the absence of food. Then the spleen is displaced, wrapped in warm, moist pads and laid on the ventral wall below the incision.

The stomach is pulled into the operative field. The greater curvature is held at multiple points so that the stomach is stretched out and the line of incision for the pouch is selected. The pouch should be made from the corpus of the stomach so that true parietal cell juice can be obtained. A line projected from the incisura angularis perpendicular to the proximal lesser curvature will generally fall across the junction between corpus and antrum. Appropriate division of the gastric branches of the right gastroepiploic artery at the lower end of the proposed line of transection clears the greater curvature for 1–2 cm. The gastroepiploic artery itself should be sectioned at this site and a long rent formed on the adjacent omentum; else the omentum vessels tend to tear during subsequent manipulations.

An index finger is then inserted through this defect dorsal to the stomach to emerge higher on the greater curvature through the gastrosplenic ligament at the upper end of the proposed line of transection. This portion of the greater curve is cleared for 1–2 cm. Von Petz clamps with their staplings are used to control bleeding and to avoid leakage of gastric content. The stomach should be kept stretched and flattened while the clamps are applied. After division between the staples, any bleeding is controlled, and the cut edges of the main stomach and pouch are then oversewn with continuous sutures of black silk. The suture should be of an inverting type. Surprisingly, leakage or excessive adhesions are not a problem when serosal apposition is neglected.

The pouch so formed is about 30 % of the corpus volume and provides adequate secretory

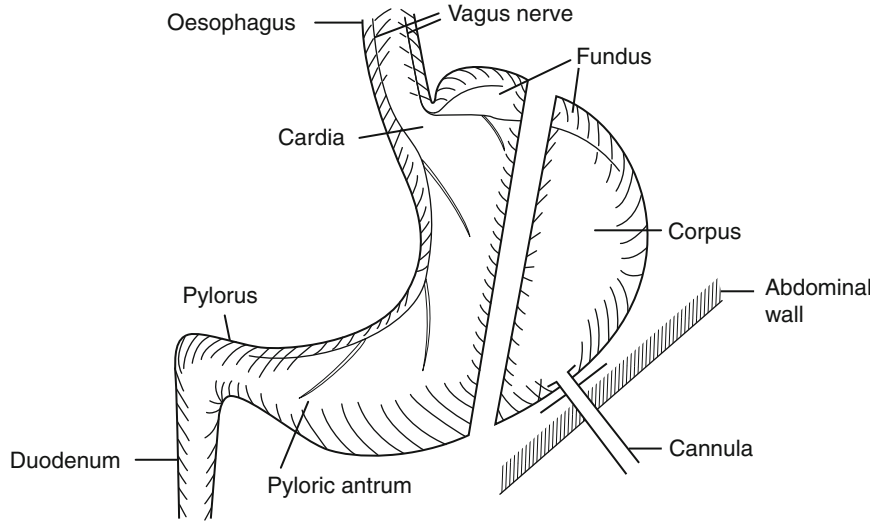

Fig. 1 Technique of Heidenhain pouch fistula in dogs

volume for further studies (Fig. 1). A cannula, made of stainless steel, 7 cm long with a beveled flange threaded at the other end, is placed in the most ventral portion of the pouch through a small incision in the anterior wall. A single purse string of silk holds it in place. A double sheet of omentum is then wrapped about the pouch and the cannula before being pulled through the abdominal wall, about 3 cm to the left of the midline subcostally. It is important that the cannula be held snugly by fascia; otherwise it will readily pull out of the pouch and abdominal wall. The linea alba is closed with a continuous suture of silk and the skin with subcuticular stitches of chromic catgut. On the outside of the cannula, a stainless steel jacket is screwed. On the outside of the cannula, a stainless steel jacket is screwed. The cannula is always open so that secreted gastric juice does not accumulate within but is drained from the pouch.

Before recovery from anesthesia, the dog receives 500 ml 5 % glucose in saline intravenously. The same volume is given for 3 days postoperatively together with oral fluid ad libitum. From the 4th day onfamong, normal food is given. A period of 7–10 days is required for full recovery from the operation. Special care has to be taken for each animal being kept separately in a suitable cage with mesh bottom.

For pharmacological studies, food is withdrawn 18 h prior to the experiment with water ad libitum. The animals are placed in Pawlow stands during the experiment of gastric secretion measurement, and a tube is fitted to the cannula to collect the gastric juice from the pouch for measurement of volume and acidity by titration. To test gastric acid secretion after administration of a test compound orally or by i.v. injection or infusion, gastric acid secretion from the pouch is measured in intervals of 15 or 30 min. The values are compared to the predrug secretion values and to a respective control group.

Evaluation

For testing the antisecretory potential, gastric acid secretion is stimulated either by i.v. infusions of histamine (0.1 mg/kg/h), carbachol (10 μg/kg/h), or pentagastrin (8 μg/kg/h). When stimulated gastric acid secretion has reached a stable plateau (after 1.5 h), the test compound is administered orally or by i.v. injection, and the secreted fluid is collected at 15 or 30 min intervals and analyzed for free HCl.

The secreted volume per time interval is measured. An aliquot is used for the determination of acidity by titration against 100 mmol/l NaOH, and total acid output per time interval is calculated. The effect on volume and HCl secretion at 15- or

30-min intervals after administration of the test compound is compared with the control values. Mean inhibition of stimulated acid secretion can be calculated according the formula:

$$\text{Mean inhibition } (\%) = -\left(\left(\left(\frac{\text{SAO}_{\text{postdrug}}}{\text{N}_{\text{postdrug}}}/\text{AOpredrug}\right) \times 100\right) - 100\right)$$

$\text{SAO}_{\text{postdrug}}$ = sum of acid output per 30 min after compound administration.

$\text{N}_{\text{postdrug}}$ = number of 30 min collection intervals after compound administration.

$\text{AO}_{\text{predrug}}$ = acid output prior compound administration.

In addition to the total acidity of the secreted juice, also pepsin total activity can be determined by appropriate enzymatic methods.

The Heidenhain pouch technique can also be used for evaluation and standardization of **gastrin analogs**. Two or more doses of the test compound and the standard are given intravenously. Dose–response curves are established using gastric juice secretion, acid secretion, or pepsin secretion, within two 15-min collection periods as parameters. Activity ratios with confidence limits can be calculated.

For evaluation of **H_2 antagonists**, a continuous infusion of histamine is given over a period of 45 min, starting with 3×10^{-8} M/kg/h. Then, the dose of histamine is doubled and infused for the next 45 min. This procedure is repeated until secretion has reached a plateau. Secretory response curves are constructed in the presence or the absence of a H_2 antagonist at a concentration between 2×10^{-6} and 5×10^{-7} M/kg/h.

To evaluate the **type of gastric acid secretion inhibition**, e.g., cholinergic, histaminergic, or gastrinergic mechanisms, either carbachol (8 μg/kg/h) or histamine (80 μg/kg/h) or pentagastrin (8 μg/kg/h) is administered as continuous i.v. infusion until a plateau of acid secretion is reached. The experiments are performed at weekly intervals with and without the administration of various doses of test compounds. Regression lines of antisecretory effects of various doses of test drugs are constructed and used for calculation of ID_{50} values. Relative potencies versus a standard, e.g., cimetidine, can be calculated by four- or six-point assays.

Critical Assessment of the Method

Due to the surgical procedure, the connections of the autonomic nervous system of the isolated pouch are interrupted from those of the main stomach. Therefore, basal gastric acid secretion from the pouch, which based mainly on the parasympathetic activity, is reduced.

Modifications of the Method

Boldyreff (1925) described a simplified method for isolation of a portion of the stomach as compared to the original method of Heidenhain (1878).

Gastric motility can be measured by balloon manometry of the Heidenhain pouch in the conscious dog. The animals are deprived of food for 18 h before the experiment, but water is allowed ad libitum. A latex balloon, connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB), is introduced through the fistula cannula into the accessory stomach. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of gastric motor activity. Secretin inhibits gastric motility dose dependent. After injection of gastrin or gastrin analogs, a dose-dependent increase of pressure is noted over a wide dose range.

Jacobson et al. (1966, 1967) studied gastric secretion in relation to mucosal blood flow by an antipyrine clearance technique in conscious dogs with vagally denervated gastric fundic (Heidenhain) pouches. A vagally denervated fundic pouch was so constructed that the entire arterial blood supply was delivered by the splenic artery. A non-cannulating transducer

(electromagnetic flowmeter) and a hydraulic occluder were implanted on the vessel.

The Heidenhain pouch preparation was used by Carter and Grossman (1978), Kauffman et al. (1980) to study the effect of luminal pH on acid secretion evoked by topical and parenteral stimulants and the effect of topical and intravenous 16,16-dimethyl prostaglandin E_2 on gastric bicarbonate secretion.

Baker (1979) and Roszkowski et al. (1986) developed a modified Heidenhain dog pouch preparation for collecting gastric juice exclusively from the pouch during experimental periods but allowed the pouch to be an integral part of the gastrointestinal tract during non-experimental periods. The pouch is prepared using conventional techniques but, instead of being fitted with a simple cannula through the abdominal wall, a three-way cannula is used which provides passage between the exterior orifice, the pouch, and the main body of the stomach. By inserting an appropriate adapter, passage is available only to the pouch and not to the main stomach or vice versa.

The Heidenhain pouch technique in dogs has been used for preclinical evaluation of various drugs, such as:

- A histamine H_2 antagonist by Uchida et al. (1993)
- Dual histamine H_2 and gastrin receptor antagonists by Kawanishi et al. (1997)
- A 5-HT_4 receptor antagonist by Bingham et al. (1995)
- Another 5-HT_4 receptor antagonist by Wardle et al. (1996)
- Inhibition of motilin-induced phase III contractions by pentagastrin by Yamamoto et al. (1994)
- Peptide YY by Zai et al. (1996)
- Reversible K^+-competitive inhibitors of the gastric H^+/K^+-ATPase by Parsons et al. (1995)
- The antiulcer agent SWR-215 by Kataoka et al. (1997)
- A selective gastrin/CCK-B receptor antagonist by Yuki et al. (1997)

Descroix-Vagne et al. (1993) used Heidenhain pouch preparations in **cats** and **rabbits** to study the effect of perfusion at pH 5.5 on acid and pepsin secretion.

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Gastrin Activity

Purpose and Rationale

Biological activity of gastrin and its analogs can be determined with a bioassay using the acid secretion as determined by a pH meter (Gosh and Schild 1955, 1958; Barrett 1966; Smith et al. 1970) or by measurement of conductivity according to Lawrence and Smith (1974).

Radioimmunoassays for gastrin have been developed (Jaffe and Walsh 1979) and are available as commercial kits.

Procedure

Male Sprague–Dawley or Wistar rats weighing about 250 g are used. They are withheld from food, but not from water, 18 h prior to the experiment. Four animals are used per dose of test drug or

a standard. Anesthesia is induced by appropriate method (e.g., pentobarbitone, ketamine, etc.). Body temperature is controlled by means of a rectal thermometer and a heated pad. The trachea is exposed and cannulated. The jugular veins are then exposed and cannulated with polyethylene tubes beveled at the tip. The abdomen is opened through a midline incision, the pyloro-duodenal junction exposed, and a catheter with two lumina is introduced through a cut in the duodenum up to the cardiac part of the stomach and secured firmly by tying a ligature around the pylorus. Care must be taken not to include blood vessels within the ligature.

Using a peristaltic pump, the stomach is perfused continuously with a phosphate–citrate buffer at 37 °C. In the effluent pH is measured with a pH meter and continuously recorded. At the beginning, gastric secretion is stimulated by an intravenous injection of 0.5 μg/kg pentagastrin. Then, the injections are repeated in 1-h intervals with alternating doses between 0.2, 0.4, 0.8, and 1.6 μg/kg of standard or test compound. Since in this dose range linearity of the response to gastrin can be assumed, a 2 + 2-point parallel assay is allowed. After each injection, pH or conductivity is measured for 45 min. The area under the curve after each dosage is evaluated by planimetry.

Evaluation

Each of four animals receives two doses of standard and test compound in the order of a Latin square. The dose differences have to follow a logarithmic scale. The evaluation is performed with the 4 × 4 assay according to Gosh and Schild (1958).

Modifications of the Method

Wan (1977) and Chang and Lotti (1986) used CF_1 female **mice**. The whole stomach was placed in tissue baths and perfused. The effluent of the perfused stomachs was collected at 15-min intervals, and the hydrogen ion concentration was determined by titration with 0.01 M NaOH to pH 7.0 or continuously recording with a pH meter.

Lotti and Chang (1989) tested the inhibition of gastrin-induced acid secretion by a selective non-peptide gastrin antagonist in mice, rats, and **guinea pigs**.

Black and Kalindjian (2002) reviewed gastrin agonists and antagonists.

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Receptor Binding for Gastrin

Purpose and Rationale

The gastrin gene in all mammals consists of three exons and encodes a 101 (human, mouse) or 104 (rat) precursor peptide with an N-terminal

signal peptide, which is removed at residues 21 or 25 to generate progastrin. The conversion of progastrin to smaller peptides is regulated by multiple mechanisms (Dockray et al. 2001).

Binding to gastrin receptors can be determined in isolated guinea pig gastric glands (Praissman et al. 1983; Gully et al. 1993).

Procedure

The gastric glands of male guinea pigs weighing between 300 and 400 g are isolated according to Berglindh and Öbrink (1976). The animals are anesthetized with 30 mg/kg Nembutal. The abdomen is opened, and the aorta is cannulated in a retrograde direction. Heparin solution (250 IU/ml) is injected forcefully through the cannula. After 1 min, the animal is bled through the cannula, and a ligature is placed around the mesenteric vessels. The chest is opened and the thoracic aorta clamped. A warm (37 °C) phosphate buffered saline solution is pumped into the aorta, whereupon the portal vein is opened to allow a free outflow of the perfusate. By this procedure, most of the solution is forced through the gastric blood vessels. When the stomach appears totally exsanguinated, it is rapidly removed, opened along the lesser curvature, and emptied. The cardial and antral regions are discarded. The corpus is rinsed several times with phosphate buffered saline solution and blotted with filter paper.

The thoroughly washed fundic mucosa from two guinea pigs is minced with fine scissors in a standard buffer consisting of 15 mM HEPES, 130 mM NaCl, 12 mM $NaHCO_3$, 3.0 mM NaH_2PO_4, 2.0 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.0 mM glucose, 4.0 mM L-glutamine, and pH 7.4. The minced tissue is washed and then incubated with 0.1 % collagenase in the abovementioned standard buffer containing 0.1 % bovine serum albumin in a shaker bath at 37 °C. After 40 min, the glands are liberated by a series of resuspensions through a 10-ml plastic pipette, filtered through 200 μ nylon mesh (Nytex), washed, and collected by centrifugation. The glands are then resuspended in 40 ml of the standard binding buffer containing 0.1 % bovine serum albumin at pH 7.4. Two-ml aliquots are transferred into 15-ml plastic centrifuge tubes. The amount of protein contained in fundic glands is determined according to Lowry et al. (1951).

[^{125}I]Gastrin binding is measured in the presence of 0.4 ml of gland suspension in triplicate tubes that contain 50 μl of either buffer, unlabeled gastrin (1 μM), or displacers at the desired concentration and 50 μl of [^{125}I] gastrin (70 pM final concentration). After 90 min of incubation at 37 °C, the mixture is layered over 1-ml ice-cold incubation buffer in microcentrifuge tubes and is centrifuged at 10,000 g for 5 min. The supernatant is discarded, and the radioactivity is measured in a γ-scintillation counter.

Evaluation

IC_{50} values and K_i constants are calculated.

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Gastrin-Releasing Peptide/Bombesin/ Neuromedin

Bombesin is a tetradecapeptide originally isolated from frog skin by the group of Erspamer (Anastasi et al. 1971). Shortly before, Nakajima et al. (1970) isolated from the skin of *Rana pipiens* a peptide called ranatensin because of its contractile effects on smooth muscle. **Gastrin-releasing peptide**, which shares a C-terminus with amphibian bombesin, has been isolated from a variety of mammalian and nonmammalian species (Spindel et al. 1993). **Neuromedin B**, a decapeptide originally isolated from porcine spinal cord (Minamino et al. 1983), also shows sequence homology to bombesin- and gastrin-releasing peptide. **Neuromedin N**, a six amino acid neurotensin-like peptide, shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1986).

The peptides which belong to the bombesin family can be classified in three subgroups according to the sequence of their C-terminal tripeptide: bombesin (−His–Leu–MethNH_2), ranatensin and litorin (−His–Phe–MethNH_2), and phyllitorin (−Ser–Phe–MethNH_2).

Bombesin and its homologues are known to affect a wide spectrum of biological processes (Tache et al. 1988; Parkman et al. 1994; Thomas et al. 1994; Konturek et al. 1995; Varga et al. 1994, 1995; Glad et al. 1996).

In the **gastrointestinal tract**, bombesin-/gastrin-releasing peptide stimulates hormone and peptide release; stimulates gastric, pancreatic, bile, and intestinal secretion; prevents gastric injury; causes smooth muscle contraction; and induces epithelial growth (Dietrich et al. 1994; Kortezova et al. 1994; Liu et al. 1995; Takehara et al. 1995; Wada et al. 1995; Weigert et al. 1995; Roberge et al. 1996; Yegen et al. 1996; Won Kyoo Cho 1997; Azay et al. 1998; Cox et al. 1998; Mercer et al. 1998; Milusheva et al. 1998; Nishino et al. 1998; Alvoro-Alonso et al. 1999; Bozkurt et al. 1999; Ladenheimer et al. 1999; Shahbazian et al. 1999).

Bombesin-related peptides inhibit **food intake** (Kirkham et al. 1994; Ladenheimer et al. 1996; Smith et al. 1997; Plamondon et al. 1998; Rushing and Gibbs 1998; Aalto et al. 1999; Edwards and Power 1999; Horstmann et al. 1999; Merali et al. 1999).

Several **central activities** of neuromedin B and gastrin-releasing peptide are reported, e.g., involvement in the hypothalamic–pituitary system (Pinski et al. 1992; Plamondon and Merali 1997; Garrido et al. 1998, 1999). Moody and Merali (2004) reviewed the behavioral implications of bombesin-like peptides and associated receptors within the brain. Roesler et al. (2004) discussed bombesin receptors as novel therapeutic targets in anxiety disorders. **Autocrine actions** of neuromedin B and gastrin-releasing peptide in small and non-small cell lung carcinomas are described (Gaudino et al. 1988; Siegfried et al. 1999).

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Bombesin Receptor Binding

Purpose and Rationale

Different types of receptors have been described for bombesin-like peptides (von Schrenk et al. 1989; Battey et al. 1991; Rouissi et al. 1991; Severi et al. 1991; Fathi et al. 1993). The mammalian bombesin receptor subfamily of G protein-coupled receptors consists of the gastrin-releasing peptide receptor (GRP-R), also called BB2; the neuromedin B receptor (NMB-R), also called BB1; and the bombesin receptor subtype 3 (BRS-3), also called bb3 (Fathi et al. 1993; Mantey et al. 1997; Donohue et al. 1999; Jian et al. 1999). A fourth subtype, bombesin receptor subtype 4, has been isolated from a cDNA library from the brain of the frog, *Bombina orientalis* (Katsuno et al. 1999). A ligand with high affinity to all four receptors has been identified (Pradhan et al. 1998).

Moody et al. (1978) studied the binding of the radiolabeled bombesin analog [^{125}I]bombesin to rat brain membranes. Functional GRP-preferring bombesin receptors were identified in human melanoma cells by Pansky et al. (1997). Benya et al. (1995) expressed and characterized clones of human bombesin receptors.

Procedure

BALB/3 T3 fibroblasts devoid of gastrin-releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R) are selected by clonal expansion after assaying for GRP-R or NMB-R by RNase protection and binding studies (Benya et al. 1992). These BALB/3T3 cells are stably transfected using a full-length human GRP-R clone (huGRP-R transfected cells) or a full-length human NMB-R clone (huNMB-R transfected cells) (Corgay et al. 1991). In both cases the receptor is subcloned into a modified version of the pCD2 plasmid and transfected using calcium phosphate precipitation. Stable transfectants are isolated in the presence of 800 μg/ml aminoglycoside G-418 (GIBCO, Waltham MA), identified by binding studies, and then maintained in DMEM containing 10 % fetal bovine serum and 270 μg/ml G-418. Cells are passaged every 3–5 days at confluence by splitting 1:4.

[^{125}I-D-Tyr^{0}]NMB (2,200 Ci/mmol), ^{125}I-GRP (2,200 Ci/mmol), and [^{125}I-Tyr^{4}]Bn (2,000 Ci/mmol) are prepared using Iodo-Gen and purified using high-pressure liquid chromatography. Binding studies are performed by suspending disaggregated cells in binding buffer containing 75 pM levels of either [^{125}I-D-Tyr^{0}]NMB or [^{125}I-Tyr^{4}]Bn and 3×10^{6} cells/ml for 30 min at 22 °C. Nonsaturable binding of either radiolabeled peptide is the amount of radioactivity associated with transfected cells when the incubation mixture contains either 1 μM NMB of 1 μM Bn.

The ability of various bombesin-related agonists or antagonists to inhibit the binding of [^{125}I-Tyr^{4}]Bn to huGRP-R transfected cells and of [^{125}I-D-Tyr^{0}]NMB to huNMB-R transfected cells is compared.

Evaluation

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment, each value is determined in duplicate, and the results are expressed as the means ± standard errors of at least three separate experiments.

Modifications of the Method

Radulovic et al. (1991) studied biological effects and receptor binding affinities of pseudononapeptide bombesin/CRP receptor antagonists.

Fanger et al. (1993) identified a 63-kDa serum protein that binds somatostatin and gastrin-releasing peptide but not bombesin.

Wada et al. (1997), Ohki-Hamazaki et al. (1999), and Yamada et al. (2000a, b) studied mice lacking the gastrin-releasing peptide receptor, the bombesin subtype-3 receptor, or the neuromedin B receptor.

Mantey et al. (2004) and Boyle et al. (2005) described selective ligands for the bombesin receptor subtype 3.

Akeson et al. (1997) identified four amino acids in the gastrin-releasing peptide receptor that are required for high affinity agonist binding.

Ryan et al. (1998) studied the intracellular signaling of the human bombesin orphan receptor BRS-3 by various bombesin receptor agonists and antagonists.

Chave et al. (2000) analyzed the expression of bombesin-like peptides and their receptor subtypes in normal and neoplastic colorectal tissue.

Sun et al. (2000) investigated the presence and characteristics of the functional receptors for bombesin/GRP in human prostate adenocarcinoma specimens by radioreceptor assay and the mRNA expression of the three bombesin receptor subtypes by RT-PCR.

Weber et al. (2000) determined the structure of the mouse gastrin-releasing peptide receptor gene and investigated its basal promoter activity.

Radioligands for the **GRP receptor** are:

- [^{125}I][D-Tyr^{6}]bombesin-6–13-methylester

[^{125}I]GRP, [^{125}I][Tyr^{4}]bombesin
For the **neuromedin B receptor:**

- [^{125}I]BH-NMB, [^{125}I][Tyr^{4}]bombesin

For the **bombesin receptor subtype 3:**

- [^{125}I][Tyr^{6}, βAla^{11},Phe^{13},Nle^{14}]bombesin-6–14 (Alexander et al. 2000)

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Evaluation of Bombesin Receptor Antagonists as Anticancer Drugs

Purpose and Rationale

Bombesin and gastrin-releasing peptide affect the growth and differentiation of lung epithelium in the fetus and the adult. Gastrin-releasing peptide is an autocrine growth factor for various small cell carcinoma cell lines. Bombesin/gastrin-releasing peptide receptors were also identified in other human cancer tissue (Halmos et al. 1995). Therefore, bombesin- and gastrin-releasing peptide-antagonists were synthesized and tested as antitumor agents (Heimbrook et al. 1991; Qin et al. 1994a, 1995; Thomas et al. 1994; Azay et al. 1996; Casanueva et al. 1996; Halmos and Schally 1997; Moody and Jensen 1998).

The procedure of Qin et al. (1994a) on the inhibitory effect of an bombesin receptor antagonist on the growth of human pancreatic cells in vivo and in vitro is described as example of various similar studies.

Procedure

Cancer Cell Line

Cancer cells of the CFPAC-1 human pancreatic cell line, originally established from a well-differentiated ductal pancreatic adenocarcinoma of a 26-year-old white male with cystic fibrosis, are routinely maintained in a monolayer culture in Costar T75 culture flasks with IMDM medium containing 10 % FCS, 0.5 g/l L-glutamine, 25 mM HEPES, 3.7 g/l $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B under humidified 5 % CO_2 at 37 °C. The cells growing exponentially are harvested by an incubation with 0.25 % trypsin–EDTA in calcium- and magnesium-free Hank's balanced salt solution for 5 min at 37 °C. For tumor cell implantation, a cell suspension is prepared in serum-free IMDM by repeatedly passing the cells through a G-22 needle; then the cells are diluted to a concentration of 5×10^6 cells/ml.

Implantation of Tumors in Nude Mice

Male athymic BALB/c (nu/nu) 6-week-old mice are housed in a laminar airflow cabinet under pathogen-free conditions throughout the experiments. Three nude mice receive s.c. injections in the flanks with 0.2 ml of cell suspension (1×10^6 cells) and serve as tumor donors. After 4 weeks, the implanted tumors grow to a size of about 5 mm in diameter and are removed from the mice. Tumor samples are dissected free of necrotic tissue and blood vessels and are cut into small fragments of about 8 mm^3. Under anesthesia, two pieces of tumor fragments are implanted s.c. by trocars on both sides of the flanks for each mouse. The mice bearing the implanted tumors are randomly divided into groups with 10 mice in each group.

The nude mice with implanted tumors start to receive injections of the bombesin antagonist or vehicle 7 days after the tumor cell injection. The treatment is continued for 25 days.

Evaluation of Tumor Growth

During the treatment, the size of the implanted tumors is measured by calipers in each mouse at 3–4 days intervals for 25 days to construct the tumor growth curve in vivo. Tumor volume is calculated by the formula: tumor volume = length $\times$ width^2 $\times$ 0.5. Tumor volume doubling time is defined as the time required for the tumors to grow from 50 to 100 mm^3 for the control group and from 35 to 70 mm^3 for the treatment group, respectively. The tumor growth delay time is estimated as the time difference for the treated tumors and the controls to reach a volume of 70 mm^3. At the end of the experiment, the animals are sacrificed by an appropriate procedure. The

tumors are removed from the animals, weighed, and immediately frozen in liquid nitrogen for measurement of DNA and protein content in tumor tissues.

Determination of DNA and Protein in Tumor Tissue

DNA in tumor tissue is determined by the method of Labara and Paigen (1979) which is based on the enhancement of fluorescence reaction upon binding bis-benzimidazole Hoechst 33528 to DNA in cell nuclei in a high ionic strength solution. Tumors collected in each group are pooled and homogenized in 10 times their volumes in a buffer consisting of 0.05-M NaH_2PO_4, 2.0 M NaCl, and 2 mM EDTA (pH 7.4). Hoechst 33528 is dissolved in the same buffer at a concentration of 1 μg/ml and filtered before use. An aliquot of tumor homogenate (0.4 ml) is suspended in 4 ml of Hoechst 33528 solution, followed by incubation in a dark room for 30 min. The reaction is measured by a fluorescence spectrophotometer at excitation and emission wavelengths of 356 and 492 nm, respectively. Calf thymus DNA type I is used as a standard.

Measurement of Cell Growth in Vitro

The effects of bombesin and the bombesin antagonist on the growth of CFPAC-1 human pancreatic cells in vitro are evaluated by direct cell counting and [^{3}H]thymidine incorporation assay.

Direct Cell Counting

CFPAC-1 cells collected from 60 % to 70 % confluent cultures are used for this study and seeded to 24-well culture plates (1×10^4 cells/well). After the cells are cultured in IMDM containing 10 % FCS for 48 h, the medium is replaced by IMDM supplemented with 2.5 % FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. Following another 24 h of incubation, the culture is terminated by aspiration of the medium from the wells and washing with PBS (0.5 ml/well). The cells are trypsinized by a 10-min incubation with (0.5 ml/well) 0.25 % trypsin–EDTA. The detached cells are dispersed by repeated pipetting using a G-22 needle and syringe. The number of cells is counted by an automated electronic cell counter (Coulter Counter Model ZF).

[^{3}H]Thymidine Incorporation Assay

Single-cell suspension is prepared in IMDM with 10 % FCS and seeded to 24-well culture plates (1×10^4 cells/well). After 46 h of culture, the medium is changed to IMDM (0.5 ml/well) containing 2.5 % FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. After 24 h of culture, [methyl-^{3}H]thymidine (radioactivity 25 Ci/mg) is added to each well (1 μCi/well) to pulse the cells. After a 4-h incubation, the medium is removed from the wells, and the cells are fixed by Camoy's solution (1 ml/well; methanol to glacial acetic acid, 3:1, v/v) for 20 min. After washing three times with PBS, the cells in each well are dissolved with 0.5 ml of 0.3-N NaOH for 15 min at room temperature. The cell lysate is collected and mixed with 3 ml of universal scintillation cocktail. The radioactivity is measured for 1 min by a liquid scintillation beta counter.

Receptor Binding Assay

Receptor binding assay is performed using intact CFPAC-1 cells in monolayer cultures. Tyr^4-bombesin is labeled with ^{125}I-Na using a Bio-Rad enzymo-bead iodination kit. Mono-^{125}I-Tyr^4-bombesin is purified by high-performance liquid chromatography resulting in a specific activity of ^{125}I-Tyr^4-bombesin of about 2,000 Ci/mmol. CFPAC-1 cells are seeded to 24-well culture plates (1×10^4 cells/well) and cultured with IMDM containing 10 % FCS for 48 h. The cells in subconfluent culture are washed once with serum-free IMDM supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM $MgCl_2$, 1 mM EGTA, 10 mM monothioglycerol, 0.25 mM phenylmethylsulfonyl fluoride, aprotinin 10,000 kallikrein inactivator units/l, and 0.1 % bovine serum albumin (pH 7.5), followed by an incubation for 2 h at 22 °C with the same medium containing (0.5 nM) ^{125}I-Tyr^4-bombesin in the presence or absence of various concentrations of bombesin, bombesin

antagonists, or structurally unrelated peptides. The binding reaction is terminated by adding 0.5 ml of ice-cold medium to each well. After washing four times with ice-cold PBS (pH 7.4), the cells in each well are dissolved with 0.5 ml of 0.3 N NaOH. The resultant cellular lysate is collected from each well for measurement of radioactivity by a gamma counter.

Evaluation

All data are expressed as the mean ± SEM of duplicate or triplicate observations from at least 2–3 repeated experiments. Mean values between the treatment and control group are analyzed by the student's *t*-test, Mann–Whitney *U*-test, or one-way analysis of variance.

Data from receptor binding assays are analyzed by a ligand-PC computerized curve-fitting program created by Munson and Rodbard as modified by McPherson (1985) to determine the types of binding sites, the dissociation constants (K_d), and the maximal binding capacity of receptors (B_{max}).

Modifications of the Method

Similar studies, mostly by Schally's group, as with tissue derived from human pancreatic cancer were performed with nitrosamine-induced pancreatic cancers in hamsters (Szepeshazi et al. 1993, 1994, 1999), with human prostate cancer (Pinski et al. 1993a, b), with rat prostate cancer (Pinski et al. 1994a), with human gastric cancer (Halmos et al. 1994; Qin et al. 1994b), with human small-cell and non-small-cell lung carcinoma (Pinski et al. 1994b; Moody et al. 1996; Koppan et al. 1998; Kiaris et al. 1999a), with human breast cancer (Yano et al. 1994; Miyazaki et al. 1998; Kahan et al. 2000), with mouse mammary cancer (Szepeshazi et al. 1992, 1997), with human colon cancer (Radulovic et al. 1994), with human glioblastoma (Kiaris et al. 1999b), and with human renal adenocarcinoma (Jungwirth et al. 1998).

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Mucus Secretion

Isolated Gastric Mucosal Preparation

Purpose and Rationale

Main and Pearce (1978) described an isolated gastric mucosal preparation from rats for studying the pharmacology of gastric secretion and the synthesis or release of endogenous substances.

Procedure

Rats of either sex weighing 100–120 g or guinea pigs weighing 400–600 g are anesthetized with 60 mg/kg pentobarbitone s.c. The abdomen is opened along the midline and the stomach exteriorized. The nonglandular portion is removed and the stomach rinsed with mucosal solution, containing 136 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2 \cdot 6H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, and 16.7 mM glucose. The muscular layer overlying the nonantral region is separated from the mucosa by blistering. The tip of a fine needle (27 gauge) is inserted just below the muscle and mucosal solution injected between the layers. The process is repeated as many times as is necessary to blister the whole area. Fine scissors are then used to cut the muscle along the greater curvature and then parallel to the cut edge. The muscle sheet is pulled back to expose the mucosa. The stomach is then removed, opened by cutting along the greater curvature, and rinsed with mucosal solution.

Two pieces are obtained from one stomach, and each is placed, mucosal surface inwards, over a 1 cm^2 opening on a polyethylene vessel (titration cup). Both preparations, one each from the ventral and dorsal surfaces, are stretched lightly over the cup and tied in place. Each tissue is placed in an organ bath, at 37 °C, containing 35 ml of serosal solution (110 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2 \cdot 6H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 26 mM $NaHCO_3$, and 16.7 mM glucose) being gassed with a 95 % O_2/5 % CO_2 mixture. The mucosal surface is superfused by means of a peristaltic pump at a rate of 0.5 ml/min with an unbuffered solution of similar ionic composition and gassed with 100 % O_2. The volume of solution on the mucosal side is kept constant by suction via the same pump using larger diameter tubing, varying between 1.6 and 2.0 ml. This small volume is used in order to follow changes in acid secretion more closely. Secretion is recorded continuously via a dual microelectrode in the mucosal solution connected with a potentiometric pen recorder. The H^+-ion concentration is noted every 15 min and expressed as apparent secretion rate. Drugs are added to the serosal solution in volumes not exceeding 1 ml. Contact

times for drugs and hormones (e.g., gastrin or histamine) are between 30 and 60 min. Recovery periods before the next response are between 45 and 60 min. Responses are readily reversible on replacing the solution in the bath with fresh, warmed serosal solution.

Evaluation

The secretory response is calculated as the increase in secretion rate at the peak of the response over the preceding basal value. Dose–response curves and time–response curves are established for histamine, gastrin, and methacholine. The effects of drugs which inhibit acid secretion are evaluated by adding them either at the peak of a response or 15–60 min prior to the secretagogue.

Modifications of the Method

Wan et al. (1974) used the fundic glandular portion of the rat stomach mounted onto a glass tube without removing the muscular layer to study the inhibition of in vitro stimulated gastric acid secretion by a histamine H_2 receptor antagonist.

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Primary Culture of Rat Gastric Epithelial Cells

Purpose and Rationale

Zheng et al. (1994) described an in vitro model for evaluation of antisecretory agents using primary cultures of rat gastric epithelial cells.

Procedure

Gastric mucosal cells from 1- to 2-week-old Sprague–Dawley rats are isolated according to Terano et al. (1982). Gastric mucosal surface is washed thoroughly with sterile cotton and Hank's balanced salt solution (HBSS) and then rinsed with HBSS before being minced into approximately 1-mm^3 pieces. The minced tissues are incubated in HBSS containing 0.1 % collagenase and 0.05 % hyaluronidase at 37 °C in a shaking water bath for 60 min and then pipetted several times and filtered through a sterile nylon mesh. The filtrate is washed twice with HBSS by centrifugation (200 g for 5 min) and resuspended in Coon's modified Ham's F-12 culture medium containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 15 mM HEPES, 2-μg/ml fibronectin, and 10 % fetal bovine serum. Cells are seeded at a density of 1.5–2 $\times$ 10^5 or 1–2 $\times$ 10^4 cells/cm^2 directly onto either 96-well plates or 6-well plates and maintained in a Steri-Cult incubator at 37 °C in a humidified atmosphere with 5 % CO_2. The medium is changed daily. The confluent monolayers are formed after 4–5 days in 96-well plates.

Drug Treatment

To measure the effects of individual drugs on the viability of gastric mucosal cells, cells are incubated with 0.02–5 mg/ml drugs in culture medium for either 2 or 48 h for the uptake of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) or up to 8 days for the colony-forming efficiency assay. To measure the cytotoxicity of drugs, e.g., indomethacin, and acidified medium to gastric mucosal cells, cells are incubated in either serum-free medium containing 0.5–10 mM indomethacin for 1 h or pH 3.5 at for 10–30 min.

To study the effect of various antacids, e.g., aluminum hydroxide or sucralfate preparations, cells are incubated for 1 h in culture medium containing these drugs. The drug suspensions are then aspirated away, followed by another hour of treatment with 3.5 mM indomethacin.

Alternatively, cells are treated with drugs and 3.5 mM indomethacin concurrently for 1 h. The effects of antacids on acid-induced damage are investigated by incubating the cells with the agents for 2 h and then exposing them to pH 3.5 medium for 10–30 min.

MTT (3-[4,5-Dimethyl-2-Thiazolyl]-2,5-Diphenyl-2H-Tetrazolium Bromide) Colorimetric Assay

Cells in 96-well plates are treated with the different drugs and then incubated in 100 µl of culture medium containing 10 µl of an MTT stock solution (5 mg/ml) for 4 h at 37 °C according to Mosmann (1983). Following the incubation, 100 100 µl mu;l acid-isopropanol (0.04 N HCl in isopropanol) is added to the wells and incubated overnight at room temperature. The color changes are recorded at 540 nm on a microplate reader. To exclude the disturbance of precipitates in some samples, the samples are centrifuged, and only the supernatants are read. For each experiment, a standard curve is generated by measuring the relationship of absorbance to a series of viable cell numbers.

Neutral Red Uptake Assay

Neutral red is prepared as a 1 % stock solution in distilled water and diluted to 0.035 % in HBSS immediately before each experiment. The cells are treated with the drugs and then stained with 0.1 ml of 0.035 % neutral red for 30 min (Parish and Müllbacher 1983). The stain is discarded, and the cells are washed twice in HBSS before the addition of 200 µl/well of acidified alcohol solution (50 %, v/v, ethanol/water, containing 0.5 % acetic acid). After a 2-h incubation period at room temperature, the color changes are measured in a microplate reader.

Colony-Forming Efficiency Assay

The cells are seeded into 6-well plates and incubated in culture medium containing 0.02–5 mg/ml of individual drugs for 8 days. Cells are then fixed in 10 % formalin and stained with 1 % aqueous crystal violet (Sundqvist et al. 1989). Colonies formed on each well are counted (crystal violet stains cell nuclei) and compared with those formed on drug-free wells.

Evaluation

Data are expressed as mean ± standard error. A one-way analysis of variance followed by Scheffe's post hoc test is used to test the significance between control and drug-treated samples. Differences are considered significant at $p < 0.05$.

Modifications of the Method

Buchan et al. (1993) used cultured human antral epithelial cells enriched for D cells to study the effect of cholecystokinin and secretin on somatostatin release.

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Gastric Motility

Measurement of Intragastric Pressure in Rats

Purpose and Rationale

Gastric motor activity can be measured by recording intragastric pressure in anesthetized rats (Holzer 1992).

Procedure

Sprague–Dawley rats of either sex weighing 220–240 g are deprived of food for 20 h prior experimentation but are allowed free access to tap water. After induction of anesthesia by i.p. injection of phenobarbital sodium (0.92 mmol/kg), the trachea is cannulated to facilitate spontaneous breathing. The left carotid artery is cannulated and connected to a pressure transducer to monitor mean arterial blood pressure. The left jugular vein is cannulated for i.v. injection of drugs and for continuous infusion of physiological saline at a rate of 0.6 ml/h to avoid dehydration of the animals. Intragastric pressure is measured by a catheter (outer diameter 1.9 mm) passed down to the stomach via the esophagus. The position of the catheter tip in the corpus region is verified at the end of each experiment. The catheter has two side holes in its tip segment and is continuously perfused with physiological saline at a rate of 0.6 ml/h. To record intragastric pressure, the catheter is connected to a pressure transducer.

Evaluation

The gastric motor effect is quantitated by calculation of the area under the curve versus baseline intragastric pressure, measured immediately before injection of the stimulant (e.g., neurokinin A). To test inhibitors, the area under the curve after the stimulant is set as 100 %, and the effect of application of the inhibitor with the stimulant is calculated as percentage.

Modifications of the Method

Lotti et al. (1986) described a simple mouse assay for the in vivo evaluation of cholecystokinin antagonists which is based on visual determination of the gastric emptying of a charcoal meal.

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Isolated Smooth Muscle Preparation of Guinea Pig Stomach

Purpose and Rationale

Boyle et al. (1993) described a novel smooth muscle preparation from the guinea pig stomach for characterization of CCK receptors by the use of selective antagonists.

Procedure

Adult male guinea pigs weighing 330–400 g are sacrificed and the stomach rapidly removed. The fundus is discarded, and the stomach is opened along the greater curvature, pinned on a Petri dish with the mucosa pointing up. The mucosal and submucosal layers are removed by dissection to reveal the underlying smooth muscle layer. Strips of circular muscle (2 × 25 mm) from the corpus region of the stomach are obtained by cutting inwards, following the striations of the lesser curvature. Muscle strips are mounted in siliconized 3 ml organ baths containing Krebs–Henseleit solution. The buffer is modified to include 5 μM indomethacin. The solution is maintained at 37 °C and continuously gassed with a mixture of 95 % O_2/5 % CO_2. Isometric contractile responses are measured with force-displacement transducers (e.g., Grass FT.03) and recorded on a polygraph (e.g., Mark VII Graphtec Linearcorder).

Tissues are placed under 1 g tension and allowed to equilibrate for 30 min after which

time they are contracted with a submaximal dose of carbamylcholine (10 nM). Using a 12-min dose cycle, concentration–response curves are established for agonists, e.g., gastrin, pentagastrin, CCK, and CCK analogs. For studies with antagonists, the tissues are exposed to antagonists for 15 min before re-exposure to agonists. The addition of 5 μM indomethacin removes the spontaneous activity due to the inherent myogenic tone existing in the tissue, but leaves the responses to the agonists unaffected.

Evaluation

Contractile responses to exogenously applied agonists are expressed as absolute changes in tension and are transformed as a percentage of the maximal response achieved for that agonist in order to obtain potency values. EC_{50} values are obtained graphically for individual concentration–response curves. Responses to agonists in the presence of antagonists are expressed as a percentage of the control maximum response obtained in the same tissue preparation. Agonist concentration–response curves in the absence and presence of increasing concentrations of antagonists are obtained. The method of Arunlakshana and Schild (1959) is used to provide Schild plots of the data and to obtain affinity constants for the antagonists.

Modifications of the Method

Riazi-Farzad et al. (1996) described an improved preparation of the **isolated rat stomach fundus strip** based on the finding that the majority of the contractile response to 5-HT and carbachol was present in the left ventral longitudinal quartile of the tissue.

Van Nueten et al. (1978), Reyntjens et al. (1984), Schuurkes et al. (1985), and Kishibayashi and Karasawa (1998) used an **isolated gastroduodenal preparation of the guinea pig**. After ligation of the esophagus, the stomach was filled with 20 ml saline and suspended in 200 ml of oxygenated Krebs–Henseleit solution, maintained at 37 °C. The duodenum was cannulated and connected with an ultrasonic transit time transducer to record changes in intraluminal volume and with a bottle of saline to ensure constant hydrostatic pressure of 6 cm H_2O. Spontaneous phasic activity was always present on the stomach and recorded as rhythmic changes in gastric volume. Gastric peristaltic waves either stopped at the pylorus or were propagated to the duodenum. Antroduodenal coordination was quantified as the relative number of antral waves that were propagated to the duodenum. After 30 min stabilization, drugs were added in varying concentrations and the effects followed for 30 min. Three parameters were determined: (1) contractile amplitude, expressed as milliliters of expelled volume; (2) frequency, measured as number of contractions per minute; and (3) percentage of antroduodenal coordination.

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Absorption

Measurement of Gastric Absorption of Drugs in Rats

Purpose and Rationale

Although the stomach is not the prime absorptive site of drugs, the absorption of some drugs from the gastric mucosa has been established (Doluisio et al. 1969; Welling 1977). Worland et al. (1983) described an in situ gastric pouch technique for direct measurement of the gastric absorption of drugs in the rat.

Procedure

Male Wistar rats weighing 220–300 are anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. A cannula is placed in the jugular vein for administration of heparinized saline (3,000 IU/kg) and whole blood replacement during the experiment. The replacement blood is collected from heparinized donor rats immediately prior to the experiment. Another cannula is placed into the carotid artery of the experimental animal for the collection of systemic blood samples. A midline incision is made in the abdominal wall. Double ligatures are placed on the superior epigastric vessels, and transverse incisions are made between the ligatures. The branches of the right gastroepiploic veins and arteries are then ligated using surgical silk, and the gastrohepatic ligament between the stomach and the posterior surface of the left hepatic lobe and the caudal hepatic lobe is separated. The gastrosplenic mesentery is also severed. A strip of gauze is employed to keep the liver lobes out of the surgical field. Using appropriately sized surgical silk, double ties are placed around the short gastric vessels and the esophagus, which are then severed between the ties. The pylorus and pyloric vessels are then ligated, and an incision is made in the forestomach to allow the removal of gastric contents.

The pouch is then rinsed with warm saline until clear, and the remaining fluid is removed using an adsorbent tissue. A Luer adapter modified from a three-way tap is tied into the incision to enable drug administration via a syringe. The gastric pouch is then transposed to the right of the animal exposing the left gastric vein. The vein is cannulated above the junction with the lienal (splenic) vein using a 21-gauge needle connected to a 15-cm length of polyethylene tubing (i.d. 0.75 mm, o.d. 1.45 mm). A small Oxford clamp with foam rubber insets is employed to prevent dislocation of the cannula during the changing of the sample vials. The drug is administered into the stomach, and the blood draining from the gastric pouch is collected over timed intervals. To determine the volume (ml) of the blood collected, the venous effluent is weighed, and hematocrit (HCT) readings are taken for each sample and converted to units using the formula:

$$\text{Volume(ml)} = \frac{\text{mass blood collected (g)}}{\text{HCT} \times \text{blood celldensity} \; + \; (1 - \text{HCT}) \times \text{plasma density}}.$$

Rat blood cell and plasma density measurements are obtained from five determinations from four rats, blood samples being separated at 3,000 g for 10 min. Samples from the gastric pouch are kept on ice until the plasma can be separated by centrifugation at 3,000 g for 10 min and the plasma frozen until assay.

Blood replacement is delivered at a rate of 0.7 ml/min from a gently oscillating reservoir using a peristaltic pump.

The compound to be tested is administered in a volume of 0.5 ml to the gastric pouch. Plasma levels are determined with a method specific for the compound under investigation. Plasma

samples are collected at 4 min intervals over a period of 30–60 min. At the end of the experiment, the fluid in the gastric pouch is collected for determination of the dose remaining in the stomach.

Evaluation

Mean values ± standard deviation of plasma concentration are calculated from 4 to 6 experiments and plotted versus time to demonstrate the absorption profile.

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Antacid Activity

Evaluation of Antacids

Purpose and Rationale

Antacids have been used for the treatment of gastroduodenal ulcerations since a long time (Konturek 1993). The main action of antacids is to reduce the acidity of the gastric content through neutralization and increasing intragastric pH. Since pepsin is not active at higher pH levels, antacids reduce peptic activity (Goldberg et al. 1968) and may also adsorb pepsin (Sepelyak et al. 1984). Binding of bile salts (Clain et al. 1977) by antacids may also have a beneficial influence on peptic ulcer disease. Most antacids contain magnesium and aluminum hydroxide, in some cases also calcium carbonate and sodium bicarbonate. The acid-neutralizing potency can be measured in vitro.

Procedure

0.1 M HCl is added to the antacid to be tested. The acid-neutralizing capacity is defined as the amount of 0.1 M HCl that can be added to a liquid antacid without reducing the pH of the mixture below pH 3.0. The determination of the acid-neutralizing capacity of antacid tablets is performed in vitro by stirring a mixture of crushed tablets and water. The time at which all antacid is consumed is much longer for antacid in tablet as compared with the same amounts of antacid in liquid form. Magnesium hydroxide is very insoluble in water but is readily soluble in hydrochloric acid. In combined preparations, the magnesium hydroxide reacts first to produce an almost immediate neutralizing effect and an increase of the pH within a few minutes. Aluminum oxide, on the other hand, has a weaker antacid activity, reacting more slow with acid (Fordtran et al. 1973; Richardson and Peterson 1988).

Evaluation

In vitro titration curves of 0.1 M HCl with 5 ml liquid antacid or one tablet are measured over 3 h and compared with the standard.

Critical Assessment of the Method

Acid-neutralizing potency may be not the only factor which contributes to the therapeutic effect of antacids. Damage and protection in the stomach are essentially represented by acid secreted by the parietal cells and by bicarbonate released by the surface epithelial cells and mucous neck cells. Among various neurohumoral factors, most important in the bicarbonate secretion appear the prostaglandins, mainly of the E series. Mucosal bicarbonate secretion may be stimulated through the activation of prostaglandins by aluminum hydroxide-containing antacids. Aluminum-containing antacids were found to protect against mucosal damage due to topical irritants and against stress or aspirin-induced lesions. Gastroprotection of aluminum-containing antacids has been attributed to the biological activity of nitric oxide interacting with mucosal prostanoids on the mucosal microcirculation. Therefore, these antacids were found to be active in various experimental ulcer models and to exert

cytoprotective activity against ethanol-induced gastric injury in rats (Szelenyi et al. 1983; Domschke et al. 1986; Hollander et al. 1986; Konturek et al. 1989, 1992; DiJoseph et al. 1989; Vergin and Kori-Lindner 1990). Konturek (1993) reported that aluminum hydroxide-containing antacids protect growth factors, involved in the healing of ulcers, against acid degradation.

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Inhibition of HCl Secretion

Anticholinergic Activity

General Considerations

Gastric motility and tonus, as well as gastric secretion, are stimulated by cholinergic impulses. Anticholinergic compounds, such as the *Belladonna* alkaloid atropine, were the first drugs used in the treatment of gastric ulcers. The doses that are necessary to reduce acid secretion also decrease mucus secretion and cause side effects, such as dry mouth, increase in heart rate, and ocular disturbances. Inhibitors of specific muscarinic receptors responsible for gastric acid secretion were found. Receptors for acetylcholine which play a major role in central and peripheral transmission have been studied extensively (Karlin et al. 1976; Hulme et al. 1990; Jones et al. 1992; Kebabian and Neumeyer 1994).

Acetylcholine Receptor Binding

Purpose and Rationale

The search of specific muscarinic antagonists for inhibition of gastric acid secretion leads to the discovery of pirenzepine, a tricyclic compound, originally tested as psychotropic agent. This

compound has a greater gastrointestinal selectivity than other muscarinic antagonists (Carmine and Brogden 1985; Longdong 1986). The activity of pirenzepine has been localized to M_1 receptors.

This, and the involvement of acetylcholine in many physiological processes, has stimulated the research on the various types of muscarinic receptors. At present, five types have been described, but further subdivisions can be envisaged (Hulme et al. 1990; Jones et al. 1992; Kebabian and Neumeyer 1994; Caulfield and Birdsall 1998; Alexander et al. 2001; Eglen et al. 2001; Ma et al. 2004). The subtypes of muscarinic receptors have been characterized by the use of organs with a predominant subtype receptor population, e.g., rabbit vas deferens stimulated with electric impulses for M_1, electrically stimulated left atria from guinea pigs for M_2, and longitudinal smooth muscle preparations of guinea pig ileum or salivary gland of the rat for M_3 (Doods et al. 1987; Lambrecht et al. 1993), or by the use of selective antagonists (Pitschner et al. 1989; Richards 1990; Svensson et al. 1992) or agonists (Lambrecht et al. 1993). Further characterization has been achieved by studies of the transduction mechanisms (Brown and Brown 1984; Brown et al. 1985; Parekh and Brading 1992) and by voltage clamp techniques (Bernheim et al. 1992). The five muscarinic receptor subtypes are referred to as M_1–M_5. The odd-numbered receptors (M_1, M_3, M_5) couple efficiently, through $G_{q/11}$, to activation of phospholipase C, which initiates the phosphatidylinositol turnover response. The even-numbered muscarinic receptors (M_2–M_4) inhibit adenylyl cyclase activity via activation of the G_i class of G proteins.

The genes for the muscarinic acetylcholine receptor subtypes have been cloned and expressed in Chinese ovary hamster cells (Buckley et al. 1989; Dörje et al. 1991a) or in fibroblasts (Kashihara et al. 1992).

Many organs, e.g., rat brain (Luthin and Wolfe 1984; El-Fakahani et al. 1986), have been used for studies on acetylcholine receptor subtypes. Only the method using Chinese ovary hamster cells is presented here.

Procedure

Preparation of Plasmid DNA

The coding sequences of the m1, m2, m3, m4, and m5 receptors are derived from a human genome library. The cDNAs are inserted into the Okayama/Berg pCD or pCD-PS expression vector (Bonner et al. 1987, 1988; Buckley et al. 1989). Plasmid DNA is isolated by two sequential density gradient centrifugations through CsCl (Maniatis et al. 1982).

Cell Culture

Chinese hamster ovary cells are incubated at 37 °C in a humidified atmosphere (5 % CO_2) as a monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 100 units/ml each of penicillin G and streptomycin, and 4 mM glutamine.

Transfection Procedures

Cells are transfected using a modified calcium phosphate procedure (Chen and Okayama 1987) involving the use of cotransfected pcDneo as a selectable marker. Selection with the neomycin analog G 418 (600 µg/ml; Gibco NY) is started 72 h after transfection and continued for 2–3 weeks. Media are changed every 3 days. Clonal cell lines are obtained by single-cell cloning and assayed for [^{3}H]NMS (*N*-methylscopolamine hydrochloride) binding capacity.

Membrane Preparation

Cells are grown to about 80 % confluency, scraped into ice-cold binding buffer and homogenized for 30 s using a Brinkman homogenizer (setting 5). Membranes are pelleted at 16,000 g for 15 min and re-homogenized. Membrane protein is determined using a Bio-Rad protein assay dye reagent. Membranes are stored frozen at −80 °C before use.

Radioligand Binding Studies

All membranes, drugs, and radioligands are made up in binding buffer consisting of 25 mM sodium phosphate (pH 7.4) containing 5 mM magnesium chloride. The assays are performed in 1 ml total

volume. Final membrane concentrations are m1 6 µg/ml, m2 10 µg/ml, m3 5 µg/ml, m4 3 µg/ml, and m5 4 µg/ml. In [^{3}H]NMS saturation experiments, 8–10 different concentrations of the radioligand (2–1,400 pM) are employed. For displacement experiments, [^{3}H]NMS in a concentration of 150 pM, and 10 different concentrations of the displacer are used. Specific binding is defined as the difference in [^{3}H]NMS binding in the absence and presence of 1 µM atropine. Alternatively [^{3}H]pirenzepine is used. Incubations are carried out at 22 °C for 3 h. Assays are terminated by filtration through a Brandel cell harvester onto Whatman GF/C filters. Membranes are washed three times with 5 ml of ice-cold binding buffer before being dried. They are transferred to 10 ml of scintillant (New England Nuclear Aquasol) and counted in a LKB β-counter.

Evaluation

Data from direct binding experiments are fitted to the equation:

$$\alpha = \frac{(B_{\max} x^n / k)}{(1 + x^n / k)}$$

to derive the Hill coefficient n and to

$$\alpha = \frac{(B_{\max} x^n / K_D)}{(1 + x / K_D)}$$

to obtain the dissociation constant K_D and the total number of binding sites $B_{\max}$ (α = [^{3}H]NMS specifically bound; x = [^{3}H]NMS concentration).

Data from displacement experiments are fitted to the equation:

$$\%[^3\mathrm{H}]\mathrm{NMS\ bound} = 100 - [100 x^n / k / (1 + x^n / k)]$$

to obtain the Hill number n and to

$$\%[^3\mathrm{H}]\mathrm{NMS\ bound} = 100 - [100 \times IC_{50} / (1 + x / IC_{50})]$$

to derive the IC_{50} value (x = concentration of the cold inhibitor).

K_i values are calculated by the method of Cheng and Prussoff (1973):

$$K_i = \frac{IC_{50}}{(1 + L / K_D)}$$

where L is the concentration of the radioligand, IC_{50} is the concentration causing 50 % inhibition of the specific radioligand binding, and K_D the dissociation constant of the radioligand receptor complex. Data are analyzed by a nonlinear least-squares curve-fitting procedure.

Results are expressed as mean values ± SEM of n experiments. Statistical significance is assessed using student's t-test or Scheffé's method. $p < 0.05$ is accepted as being significant.

Modifications of the Method

The selectivity towards muscarinic receptor subtypes can be tested by radioligand binding assays using either selective ligands or tissues possessing only one receptor subtype (Giachetti et al. 1986; Pitschner et al. 1989; Bickel et al. 1990). M_1 receptors from bovine cortex which has also M_2 and M_3 receptors are tested with the M_1-selective radioligand ^{3}H-pirenzepine. M_2 receptor from porcine heart possessing only this receptor type can be tested with the unselective ligand ^{3}H-*N*-methylscopolamine. M_3 receptors from rat submaxillary gland are likewise labeled with the unselective ligand ^{3}H-*N*-methylscopolamine, because this subtype is present predominantly in this tissue.

Measurement of the contractions of rabbit vas deferens after electrical stimulation was used to study the effects of prejunctional M_1 heteroreceptors and postjunctional M_2 receptors (Eltze et al. 1988; Dörje et al. 1991b) of guinea pig atria for M_2 receptors and of guinea pig ileum for M_3 receptors (Lambrecht et al. 1989, 1995). Cardiac muscarinic $M_{2\alpha}$ receptors have been discussed (Wess et al. 1988).

Coexistence of M_2 and M_3 subtypes of muscarinic receptors in canine colonic circular smooth muscle was reported by Zhang et al. (1991).

Investigations on the nature of muscarinic receptors present on parietal cell membranes using

binding studies and polymerase chain reaction amplification or parietal cell messenger RNA revealed the existence of only a M_3 receptor responsible for acid secretion (Kajimura et al. 1992).

Spalding et al. (2002) described a novel muscarinic agonist, AC-42, and demonstrated its selectivity for the M_1 muscarinic subtype.

The phenotypes of knockout mice or the responses lost in these animals were used to characterize the properties of the subtypes of muscarinic receptors (Birdsall et al. 2001). Champtiaux et al. (2003) investigated the subunit composition of functional nicotinic receptors in dopaminergic neurons with knockout mice.

Owicki et al. (1990, 1992) and McConnell et al. (1991, 1992) used a special apparatus, the "cytosensor microphysiometer" which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells were transfected with the m_1 muscarinic acetylcholine receptor. Sequential addition of increasing doses of carbachol every 2.5 min induced an increasing acidification allowing the determination of an EC_{50} value. The effect was antagonized by atropine.

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H_2 Antagonism

General Considerations

Three classes of histamine receptors with subtypes for the H_2 receptor have been identified by selective antagonists (Haaksma et al. 1990;

Hill 1990; West et al. 1990; Clapham and Kilpatrick 1992). H_2 antagonists inhibit competitively the interaction of histamine with H_2 receptors responsible for acid secretion in the stomach. Although H_2 receptors are present in many tissues, including vascular and bronchial smooth muscle and the right atrium, H_2 antagonists interfere remarkably little with physiological functions other than gastric secretion.

The H_2 receptor was reported to be spontaneously active in transfected CHO cells (Smit et al. 1996). Based on this concept, the H_2 antagonists were reclassified; cimetidine, ranitidine, and famotidine are in fact inverse agonists, whereas burimamide acts in this model system as a neutral agonist.

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Histamine H_2 Receptor Binding

Purpose and Rationale

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H_1 receptor couples mainly to $G_{q/11}$, thereby stimulating phospholipase C, whereas the H_2 receptor interacts with G_s to activate adenylyl cyclase. The histamine H_3 and H_4 receptors couple to G_i proteins to inhibit adenylyl cyclase, and to stimulate MAPK.

Histamine H_2 receptor binding can be determined using homogenates from guinea pig cerebral cortex and ^{3}H-tiotidine as labeled ligand (Gajtkowski et al. 1983; Norris et al. 1984; Hill 1990). Using the polymerase chain reaction, the gene encoding the histamine H_2 receptor has been cloned (Gantz et al. 1991).

Procedure

Preparation of Membranes

Guinea pigs of either sex weighing 400–600 g are sacrificed by exsanguination and the brains rapidly removed. The cerebral cortex is dissected away from the rest of the brain and homogenized in 50 mM sodium–potassium buffer, pH 7.4, using a Potter homogenizer. The homogenate is then centrifuged at 50,000 g for 10 min at 4 °C. The resulting pellet is washed three times by being resuspended in phosphate buffer followed by recentrifugation. The pellet is finally resuspended in phosphate buffer, pH 7.4, at a protein concentration of 5 mg/ml.

Assay

An aliquot of 100 µl of the homogenate is incubated with 2 nM ^{3}H-tiotidine and varying

concentrations of competing test substance, in triplicate, in a total volume of 250 µl, for 30 min at room temperature. The reaction is stopped by the addition of 2 ml of ice-cold phosphate buffer and immediately filtered under reduced pressure through Whatman GF/B glass fiber filters, followed by three times 3 ml washes with room temperature buffer.

Radioactivity is determined by allowing the filters to remain for at least 18 h in NE 260 scintillator (Nuclear Enterprise), followed by liquid scintillation counting.

Evaluation

K_i values (µM) are calculated for displacement of specific H_2 binding from the relationship

$$K_i = \frac{IC_{50}}{\left(1 + [L]K_d^{-1}\right)}$$

where IC_{50} is the concentration of the drug required for 50 % inhibition of specific binding, [L] is the concentration of ^{3}H-tiotidine in the assay, and K_d is the dissociation constant for ^{3}H-tiotidine.

Data can be analyzed using a computer program as described by McPherson (1985).

Modifications of the Method

Hirschfeld et al. (1992) performed photoaffinity labeling studies of the H_2 receptor using the radioactive probes [^{125}I]iodoaminopotentidine and its photolabile azido analog [^{125}I] iodoazidopotentidine.

Martinez-Mir et al. (1990) studied the distribution of histamine H_1, H_2, and H_3 receptors in postmortem human and rhesus monkey brain by receptor autoradiography using [^{125}I] iodobolpyramine, [^{125}I]iodoaminopotentine, and [^{3}H](R)α-methylhistamine as ligands to label H_1, H_2, and H_3 receptors, respectively.

Traiffort et al. (1992) used [^{125}I]iodoaminopotentidine for pharmacological characterization and autoradiographic localization of histamine H_2 receptors in human brain.

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H_2 Antagonism in Isolated Guinea Pig Right Atria

Purpose and Rationale

H_2 antagonism can be determined in isolated guinea pig right atria which contain predominantly H_2 receptors. Compounds that inhibit the positive chronotropic effect mediated by histamine H_2 receptors in the isolated right guinea pig atrium (Reinhardt et al. 1974) can be classified as specific histamine H_2 antagonists. The test can be used as screening method for H_2 antagonists.

Procedure

Male guinea pigs, e.g., Hartley strain, are sacrificed by exsanguination. The right atria are dissected and suspended at 0.7 g tension in a 25-ml organ bath with Tyrode's solution bubbled with carbogen (5 % CO_2/95 % O_2) at 38 °C. After a stabilization period of 30 min, the contractions are recorded on a polygraph with a force-displacement transducer through a strain gauge. Cumulative concentration–response curves are obtained after sequential additions of histamine (10^{-7} to 10^{-4} M) in the absence of test drugs and the equilibration of test drugs or the standard (cimetidine).

Evaluation

pA_2 values and relative potencies are calculated by a Schild plot (Arunlakshana and Schild 1959).

Modifications of the Method

Hattori et al. (1990) studied the inotropic, electrophysiological, and biochemical responses to histamine in rabbit papillary muscles and found evidence for coexistence of H_1 and H_2 receptors. Histamine increases force of contraction and decreases action potential duration in rabbit papillary muscles. H_2 antagonists antagonize the histamine-induced decrease in action potential duration and, however, are less effective in antagonizing the increase in force of contraction produced by histamine. The positive inotropic response to histamine is abolished by sequential addition of a H_1 antagonist.

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H_2 Antagonism in Isolated Rat Uterus

Purpose and Rationale

Histamine inhibits spontaneous and electrically stimulated contractions of rat uterus horns. This effect can be antagonized by H_2, but not by H_1 antagonists.

Procedure

Adult, virgin Wistar rats weighing 180–200 g are used. By microscopic examination of vaginal smears, the stage of the estrus cycle is determined. Only animals in natural proestrus or metestrus are chosen. After sacrifice, the two uterus horns are removed and placed in modified de Jalon solution at room temperature and cleaned of mesenteric fat and connective tissue. Paired preparations from the same animal are used in parallel experiments. Two segments, about 2 cm in length, are taken from the ovarian end of the uterus horns and mounted separately on Perspex organ holders between two steel electrodes arranged at the end of the muscle. The organs are immediately superfused with modified de Jalon solution at 35 °C at a rate of 2–3 ml/min being continuously gassed with air. Basal muscle tension is maintained at 0.5 g. The contractions of the preparations are recorded isometrically with a force-displacement transducer linked to a multipen recorder. The intrinsic rhythm of spontaneous and regular contractions appears within 5–10 min after mounting the tissues in their holders.

Organs showing a frequency of only one contraction every 2 min or less are electrically stimulated with square wave impulses of 2 ms duration for 1 s every 1 min at 10 V and 80 Hz with a suitable stimulator. The organs are linked via a four-way stopcock to a set of reservoirs of modified de Jalon solution containing different concentrations of agonist and test substance. The preparations are superfused for 20–30 min with nutrient solution until mechanical activity of the uterus horns is stabilized in frequency and contraction height before addition of drugs.

The time interval between applications of drugs is 15–20 min; the time of contact of the drugs with the uterus segments is about 4–5 min, being interrupted as soon as the contractions of the muscles reach a minimum. Logarithmic dose–response curves for histamine are constructed from mean effects of single doses, taking the average amplitude of five contractions immediately preceding the addition of histamine as 100 % control activity. In experiments involving antagonists, a standard dose of histamine is used giving a response within the linear region of the log dose–response curve and is set as 100 %. Reduction of histamine-induced inhibition is studied by superfusing increasing concentrations of antagonists in addition to the standard dose of histamine.

Evaluation

Results are expressed in percentage of the reversal of initial depression of contraction height after histamine and plotted against –log mol/l concentration of the antagonist. pA_2 values are calculated according to Ariëns and van Rossum (1957).

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Activity at Histamine H_1 and H_2 Receptors In Vivo

Purpose and Rationale

Owen and Pipkin (1985) described a technique that allows simultaneous and quantitative assay of the action of agonists and antagonists at histamine H_1 and H_2 receptors in anesthetized guinea pigs. The principle of the technique is based on H_1-receptor-caused bronchoconstriction and H_2-receptor-caused tachycardia.

Procedure

Guinea pigs of either sex weighing about 500 g are anesthetized with 90 m/kg sodium pentobarbitone i.p. The trachea is cannulated to permit artificial respiration. Blood pressure is measured from a catheter tied into one carotid artery. Catheters are tied into one jugular and one femoral vein for the administration of drugs.

Airway resistance is measured using a modification of the Konzett–Rössler technique. By means of a small animal respiration pump, the lungs are inflated at a rate of 40 breaths/min. The inflow arm of the circuit includes a side arm, the outlet of which is placed below 12 cm of H_2O. The volume of air used in the study has to be that which fills the lungs at a pressure of 12 cm H_2O, selected by adjusting the volume until no air escapes through the H_2O trap each time the lungs are filled. The side arm is then clamped, and the animal is respired with the selected volume for the duration of the study. Airway resistance to inflow is measured using a pressure transducer connected to a second arm of the inflow circuit. Resistance is proportional to the maximum pressure required to inflate the lungs. Inflow pressure is registered on an electronic recorder.

Heart rate is measured from the blood pressure pulse using an instantaneous rate meter and is registered on an electronic recorder.

Intravenous injection of histamine causes simultaneously bronchoconstriction and tachycardia. The threshold dose needed to cause tachycardia may be less than that for bronchoconstriction, but both responses will be apparent over the dose range 1×10^{-8} to 5×10^{-7} mol/kg.

Evaluation

Agonists

Dose–response curves for agonists compared to the dose–response curve for histamine for both parameters, bronchoconstriction and tachycardia, allow the calculation of potency ratios. Specific H_1 receptor agonists are more potent to cause bronchoconstriction than tachycardia, whereas H_2 agonists provoke tachycardia but are less active or inactive causing bronchoconstriction.

Antagonists

To evaluate antagonists, various doses of the test compound are injected i.v., and dose–response curves of histamine are established for both parameters to be compared with the dose–response curve of histamine without pretreatment. From the ratios of shift to the right, dose–response curves for both parameters can be established. These are similar for mixed antagonists, but a definitively higher potency is shown for H_1 antagonists in bronchoconstriction and a higher potency for H_2 antagonists in tachycardia.

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Inhibition of Histamine Stimulated Adenylate Cyclase from Gastric Mucosa

Purpose and Rationale

The in vitro potencies of H_2 antagonists can be evaluated using determinations of adenylate cyclase activity in membrane preparations of guinea pig mucosa after stimulation with histamine.

Procedure

Preparation of Membranes

Guinea pigs of either sex weighing 400–600 g are sacrificed. The fundic portion of the stomach is rapidly removed. Food particles are washed away with ice-cold 50 mM Tris buffer, pH 7.4,

containing 4 mM EDTA and 0.25 M sucrose (homogenization buffer). The tissue is stretched, mucosal side up, on a glass Petri dish supported on ice, and the mucosal layer is scraped off the muscle layer using a scalpel blade. The mucosal scrapings are transferred to 10 ml ice-cold homogenization buffer in a glass homogenization tube and homogenized using an Ultra-Turrax homogenizer followed by four strokes using a Potter Teflon homogenizer. The resulting suspension is centrifuged at 700 g for 10 min at 4 °C. The supernatant is discarded, and the pellet resuspended in 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA followed by a further period of centrifugation. The resulting pellet is finally resuspended in Tris/EDTA buffer at a protein concentration of 2.4 mg/ml as determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Stimulation of Adenylate Cyclase

Adenylate cyclase activity is measured according to Hegstrand et al. (1976). Aliquots (120 µg) of fundic mucosal homogenate are incubated in 100 mM Tris buffer, pH 7.8, containing 0.6 mM EGTA (ethylene-glycol-bis-(*β*-aminoethyl ether)-*N*,*N*′-tetra-acetic acid), 1 mM IBMX (isobutylmethyl xanthine), 2 mM $MgCl_2$, 0.1 mM GPT (guanosine triphosphate), histamine at various concentrations between 0.1 µM and 100 µM, and the compound under investigation at varying concentrations between 0.1 µM and 1 mM for 10 min at 0 °C. The reaction is initiated by the addition of 1 mM ATP followed by a 10 min incubation at 30 °C. The reaction is terminated by placing the assay tubes in a boiling water bath for 3 min. After cooling, 40 mg of Alumina 90 (E. Merck, 70–230 mesh) are added to each tube prior to mixing and centrifugation at 700 g for 15 min at 4 °C.

Assay of Cyclic AMP

The cyclic AMP content of each sample is determined according to Brown et al. (1971) using ^{3}H-cyclic AMP in a competitive protein binding assay. Fifty microliter aliquots from the adenylate cyclase assay are incubated with 50 µl of ^{3}H-cyclic AMP (14 nC at a specific activity of 62 Ci/mmol) and 100 µl of previously prepared cyclic AMP binding protein at 4 °C. The bound and free ^{3}H-cyclic AMP are separated by the addition of 100 µl of charcoal reagent (2 g bovine serum albumin, 2.5 g Norit GSX charcoal in 100 ml of 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA), followed by mixing and centrifugation at 1,000 g for 15 min. Two hundred microliter aliquots of the supernatant are decanted into scintillation vials containing 10 ml of NE 260 scintillator. Radioactivity is determined by liquid scintillation counting.

Evaluation

Results are calculated from a standard curve using cyclic AMP as the standard. From these data, IC_{50} values (µM) are derived.

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H^+/K^+-ATPase (Proton Pump) Inhibition

General Considerations

The parietal cell of the stomach is activated by three major stimuli: histamine, acetylcholine, and gastrin. In addition to the direct action of gastrin and acetylcholine on the parietal cell, these two

agents may release histamine from a histamine storage in the gastric mucosa. In this manner, histamine would act as the final mediator of acid secretion. One of the first events leading to acid secretion is a massive membrane transformation that occurs in the parietal cell. When the cell is stimulated, the tubulovesicles in the cytoplasma of the cell fuse and form an expanded secretory cannaliculus in the apical membrane where the enzyme H^+/K^+-ATPase is located.

In this way, the ultimate mediator of acid secretion in the stomach is the proton pump H^+/K^+-ATPase which transports hydrogen in exchange for potassium. The rat stomach H^+/K^+-ATPase has been cloned by Shull and Lingrel (1986). Development of specific inhibitors of this enzyme is an approach to suppress acid secretion and ulcer formation since ulcers only exist in acidic medium.

Alderuccio et al. (1993) described an experimental autoimmune gastritis in BALB/c mice as a $CD4^+$ T cell-mediated organ-specific autoimmune disease induced by neonatal thymectomy. Transgenic expression of the gastric H^+/K^+-ATPase β subunit specifically prevented the onset of this form of autoimmune gastritis.

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H^+/K^+-ATPase Inhibition in Membrane Vesicles of Stomach Mucosa

Purpose and Rationale

Several H^+/K^+-ATPase inhibitors contain a sulfhydryl group. At lower pH values, the compounds are protonated and rearrange to a sulphenic acid and a sulphenamide that react with sulfhydryl groups in the enzyme. Therefore, the in vitro assays are performed both at neutral and at acidic pH levels.

Procedure

Membrane vesicles containing H^+/K^+-ATPase are prepared from pig stomachs obtained from the local slaughter house (Ljungström et al. 1984). Pigs are fasted overnight before slaughter. The gastric mucosa of four stomachs is rinsed with cold saturated NaCl solution for 3–5 min. The superficial cells, cell debris plus the mucus are wiped off with the edge of a plastic ruler and with paper towels. The mucosa is scraped off. About 100 g scrapings are divided into portions of 10 g and homogenized in 0.25 M sucrose with seven strokes in a Potter–Elvehjem Teflon glass homogenizer. The total volume is 600 ml which is centrifuged at 20,000 g for 40 min. The pellet is discarded. The supernatant is centrifuged at 75,000 g for 1 h. The resulting microsomal pellet is homogenized in 30 ml 0.25 M sucrose.

Aliquots of 15 ml are transferred to 100-ml centrifuge tubes and layered on top of step gradients, from the bottom comprising 25 ml 37 % sucrose (w/v) and 45 ml 7.5 % Ficoll (w/v) in 0.25 M sucrose. The tubes are centrifuged at 75,000 g for 1 h in a 6 × 100 ml ME angle rotor at 4 °C. The gradient is then fractionated by pumping Fluorinert 70 through a narrow tubing in a fractionating cap down to the bottom of the tube. Fractions are collected from top through a center hole in the fractionating cap. The yield of vesicles in a typical preparation is about 50–75 mg protein. In order to maintain a stable vesicular structure for a long period of time, the vesicles

are frozen at −70 °C under nitrogen. They can then be kept for several months without decrease of H^+/K^+-ATPase activity.

The ATPase activity is measured at 37 °C as the release of inorganic phosphate (P_i) from ATP. The test drug and the standard (omeprazole) are preincubated in concentrations of 0.01–100.0 μM in enzyme containing buffers in parallel at pH 6.0 and 7.4 for 30 min at 37 °C. Then, the medium of pH 6.0 is adjusted with HEPES/Tris buffer to pH 7.4. The enzyme reaction is started by addition of nigericin and Tris/ATP. The total reaction volume is 1 ml, containing 20 μg vesicular protein, 4- mM $MgCl_2$, 10 mM KCl, 20 μM nigericin, 2 mM Tris/ATP, 10 mM HEPES, and additionally 2 mM Pipes for the preincubation medium at pH 6.0.

After 4 min at 37 °C, the reaction is stopped by the addition of 10 ml of 50 % trichloroacetic acid. The denaturated protein is spun down, and the P_i content is determined according to LeBel et al. (1978) based on the reduction of a phosphomolybdate complex by *p*-methylaminophenol sulfate in a copper acetate buffer or according to Carter and Karl (1982) based on the reaction of phosphomolybdate with the basic dye malachite green.

Evaluation

IC_{50} values are calculated by probit analysis, whereby 0 % corresponds to 4 mM Mg^{2+}-dependent and 100 % to 4 mM Mg^{2+} plus 10 mM K^+-dependent ATP hydrolysis. IC_{50} values of the test compound at different pH values are compared with IC_{50} values of the standard. Statistical differences ($p < 0.05$) are calculated by student's *t*-test.

Modifications of the Method

Proton transport in gastric vesicles can be measured by acridine orange fluorescence quenching (Lee and Forte 1978; Beil et al. 1990). Membrane protein (0.12 mg) is incubated at 37 °C in a volume of 2 ml containing: 10 mM pipes/Tris buffer, pH 7.0 in the presence of 150 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, and 10 μM acridine orange. The pump reaction is started by the addition of valinomycin (ionophore for K^+). The decrease of fluorescence is studied at 530 mm as a measure for the intravesicular proton uptake.

Critical Assessment of the Method

The preincubation period at the lowest possible pH of about 6 is used to initiate the acidic conversion of the test compound into its active principle. This reflects more the chemical instability of the test compound at neutral pH values than its effect during conditions of much higher acidity within the secretory cannaliculus of the parietal cell during acid secretion. Many chemically labile inhibitors are therefore very active in this test system. However, they do not cause an inhibition in more complex test systems and, therefore, are without any practical usefulness (Lindberg et al. 1990). Proton transport studies in gastric vesicles, using the acridine orange fluorescence quenching technique, where a pH gradient similar to in vivo conditions is formed (Lee and Forte 1978; Beil et al. 1990) are more suitable for studying the mechanism of action, acid conversion, and structure–activity relationship of K^+/H^+-ATPase inhibitors (Herling and Weidmann 1994).

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Effect of H^+/K^+-ATPase Inhibitors on Serum Gastrin Levels

Purpose and Rationale

It is known from the H^+/K^+-ATPase inhibitor omeprazole that the total acid blockade initiates a gastric antral feedback mechanism resulting in an excessive hypergastrinemia (Arnold et al. 1986; Creutzfeldt et al. 1986; Larsson et al. 1986) which is believed to cause diffuse endocrine cell hyperplasia, characterized as carcinoids, in the gastric corpus after 2 years of treatment in the rat (Ekman et al. 1985).

Procedure

Groups of 10–15 female Wistar rats weighing 90–110 g are treated daily for 10 weeks with omeprazole (10 or 30 mg/kg p.o.) or the test compound or serve as controls. The compounds are suspended in potato starch mucilage (20 mg/ml) and administered in a volume of 2 ml/kg. On days 1–3, the rats receive the H^+/K^+-ATPase inhibitors by intraperitoneal injection in order to cause gastric acid inhibition and therefore to reduce the gastric acid degradation of subsequent oral doses. After treatment for 2, 4, 7, and 10 weeks, blood samples are collected under anesthesia by retro-orbital puncture. Gastrin is determined by radioimmunoassay using a commercially available kit, e.g., Gastrin RIAKit II; Dainabot Co., Ltd. At the end of the study of 10 weeks, the animals are studied for their gastric acid output using the pylorus ligation (Shay technique).

Evaluation

Serum gastrin levels are determined as pg/ml by using a commercial gastrin ELISA or RIA. Statistical differences ($p < 0.05$) are calculated using student's *t*-test.

Modifications of the Method

Katz et al. (1987) described a five-day test to predict the long-term effects of gastric antisecretory agents on serum gastrin in rats.

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(^{14}C)-Aminopyrine Uptake and Oxygen Consumption in Isolated Rabbit Gastric Glands

Purpose and Rationale

Isolated gastric glands from rabbits and other species can be used in studying the control of mechanism of gastric H^+ secretion (Berglindh and Öbrink 1976; Berglindh et al. 1976; Sack and Spenney 1982; Sewing et al. 1983; Herling et al. 1987, 1988, 1990). In isolated gastric glands and parietal cells, H^+ secretion cannot be directly measured by titration; therefore, accumulation of weak bases such as aminopyrine is used as an indirect probe of H^+ secretion. Moreover, glandular oxygen consumption can be measured with the Warburg technique.

Procedure

Preparation of Gastric Glands

Rabbits are anesthetized with 40 mg/kg pentobarbital i.v. The abdomen is opened, and the aorta is cannulated in a retrograde direction. Five milliliter of a heparin solution (250 IU/ml) is injected with force through the cannula. After 1 min, the rabbit is bled through the cannula, and a ligature is placed around the mesenteric vessels. The chest is quickly opened and the thoracic aorta clamped. Phosphate buffered saline solution (containing 149.6 mM NaCl, 3 mM K_2HPO_4, 0.64 mM NaH_2PO_4, pH 7.3) at 37 °C is pumped through the aorta, whereby the portal vein is opened to allow free outflow of the perfusate. By this procedure, most of the solution is forced through the mesenteric vessels. The perfusion pressure, as measured proximal to the cannula, can be up to 600 mmHg.

The stomach appears totally exsanguinated after perfusion with about 500 ml phosphate buffered saline solution and is then removed, cut open along the lesser curvature, and emptied. The cardial and antral regions are discarded. The corpus is rinsed several times with phosphate buffer solution and finally blotted with filter paper, whereby the remaining gastric content as well as some surface epithelial cells are removed. By blunt dissection, the mucosa can easily be separated from the muscular and submuscular layers.

The mucosa is then minced into small pieces with a pair of scissors. The pieces are washed twice in warm oxygenated phosphate buffer solution and transferred to a 200-ml flask with 50 ml of a freshly prepared collagenase enzyme solution containing 1 mg/ml collagenase (type I, Sigma), 1 mg/ml rabbit albumin (Sigma), 2 mg/ml glucose in 130 mM NaCl, 10 mM $NaHCO_3$, 3 mM NaH_2PO_4, 3 mM Na_2HPO_4, 3 mM K_2HPO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mg/l phenol-red at pH 7.4. The flask is gassed with 100 % oxygen, sealed, and kept in a 37 °C water bath under gently stirring with a magnet for 90 min. The cloudy suspension containing the separated glands and some cells is filtered through a nylon mesh into 15-ml test tubes with conical bottoms. The glands rapidly sediment to the bottom, while the free cells remain in the solution. In this way centrifugation can be avoided, and the glands can easily be washed free from isolated cells and collagenase by three washings with phosphate buffer at room temperature.

^{14}C-Aminopyrine Accumulation in Gastric Glands

The ability of gastric glands to form acid is measured based on aminopyrine accumulation. The glands are diluted to a final concentration of 2–4 mg dry weight/ml in a medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20.0 mM $NaHCO_3$, 20.0 mM HEPES, 2 g/ml glucose, 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4. Samples of 1 ml gland suspension are equilibrated in a 1-ml medium containing 0.1 μCi/ml [^{14}C]aminopyrine at 37 °C in a shaking water bath together with the agent to be tested. After 20 min, either histamine or dibutyryl-cyclic-AMP is added, followed by a 30 min incubation period for histamine and a 45 min incubation period for dibutyryl-cyclic-AMP.

The glands are then separated from the medium by brief centrifugation. The supernatant is withdrawn, and the pellets are dried at 80 °C for

50 min, weighed, and dissolved in 200 µl 1 M NaOH. Aliquots of the supernatant and the digested gland pellet are examined in a liquid scintillation counter. The ratio of intraglandular to extraglandular radioactivity is calculated. All determinations are made in triplicate.

Respiratory Studies

Glandular oxygen consumption is measured at 37 °C using a Warburg respirometer and air as the gas phase. The 15-ml flasks with a central well 20 % containing KOH solution on a filter paper as CO_2 absorber are filled with 1-ml medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20.0 mM $NaHCO_3$, 20.0 mM HEPES, 2 g/ml glucose, and 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4 and 1 ml gland suspension. After 20 min equilibration, the test compound and dbcAMP are added, and the oxygen consumption is measured at 15 min intervals for the following 45 min. The recorded oxygen consumption is corrected according to the following formula:

$$O_2\,\text{consumption} = O_2\,\text{recorded} \times K$$

where

- $K = 273 \times (Pb/t + 273) \times 760$
- t = ambient temperature in °C around the manometers.
- Pb = atmospheric pressure (mmHg).

The respiratory activity is expressed in µl O_2 consumed per mg dry weight and time.

Evaluation

IC_{50} values are calculated by probit analysis. Statistical differences ($p < 0.05$) are assessed by student's t-test, n = the number of different gland preparations.

Critical Assessment of the Method

Studies on (^{14}C)-aminopyrine uptake and oxygen consumption in isolated rabbit gastric glands have become a valuable approach for studying the effect of various H_2 receptor antagonists and proton pump inhibitors. When studying gastric acid production with the ^{14}C-AP accumulation technique, the addition of basic drugs can be problematic (Fryklund and Wallmark 1986). The basic nature of a test compound can compete with accumulation of ^{14}C-AP. Nevertheless, it is generally accepted that oxygen consumption of gastric glands correlates well with acid formation (Berglindh et al. 1976).

Modifications of the Method

Soll (1978) studied the actions of secretagogues on oxygen uptake by isolated mammalian parietal cells. Parietal cells were prepared from the stomach of dogs by collagenase digestion and counterflow centrifugation. Oxygen consumption was determined by polarography. Isobutyl methyl xanthine (IMX), carbamylcholine, histamine, and gastrin each independently stimulated oxygen uptake. The specificity of these responses was tested by the use of an H_2 histamine receptor antagonist or atropine as anticholinergic agent.

Stoll (1980) investigated the [^{14}C]aminopyrine accumulation in isolated *canine* parietal cells when treated with histamine, gastrin, and carbachol and the displacement by cimetidine or atropine.

Sewing et al. (1983, 1986) studied the effect of several benzimidazole derivatives on isolated and enriched guinea pig parietal cells using the ^{14}C-aminopyrine accumulation.

Schepp et al. (1994) determined the effects of exendin-4, a peptide from *Heloderma suspectum venom*, and exendin-(9–39)NH_2 on [^{14}C]aminopyrine accumulation in isolated rat parietal cells and compared these with those of glucagon-like peptide-1-(7–36)NH_2.

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Gastric Mucosal Blood Flow

Purpose and Rationale

Hydrogen gas clearance has been used to measure blood flow in the basal portion of gastric mucosa in anesthetized rats (Leung et al. 1984, 1986; Pique et al. 1988).

Procedure

Male Sprague–Dawley rats are anesthetized appropriately. A tracheotomy is performed, and a PE-250 tubing is inserted into the trachea to facilitate spontaneous breathing and for administration of 3 % hydrogen in air. The right carotid artery is cannulated for blood pressure monitoring. A midline laparotomy is then performed and the stomach exteriorized. Through an incision in the forestomach, the gastric contents are gently washed out with physiological saline. A double-lumen cannula (outer, Tygon with a diameter of 7 mm; inner, polyethylene with a diameter of 2 mm) is inserted into the stomach and secured by a ligature at the forestomach. The pylorus is ligated, and a 0.9 % sodium chloride solution is infused through the inner cannula at a rate of 0.8 ml/min and drained from the outer tubing. The gastric effluent is collected at 15-min intervals. Acid output (in microequivalents per min) is determined in the perfusate with 0.2-N NaOH with an automatic titrator.

An incision in the serosa and the muscularis externa is made in the gastric wall of the corpus, exposing 3–4 mm of the submucosa. Through this hole, a platinum electrode is placed in contact with the exposed basal portion of the mucosa. The electrode is made with a ring of platinum wire, 125 μm in diameter, wound around a glass capillary tube and held in place inside a Teflon tube by epoxy. A Ag-AgCl reference electrode is placed inside

the peritoneal cavity. The laparotomy incision is covered by Parafilm to minimize evaporation.

One femoral vein is cannulated for the infusion of saline or drugs. The rat is kept warm with a heat lamp to maintain rectal temperature at 37 °C.

Hydrogen Gas Clearance Technique

Current is generated at the surface of a platinum electrode by oxidation of molecular hydrogen to hydrogen ions and electrons. This current, which is measured using a polarographic and amplifying unit, is proportional to the hydrogen tension gradient at the surface of the platinum electrode. When the experimental animal breathes 3 % hydrogen in air, the current tracing, graphed on a recorder, gradually rises and reaches a plateau as the tissue adjacent to the electrode is saturated with hydrogen. After the external hydrogen source is removed, the current tracing gradually falls because of removal of tissue hydrogen by blood flow. The rate of dissipation of hydrogen estimates the tissue blood flow (Aukland et al. 1964).

The exponential decrease of the hydrogen gas clearing curves is evaluated by a computer program (Livingston et al. 1986). Current from the platinum electrode is passed through an ADALAB analog-to-digital converter and discrete digitized values are sampled every 5 s. Blood flow is determined by an Newtonian–-Gaussian nonlinear iterative regression program by means of a biexponential formula:

$$f(x) = A + B\,\mathrm{e}^{-k1t(t-T)} + B\,\mathrm{e}^{-k2t(t-T)},$$

where *f(x)* is the electrode current, A is the baseline current, B is the initial current, e is the base of natural logarithms, k_1 is the rate constant for the fast component, k_2 is the rate constant for the slow component, t is the time at which the current is observed, and T is the time when hydrogen gas was removed.

Mucosal blood flow is expressed in milliliters per minute per 100 g of tissue.

Inhibition of Acid Secretion

Intravenous saline is infused during the first 45 min of the study. After this period, an infusion of 80 μg/kg h of pentagastrin is administered for 135 min. During the last 75 min, an intravenous infusion of the inhibitor or vehicle is administered simultaneously with pentagastrin. Corpus mucosal blood flow measurements are obtained during the resting period, 45 min after start for the pentagastrin infusion, and during the last 15 min of combined infusion of pentagastrin and inhibitor. Acid output is measured at 15 min intervals throughout the experiments.

Evaluation

All data are expressed as mean ± standard error. The data are analyzed using a paired t-test for comparison of basal versus stimulated condition within the same animal, analysis of variance with contrasts, and linear and polynomial regression analysis for comparison between animals in different groups. A probability level of $p < 0.05$ is considered significant.

Modification of the Method

The hydrogen gas clearance technique has been used by many authors in experimental gastroenterology, e.g., Hirose et al. (1991), Holzer and Guth (1991), Lippe and Holzer (1992), Pique et al. (1992), Tsukamoto et al. (1992), Lazaratos et al. (1993), Petho et al. (1994), Tanaka and Guth (1994), Goldin et al. (1996), Hisanaga et al. (1996), Doi et al. (1998), and Heinemann et al. (1999).

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Antiulcer Activity

Pylorus Ligation in Rats (SHAY Rat)

Purpose and Rationale

A simple and reliable method for production of gastric ulceration in the rat based on ligature of the pylorus has been published by Shay et al. (1945). The ulceration is caused by accumulation of acidic gastric juice in the stomach.

Procedure

Female Wistar rats weighing 150–170 g are starved for 48 h having access to drinking water ad libitum. During this time, they are housed single in cages with raised bottoms of wide wire mesh in order to avoid cannibalism and coprophagy. Ten animals are used per dose and as controls. Under anesthesia, a midline abdominal incision is made. The pylorus is ligated, care being exercised that neither damage to the blood supply nor traction on the pylorus occurs. Grasping the stomach with instruments is to be

meticulously avoided; else ulceration will invariably develop at such points. The abdominal wall is closed by sutures. The test compounds are given either orally by gavage or injected subcutaneously.

The animals are placed for 19 h in plastic cylinders with an inner diameter of 45 mm being closed on both ends by wire mesh. Afterwards, the animals are sacrificed in CO_2 anesthesia. The abdomen is opened, and a ligature is placed around the esophagus close to the diaphragm. The stomach is removed, and the contents are drained in a centrifuge tube. Along the greater curvature, the stomach is opened and pinned on a cork plate. The mucosa is examined with a stereomicroscope. In the rat, the upper two fifths of the stomach form the rumen with squamous epithelium and possess little protective mechanisms against the corrosive action of gastric juice. Below a limiting ridge, in the glandular portion of the stomach, the protective mechanisms are better in the mucosa of the medium two fifths of the stomach than in the lowest part, forming the antrum. Therefore, lesions occur mainly in the rumen and in the antrum. The number of ulcers is noted and the severity recorded with the following scores:

- 0 = no ulcer
- 1 = superficial ulcers
- 2 = deep ulcers
- 3 = perforation.

The volume of the gastric content is measured. After centrifugation, acidity is determined by titration with 0.1 n NaOH.

Evaluation

An ulcer index U_I is calculated:

$$U_I = U_N + U_S + U_P \times 10^{-1}$$

- U_N = average of number of ulcers per animal.
- U_S = average of severity score.
- U_P = percentage of animals with ulcers.

Ulcer index and acidity of the gastric content of treated animals are compared with controls. Using various doses, dose–response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0 % corresponds to no and 100 % to maximal stimulated gastric acid output.

Critical Assessment of the Method

The "Shay rat" has been proven to be a valuable tool to evaluate antiulcer drugs with various mechanisms of action.

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Indomethacin-Induced Ulcers in Rats

Purpose and Rationale

Nonsteroidal anti-inflammatory agents, like indomethacin and acetyl-salicylic acid, induce gastric lesions in man and in experimental animals by

inhibition of gastric cyclooxygenase resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

Procedure

Groups of 8–10 Wistar rats weighing 150–200 g are used. The test drugs are administered orally in 0.1 % Tween 80 solution 10 min prior to oral indomethacin in a dose of 20 mg/kg (4 mg/ml dissolved in 0.1 % Tween 80 solution). Six hours later, the rats are sacrificed in CO_2 anesthesia and their stomachs removed. Formol saline (2 % v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greater curvature, then washed in warm water, and examined under a threefold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated.

Evaluation

The mean score in control rats is about 25 (range 20–28). Inhibition of the lesion production is expressed as percentage value.

Modification of the Method

Dose and time dependency of the ulcerogenic action of indomethacin was studied by Djahanguiri (1969).

Instead of indomethacin, gastric lesions can be induced by intravenous or oral doses of aspirin which can be prevented by exogenous PGE_2 or PGI_2 (Konturek et al. 1981). Furthermore, reserpine at a dose of 8 mg/kg i.p. or cysteamine hydrochloride at a dose of 400 mg/kg s.c. was given in order to induce ulcers in rats (Tarutani et al. 1985).

Kitajima et al. (1993) studied the role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats. Four hours after subcutaneous injection of 25 mg/kg indomethacin, the rats were sacrificed after anesthesia, and the stomach was removed. The stomach was filled with 1.5 ml of 2 % buffered formalin for 10 min and then opened along the greater curvature. The total length of the lesions was measured.

Wallace et al. (1989) studied the ulcerogenic activity of endothelin in indomethacin-pretreated rats using an ex vivo gastric chamber.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement and recording gastric potential difference in the rat.

Critical Assessment of the Method

According to West (1982), the cold stress-induced ulcer formation, but not the indomethacin- or aspirin-induced ulcers, is inhibited by H_2 receptor antagonists, whereas other authors reported protective effects of H_2 receptor antagonists under these conditions (Tarutani et al. 1985).

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Ethanol-Induced Mucosal Damage in Rats (Cytoprotective Activity)

Purpose and Rationale

Intragastric application of absolute ethanol is a reproducible method to produce gastric lesions in experimental animals (Robert et al. 1979; Szabo et al. 1981). These lesions can be at least partially inhibited by various drugs, such as some prostaglandins. The protective effect against various irritants has been called cytoprotective activity (Robert 1979; Robert et al. 1979). The method has been modified by several authors. Witt et al. (1985) described a method to objectively quantify the extent of ethanol-induced gastric lesions utilizing a transmission densitometer to measure the optical density of the photographic negative of the stomach mucosa.

Procedure

Male Wistar rats weighing 250–300 g are deprived of food 18 h prior to the experiment but are allowed free access to water. During this time, they are kept in restraining cages to prevent coprophagy. The rats are administered either the appropriate vehicle or the cytoprotective drug, e.g., a prostanoid, intragastrally 30 min prior to administration of 1 ml absolute ethanol. Untreated animals are included as controls. One hour after administration of ethanol, the animals are euthanized with CO_2, the stomachs are excised, cut along the greater curvature, and gently rinsed under tap water. The stomachs are stretched on a piece of foam core mat, mucosal site up.

The subjective scores of the treated tissues are recorded; the graded response is reflecting the least (0) to most (3) damage. A circular full-thickness area, about 13 mm in diameter, is cut with a cork borer from each lobe of the fundus just below the ridge dividing the glandular from the nonglandular portion of the stomach. A Plexiglas template (19 × 14 × 0.3 cm), burnished on one side with emery cloth and with four rows with six holes 13 mm in diameter, is placed on a sheet of clear glass, burnished side up, and bound to the glass with photographic tape along the periphery. The excised pairs of tissue from each stomach are placed into the holes of the template.

Pairs of tissue from each stomach are examined to minimize sampling errors. The template is positioned on a rectangular central open area of an Aristo Model T-16 cold cathode transilluminator (38 × 38 cm) containing a W-45 blue-white lamp. A camera is mounted on a copy stand directly above the template. Photographs are taken, the film processed in a standard manner, and a contact sheet is made from the negatives. A light transmission densitometer (e.g., MacBeth model TD-501) is used to evaluate the negatives. The optical density of the test tissues is determined by placing each area of the negative in sequence over the aperture through which the light is transmitted. The optical density is displayed on a digital read out and recorded. Hemorrhagic or damaged areas appear bright on the negative, whereas undamaged tissue appears dark. Hence, lower optical density values are indicative of damage, while higher optical densities are associated with little or, as in the case of control, no damage.

Evaluation

The significance of differences in optical density between control and ethanol-treated tissue is evaluated by nonpaired single-tail student's *t*-test.

Modifications of the Method

Cytoprotection by prostaglandins was studied in rats by prevention of gastric necrosis produced by various agents such as alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury (Robert et al. 1979) and against gastric injury produced by nonsteroidal anti-inflammatory compounds (Robert 1979; Franzone et al. 1988). The animals are fasted 48 h prior to the experiment and placed

18 h before the administration of drugs into plastic tubes to prevent coprophagy. Fifteen minutes after application of the test drug, the animals are given 1 ml of the irritant orally. After an additional hour, the animals are sacrificed, the stomachs removed and immediately opened along the greater curvature. Lesions are counted and scored (0 = no lesion; 1 = mild lesions; 2 = severe lesions; 4 = necrosis).

Starrett et al. (1989) employed 3.0 ml/kg ethyl alcohol (100 %) or 3.0 ml/kg 0.75 N HCl as necrotizing agent.

Borella et al. (1989) studied the cytoprotective and antiulcer activities of the anorganic antacid magaldrate in the rat using absolute ethanol as irritant.

Masuda et al. (1993) investigated the role of endogenous endothelin in the pathogenesis of ethanol-induced gastric mucosal injury in rats.

Critical Assessment of the Method

Several prostaglandins provide cytoprotection, particularly in rats, in a dose range which has no antisecretory activity. However, clinical experience with prostaglandins showed that ulcer healing is only achieved at antisecretory doses (Lindberg et al. 1990). Therefore, it seems very likely that the cytoprotective property of a compound in rats has very limited relevance to prediction of its ulcer healing potential in humans if cytoprotection is really separated from its antisecretory potential (Herling and Weidmann 1994).

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Subacute Gastric Ulcer in Rats

Purpose and Rationale

Ezer (1988) described a method for producing standard subacute gastric ulcers in rats and for the quantitative evaluation of the healing process.

Procedure

Female Wistar rats weighing 120–150 g are fasted for 24 h having access to water at libitum in cages with wire sieves at the bottom. The rats are anesthetized appropriately, and a polyethylene catheter including a fine steel wire with a needle tip (1.2 mm diameter) at the lower end is orally inserted into the stomach. After the cannula reaches the gastric wall, the upper end of the steel wire is pressed in a definitive manner, so as to puncture the gastric wall. Each rat is kept in the same position during the intervention in order to localize the puncture at nearly the same region of the glandular part of the stomach. The test substances are administered orally, 30 min or 24 h after puncture. Free access to food and water is provided from 2 h up to the end of the experiment. Each group consists of 8–15 rats.

The animals are sacrificed by overdose of isoflurane at definitive time intervals after puncture. The stomach is dissected and opened along the lesser curvature, extensively rinsed in tap water and fixed to the end of a polyethylene tube of 10 mm diameter (plastic tip of an automatic pipette) in a position with the punched ulcer in the center. The end of the tube with the gastric wall is suspended in a beaker containing physiological saline, and the pressure in the tube is gradually increased with a valved rubber ball connected to the other end of the tube. The third part of the system is a tonometer calibrated up to 1 bar. The value of tension at which bubbles appear at the ulcerous gastric wall is noted. This value is termed as tensile strength and can be expressed in mmHg.

Evaluation

The extent of the healing of gastric ulcers can be characterized by the healing rate (*HR*) according the following equation:

$$HR = \frac{(A - B)}{C(\text{mmHg/h})}$$

with

- A = tensile strength (mmHg) at C time point after puncture.
- B = tensile strength 30 min after puncture (the average value is 143 mmHg).
- C = time course (h) of the experiment.

Antiulcer drugs, such as H_2 antagonists, significantly increase the healing rate, which is decreased by nonsteroidal anti-inflammatory drugs.

Critical Assessment of the Method

Similarly to the method of Takagi et al. (1969) who injected 50 µl of acetic acid into the stomach wall (Szelenyi et al. 1982), the method of Ezer (1988) allows to judge the time course of healing of the ulcers.

Modifications of the Method

Okabe and Pfeiffer (1972) induced chronic gastric ulcer in rats by temporary instillation of acetic acid. In pentobarbital anesthesia, a cylindrical glass tube of 6 mm in diameter was tightly placed upon the anterior serosal surface of the glandular portion of the stomach 1 cm away from the pyloric end. A dose of 0.06 ml/animal of 50 % acetic acid was instilled into the tube and allowed to remain 1 min on the gastric wall. After removal of the acid solution, the abdomen was closed in two layers and the animals brought back to their cages and fed normally. Test drugs were given orally on day 1 twice daily, 4 h after application of acetic acid and continued up to 10 days after induction of ulcer. The animals were sacrificed after 18 h of the last dose to assess ulcer size and healing. Ulcer index was calculated upon the product of length and width of ulcers.

Karmeli et al. (1996) induced gastric mucosal erosions in rats by addition of 0.1 % iodoacetamide to the drinking water. The animals were sacrificed after various time intervals, the stomach was resected, washed, lesion area assessed, and mucosal inflammatory mediators determined. Myeloperoxidase was increased and nitric oxide synthase activity decreased. The damage induced by iodoacetamide was significantly ameliorated by treatment with a free radical scavenger (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, TEMPOL).

Piqueras et al. (2003) described gastric hypersecretion in **mice** due to mast cell activation associated with mild gastritis induced by 0.1 % iodoacetamide administered intragastrally and added to the drinking water for a 6-day period.

Marchetti et al. (1995) and Konturek et al. (1999) described a mouse model of *Helicobacter pylori* infection. Gastric function and healing of chronic acetic acid-induced ulcers in BALB/c mice were studied after inoculation with CagA- and VacA-positive (type I) or CagA- and VacA-negative (type II) *Helicobacter pylori* strains. This infection caused immediate suppression of gastric secretion and delayed the healing of ulcers.

Protell et al. (1976) described a reproducible model of acute bleeding ulcer in **dogs** – the "ulcer maker." An instrument has been developed for endoscopy or laparotomy, which creates gastric ulcers of reproducible diameter and depth.

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Gastric Ischemia–Reperfusion Injury in Rats

Purpose and Rationale

Hassan et al. (1997) described the effect of an endothelin-converting enzyme inhibitor on local gastric ischemia–reperfusion injury in rats. Endothelin-1 has potent ulcerogenic effects in the stomach (Wallace et al. 1989). Endogenous endothelin-1 has been implicated for ethanol-, indomethacin- and hemorrhagic shock-induced gastric ischemia–reperfusion injuries (Masuda et al. 1993; Kitajima et al. 1993; Michida et al. 1994; Kitajima et al. 1995).

Procedure

Male Wistar rats weighing 200–250 g are fasted for 24 h with free access to water. The rats are anesthetized appropriately. The stomach is exposed by a medial laparotomy and instilled with 0.15 M HCl (1 ml/100 g) via the forestomach. The left gastric artery is clamped by a small vascular clamp for 5 min to induce ischemia, and 30 min of reperfusion is done by releasing the clamp. Pretreatment with test drug or standard is given to groups of five rats immediately before the induction of ischemia. At the end of the experiment, the rats are sacrificed by cervical dislocation. The stomach is fixed with 10 % buffered formalin and photographed for macroscopic evaluation of injuries. For the assessment of microscopic injuries, a sample of corpus 0.5 cm below the limiting ridge containing the entire width of the anterior wall is taken

from each stomach and processed for subsequent histological evaluation.

A planimeter attached to a computer is used to trace the macroscopic mucosal injury from color photographs. The results are expressed as a percentage of the total glandular mucosal area.

Each histological section is stained with hematoxylin/eosin and examined under light microscope. An one cm length of each histological section is assessed for epithelial damage (score = 1), glandular disruption, vasocongestion or edema in the upper mucosa (score = 2), hemorrhagic damage in the mid to lower mucosa (score = 3), and deep necrosis and ulceration (score = 4). Each section is evaluated on a cumulative basis to give the histological index, the maximum score thus being 10.

Evaluation

Data are expressed as mean ± SEM. Comparisons between different groups are made by one-way analysis of variance followed by Fisher's least significant difference test. p-values of <0.05 are considered as statistically significant.

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Pharmacological Effects on Intestinal Functions

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Intestinal Secretion

Laxative Activity in Rats

Purpose and Rationale

Laxatives of the sennoside type act mainly by acceleration of large intestine transit and inhibition of fluid absorption in the colon (Leng-Peschlow 1986a, b).

Procedure

For **measurement of large intestinal transit time**, female Wistar rats weighing approximately 200 g are anesthetized. A PVC catheter is implanted into the cecum with the distal end fixed on the animal's neck. The animals are allowed to recover and are placed individually in a wire-mesh cage to enable the feces to fall through onto blotting paper. Carmine red (10 mg in 0.4 ml distilled water per animal) is injected through the catheter immediately after administration of the test substance. The time until appearance of the first colored feces is registered.

For **measurement of fluid absorption in the colon**, female Wistar rats weighing approximately 200 g are anesthetized (e.g., with 50 mg/kg pentobarbitone sodium). The colon is ligated and cannulated distal to the cecocolic junction (PE tube, i.d. 1 mm), and, after a thorough rinse with 50 ml physiological saline to remove all contents, a second cannula (silicone, i.d. 3 mm) is inserted proximal to the rectum for fluid outflow. Four and 6 h after oral administration of the test compounds, an open perfusion with an electrolyte solution (NaCl 6.72 g/l, KCl 0.37 g/l, $NaHCO_3$ 2.1 g/l, polyethylene glycol (PEG, mol wt 4,000) 2.0 g/l, [^{14}C]PEG 5 μCi/l; pH 6.5, osmolality 275 milliosmol/kg) is started at a rate of 12 ml/h for two consecutive 2-h periods. [^{14}C] PEG activity is measured by liquid scintillation counting, Na^+ and K^+ by flame photometry, Cl^- by coulometric titration, osmolality by freezing point depression, and mucus as protein-bound total hexoses by the orcinol–sulfuric acid method. Net H_2O, Na^+, K^+, and Cl^- transports are calculated and expressed as ml or μmol/h and per 10-cm colon length.

Evaluation

All values are expressed as mean ± standard deviation. Statistical significance is assessed with Student's *t*-test.

Modifications of the Method

Ogunti and Elujoba (1993) tested the laxative activity of *Cassia alata* in rats. Male Charles River rats were kept in individual cages during 1 week. Any rat producing wet feces was rejected. After administration of the test compounds to groups of 5 rats per dose, the feces were examined for wetness hourly for 12 h. The results were expressed as the mean percent of total feces that were wet per kg rat.

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Enteropooling Test

Purpose and Rationale

The enteropooling assay in rats has been developed by Robert et al. (1976) to test the diarrheogenic property of prostaglandins for prediction of this clinically relevant side effect of several synthetic prostaglandins.

Procedure

Female Sprague Dawley rats weighing 190–215 g are used. The animals are fasted overnight having free access to water. The test compounds are administered orally, and the animals, 12 per group, are sacrificed one hour later. The fluid accumulation occurs in the small intestine which is cut at the pylorus and the ileocecal junction, and its

contents, consisting of a thick fluid (in controls) and a very watery fluid (in prostaglandin-treated animals), are collected into a graduated test tube by milking the whole length of the small intestine with the fingers. The volume of fluid is recorded.

Evaluation

Using various doses, dose–response curves can be established and potency ratios calculated. 16,16-Dimethyl PGE_2 was found to be the most active compound.

Critical Assessment of the Method

Some other diarrheogenic agents, like $MgSO_4$, castor oil, bile, taurocholate, and taurochenodeoxycholate, cause enteropooling, whereas mineral oil and tragacanth are ineffective. The anticholinergic agent methylscopolamine partially counteracted the enteropooling. The assay, therefore, can be used to test the laxative or the antidiarrheal activity of compounds (Shook et al. 1989).

Modifications of the Method

Beubler and Badhri (1990) used the PGE_2-induced net fluid secretion in the jejunum and colon in the rat to evaluate the antisecretory effects of antidiarrheal drugs. Polyethylene catheters were placed into the jejunum and colon and Tyrode solution was instilled into the loops. Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution.

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Inhibition of Chloride Secretion in Rabbit Colon

Purpose and Rationale

Epithelia have the ability to reabsorb or to secrete fluids and electrolytes. In 1958, Koefoed-Johnsen and Ussing first published a model of ion transport across frog skin epithelium. Ussing also introduced the measurement of the short-circuit current as a means of defining active ion transport (Koefoed-Johnsen and Ussing 1958). Mammalian colon is an example of epithelium which has the capacity to absorb and to secrete electrolytes.

Previous studies have shown that mammalian colon actively secretes chloride when exposed to prostaglandins and vasoactive intestinal peptides, which increase the cellular concentration of cAMP resulting in an electrogenic chloride secretion (Frizzell et al. 1976). The elevation of cAMP causes the opening of chloride channels, whereas the colonic epithelium absorbs sodium and chloride ions by an electroneutral mechanism under control conditions. In general it is assumed that absorption takes place mainly in the surface cells, whereas the crypts are the predominant site of secretion (Greger et al. 1985; Binder and Sandle 1987).

Procedure

Rabbits of either sex (2.0–4.0 kg body weight) are killed by cervical dislocation. The distal colon is removed, immediately opened into a flat sheet, and washed in the standard electrolyte solution. The epithelium with an area of 1.0 cm^2 is stripped from its underlying musculature and mounted in an Ussing chamber. The tissue is mounted vertically and bathed on both sides by electrolyte solutions, which are circulated and oxygenated by a water-jacketed bubble-lift apparatus maintained at 37 °C. The carbogen gas used for bubbling contains O_2 and CO_2 in a mixture of 95 % and 5 %, respectively. The solutions used on the two sides

have the following composition (in mmol/l): NaCl 120, $NaHCO_3$ 21, Na_2HPO_4 0.4, K_2HPO_4 1.6, $MgCl_2$ 1.2, and glucose 5.

Tissues are continuously short-circuited by a four-electrode automatic voltage-clamp apparatus (AC Microclamp, Aachen, Germany) which measures short-circuit current (I_{sc}) and automatically subtracts chamber fluid resistance. Transepithelial electrical potential difference is measured between Ag–AgCl electrodes, which make contact with the bathing solutions via agar bridges (5 % agar in glucose-free standard electrolyte solution). I_{sc} is measured by passing sufficient current through Ag–AgCl electrodes to reduce the spontaneous transepithelial electrical potential difference to zero. Transepithelial conductance (G_t) is determined by passing 100-ms bipolar current pulses through the tissue.

The standard protocol consists of an initial equilibration period of 20 min. The I_{sc} measured in the presence of indomethacin (1 µmol/l, serosal and mucosal solution) corresponds mostly to the rheogenic reabsorption of sodium. In the next step, amiloride is added at 0.1 mmol/l to block sodium reabsorption. Then PGE_2 (1 µmol/l) is added to the serosal solution in the presence of amiloride in order to stimulate chloride secretion. After a further equilibration period of 20 min, putative blockers of chloride secretion are added to the mucosal or serosal solution.

Evaluation

Each compound is examined at least three times and at three different concentrations (usually 1, 10, and 100 µmol/l). From the mean values, a concentration–response curve is constructed, and from this curve, the IC_{50} value is read as the concentration producing 50 % inhibition of the stimulated I_{sc}.

Data are presented as means ± SEM. Paired *t*-test with a significance level of $p < 0.05$ may be used.

Critical Assessment of the Method

The method can be used to study the antidiarrheal activity of a test compound but also generally its influence on active electrolyte transport across the cell membrane.

Modifications of the Method

Warhurst et al. (1996) studied the effects of somatostatin analogues on electrogenic ion secretion in isolated rat colonic mucosa mounted in Ussing chambers.

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Antidiarrheal Effect

Castor Oil-Induced Diarrhea

Purpose and Rationale

The induction of diarrhea with castor oil results from the action of ricinoleic acid formed by hydrolysis of the oil (Iwao and Terada 1962; Watson and Gordon 1962). Ricinoleic acid produces changes in the transport of water and electrolytes resulting in a hypersecretory response (Ammon et al. 1974). In addition to hypersecretion, ricinoleic acid sensitizes the intramural neurons of the gut.

Procedure

Female Wistar rats weighing 210–230 g are used after overnight food deprivation. For the

experiment, the rats are housed in individual cages with no access to drinking water. The potential antidiarrheal agents are administered orally by gavage in various doses. Controls receive the solvent only. Each dose is given to 10 animals. One hour after dosage, 1 ml of castor oil is administered orally. Stools are collected on non-wetting paper sheets of uniform weight up to 24 h after administration of the castor oil. Every 15 min during the first 8 h, urine is drained off by gravity, and the net stool weight, termed early diarrheal excretion, is recorded. The diarrhea-free period is defined as the time in minutes between castor oil administration and the occurrence of the first diarrheal output. The acute diarrheal phase is the time between the first and the last diarrheal output of the 8-h observation period. Stools occurring between 8 and 24 h after castor oil administration are called late diarrheal excretion.

Evaluation

With antidiarrheal agents, dose–response curves for decrease of hypersecretion (stool weight) and for increase of the diarrhea-free period are obtained. Inhibitors of prostaglandin biosynthesis increase the diarrhea-free period but do not affect early diarrheal secretion (Niemegeers et al. 1984).

Modifications of the Method

Inhibition of castor oil-induced diarrhea in **mice** was tested by Bianchi and Goi (1977).

Dajani et al. (1977) tested antidiarrheal activity in castor oil-treated **monkeys**.

Mannitol-induced diarrhea was used as a model in **calves** (Fioramonti and Buéno 1977) and in **pigs** (Théodorou et al. 1991).

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Antidiarrheal Effect in Cecectomized Rats

Purpose and Rationale

Evaluation of antisecretory antidiarrheal agents in animal models is limited primarily to extrapolations of efficacy from enteropooling studies in vivo (DiJoseph et al. 1984), isolated intestinal loops (Nakaki et al. 1982), and Ussing flux chamber preparations in vitro (Dharmsathaphorn et al. 1984). These studies do not mimic secretory diarrhea. The method of Magnus (1915) using chronic diarrhea in cats induced by continuous milk diet and thereby proofing the antidiarrheal effect of morphine is of historical interest only.

Fondacaro et al. (1990) developed a model of secretory diarrhea utilizing conscious cecectomized rats by surgical resection of the cecum and by use of potent intestinal secretagogues. The rat has a pronounced cecum as part of its gastrointestinal tract. The rat cecum is not only a reservoir for intestinal contents where high concentrations of various microbial floras assist in the digestion of carbohydrates, cellulose, and peptides through microbial fermentation processes (Ambuhl et al. 1979; Williams and Senior 1982) but also plays a role in handling of excess intestinal fluid.

Procedure

Cecectomies are performed in unfasted rats weighing 200–250 g. Under general anesthesia (e.g., isoflurane inhalation, ketamine/xylazine, etc.), cecectomy is initiated with a 2-cm midventral incision. The cecum is lifted from the abdominal cavity and exteriorized onto a gauze drape. The cecal apex is freed by severing the avascular area of the mesocecum. A ligature of no. 1 silk suture is positioned so as to occlude the cecum and its vasculature without compromising ileocolonic patency. After the ligature is secured and ileocolonic patency confirmed, the cecum is resected, and the remaining exposed cecal mucosa is washed with saline and cauterized. The intestinal segment is then returned to the abdominal cavity and the abdominal muscle fascia closed with sutures. The dermal incision is closed with wound clips that are removed about 1 week postsurgery. Immediately following the surgical procedure, the animals are returned to their cages and allowed free access to food and water. The animals are permitted at least a 48-h recovery period before being used in an experiment.

For the diarrhea assay, cecectomized rats are put into individual wire-bottomed cages placed over sheets of clean paper and deprived of food and water for the duration of the assay. Rats are given a two hour's acclimatization period prior to the start of the assay in order to eliminate sporadic episodes of anxiety-induced defecation. During this period, they are observed also for consistent occurrences of pelleted feces; an animal producing other than pelleted stool is disqualified from the study. Diarrhea is induced with oral administration of secretagogues: either 16,16 dimethyl prostaglandin E_2 (0.3 mg/kg) in 3.5 % ethanol, carbachol (15 mg/kg) in water, or cholera toxin (0.5 mg/kg) in an aqueous vehicle of 2 % $NaHCO_3$ plus 2 % casamino acids. Antidiarrheal agents are administered by gavage after the onset of diarrheal episodes. The cage papers are removed and examined at 15-min intervals for carbachol-induced diarrhea, 30-min intervals for 16,16 dimethyl prostaglandin E_2-induced diarrhea, and hourly when cholera toxin is used as secretagogue. Fetal output is recorded at each interval and scored as follows:

- 1 = normal pelleted stool
- 2 = soft formed stools
- 3 = watery stool and/or diarrhea

Known antidiarrheal agents, such as chlorpromazine (10 mg/kg p.o.), or the alpha-2 receptor agonist clonidine (1.0 mg/kg p.o.), or morphine (10 mg/kg p.o.) reduce the fecal output and induce a cessation of diarrhea.

Evaluation

The fecal output index is defined as the summation of the number of defecation periods and their ranked consistency score within an observation period and is expressed as mean ± SEM for each group. Student's *t*-test and analysis of variance are used for statistical comparisons of data points. Significance is accepted at $p < 0.05$ or less.

Critical Assessment of the Method

The model of diarrhea induced by secretagogues in cecectomized rats has the advantage to mimic secretory diarrhea in man.

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Evaluation of Antidiarrheal Effect in Cold-Restrained Rats

Purpose and Rationale

Barone et al. (1990) tested the effect of various antidiarrheal and other drugs on increased fecal pellet output in cold-restrained rats resembling clinical observations that stressful situations can produce diarrhea in humans.

Procedure

Male Sprague Dawley rats weighing 260–310 g are maintained on Purina lab chow and water. Since gastric ulcers are reduced if cold-restrained rats are allowed free access to food and water, for studies on fecal output, food was not withdrawn prior to the experiment. The rats are studied in normal living cages at room temperature (control, non-stressed animals) or in wire-mesh restraining cylinders placed in a cold (4 °C) environment. Test drugs are administered by appropriate routes over optimal effective dose ranges for their activities and at optimal pretreatment times to maximize their effects. The number of pellets expelled by each animal is measured at 1 and 3 h (fecal pellet output). Generally, the fecal pellets of stressed animals are less firm. Fecal pellet fluid content is determined by weighing fecal pellets, drying them in an oven at 37 °C, and weighing them again.

Evaluation

The dose in mg/kg that inhibits the cold-restrained stress-induced increase in fecal pellet output by 50 % (ID_{50}) is determined using least-squares fit analysis directly from the regression line. If fecal pellet output is decreased by a drug but no clear dose-related effects occur, the maximum percent decrease is determined.

Modifications of the Method

For colonic transit studies, rats are implanted with indwelling catheters in the proximal colon. Animals are anesthetized with 60 mg/kg pentobarbital i.p., and a chronic colonic catheter is positioned to enter the proximal colon 2 cm from the ileocecal junction. A catheter of about 20 cm of silicone tubing prepared with several drops of silicon rubber adhesive coating a 1-cm length along the catheter is positioned into the colon. The colon end of the tubing also is sealed with a 1-cm plug of petroleum jelly intraluminally. A small incision is made in the proximal colon, and the adhesive-coated portion of the Silastic tubing is tied in place with the use of a purse-string suture. The tubing is brought through the abdominal wall, led subcutaneously through the skin in the midscapular region, and secured on the back of the neck with the use of a wound clip. The abdominal incision is closed with sutures and wound clips.

Experiments are performed in conscious animals 48–72 h after surgical preparation. At this time, the radiolabeled marker (^{51}Cr as sodium chromate) is instilled into the proximal colon via the indwelling catheter. After 35, 60, or 120 min, cold-restrained rats and controls are sacrificed and their colons and large intestines are removed. The cecum and equal segments of the colon are dissected, placed into vials, and subjected to gamma counting.

Ikeda et al. (1995) investigated the effect of a neurokinin$_1$ receptor antagonist on stress-induced defecation in rats placed in special restraint cages.

Kishibayashi et al. (1993) studied distal colonic function using wrap-restrained stress-induced defecation as described by Williams et al. (1988). The rats were lightly anesthetized with ether, and the foreshoulders, the upper forelimbs, and the thoracic trunk were wrapped in paper tape to restrict, but not prevent, movement. The animals recovered from anesthesia within 2–5 min and immediately moved around in cages and ate and drank but had been restricted from mobility of forelimbs, which prevented them from grooming the face, the upper head, and the neck. Fecal pellet output induced by wrap-restrained stress was weighed during the first hour after stress. The test drugs were given p.o. 1 h before stress. The ID_{50} values were calculated as the doses that reduced stress-induced defecation by 50 %.

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Gut Motility

Isolated Ileum (Magnus Technique)

Purpose and Rationale

The isolated ileum, as first described by Magnus (1904), is probably the most widely used model in experimental pharmacology. Magnus already studied simultaneously the spontaneous contractions of the longitudinal and circular musculature and the inhibiting effect of atropine. The method has been used for many purposes, such as the study on the effects of adrenaline on the lower segments causing contraction and on the segments of the upper end causing relaxation by Munro (1951) or the study on the origin of acetylcholine released from guinea pig intestine and longitudinal muscle strips by Paton and Zar (1968) either retaining or being denervated from Auerbach's plexus. The model is used as a basic screening

procedure for spasmolytic activity, whereby an anti-acetylcholine or anticarbachol effect indicates antimuscarinic activity and an anti-$BaCl_2$ effect indicates a musculotropic, papaverine-like effect. In addition to the isolated ileum, other parts of the gut such as the isolated duodenum and colon have been used widely.

Procedure

Guinea pigs of either sex weighing 300–500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, with the mesentery being cut away as necessary. When the colon is reached, the intestine is cut. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 2–3-cm length are cut. Preferably the most distal piece is used, being the most sensitive one. This piece is fixed with a tissue clamp and brought into a 15-ml organ bath containing Tyrode solution at 37 °C being oxygenated with 95 % O_2/5 % CO_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard, USA). A preload of 1 g is chosen. Responses are recorded on a polygraph. After a preincubation time of 30 min, the experiment is started.

The following agonists and antagonists (standards) are used (concentrations in g/ml bath fluid):

Agonist	Antagonist
Acetylcholine 10^{-7} g/ml	Atropine 10^{-8}–19^{-9} g/ml
	Scopolamine 10^{-8}–10^{-9} g/ml
Carbachol 10^{-7} g/ml	Atropine 10^{-8}–10^{-9} g/ml
Histamine 10^{-6} g/ml	Histamine antagonists
$BaCl_2$ 10^{-4} g/ml	Papaverine 10^{-5}–10^{-6} g/ml
Serotonin 10^{-6} g/ml	Serotonin antagonists
PGE_2 2×10^{-7} g/ml	PG antagonists

Evaluation

Several methods for the quantitative evaluation of an antagonistic effect are available. One approach is the determination of pD'_2 values according to van Rossum and van den Brink (1963). Acetylcholine or histamine is added in 1/2 $\log_{10}$ concentration increments until a maximum response is obtained. Control curves are recorded at 30-min intervals. After uniform control responses are obtained, the potential antagonist or the standard is added 5 min before the concentration–response curve is re-obtained. The potency of the antagonist is obtained by calculating the pD'_2 value which is defined as the negative logarithm of the molar concentration of an antagonist that causes a 50 % reduction or the maximal response obtained with an agonist.

Modifications of the Method

Many modifications of the Magnus technique have been described in the literature, mainly with the isolated ileum (e.g., Koelle et al. 1950).

Okwuasaba and Cook (1980) dissected the myenteric plexus and longitudinal muscle free of the underlying circular muscle according to the method of Paton (1957) and Paton and Zar (1968) and stimulated the preparation with trains of supramaximal rectangular pulses of 1.0-ms duration at a frequency of 0.2 Hz.

Kilbinger et al. (1995) studied the influence of 5-HT_4 receptors on [^{3}H]-acetylcholine release from guinea pig myenteric plexus.

De Graaf et al. (1983) described a fully automated system for in vitro experiments with isolated tissues. The apparatus consists of an organ bath equipped with (a) a gradient pump supplying a logarithmic concentration–time gradient of agonist; (b) pumps and valves for dispensing bath fluid, antagonist solutions, and an oxygenation gas mixture; and (c) a transducer with automatic baseline adjustment. The information coming from the preparation is fed into a minicomputer. The data of various experiments can be accumulated and Schild plots obtained.

Furukuwa et al. (1980) studied the effects of thyrotropin-releasing hormone on the isolated small intestine and taenia coli of the guinea pig.

Paiva et al. (1988) studied the role of sodium ions in angiotensin tachyphylaxis in the guinea pig ileum and taenia coli.

Barnette et al. (1990) used electrically stimulated strips of circular smooth muscle from the

lower esophageal sphincter of dogs to study the inhibition of neuronally induced relaxation by opioid peptides.

Bradykinin antagonism can be studied in the isolated guinea pig ileum bathed in a solution containing atropine (1.5 mM), diphenhydramine (3.4 mM), indomethacin (2.8 mM), and captopril (0.9 mM) (Rubin et al. 1978; Kachur et al. 1987).

Griesbacher and Lembeck (1992) used the isolated guinea pig ileum for analysis of bradykinin antagonists.

Hew et al. (1990) used field-stimulated (95 % of maximum voltage, 0.1 Hz, 0.5 ms) guinea pig ileum, bathed in physiological salt solution at 37 °C in the presence of 1 mM mepyramine for determination of histamine H_3 bioresponse. Reduction of contractile response by the test substance (>50 % relative to control 0.3 mM R-α-methylhistamine) indicates possible H_3 agonism. At a test concentration where no significant activity is seen, the ability to inhibit (>50 %) R-α-methylhistamine-induced contractile reduction indicates antagonistic activity.

Feniuk et al. (1993) used the guinea pig isolated ileum, vas deferens, and right atrium to characterize somatostatin receptors. Transmural electrical stimulation was applied to the guinea pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Radimirow et al. (1994) investigated opioid effects of short enkephalin fragments containing the Gly-Phe sequence on contractile responses of the guinea pig ileum after addition of 10 nM acetylcholine or after electrical stimulation.

Coupar and Liu (1996) described a simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle in **rats**. The preparation consists of a segment of rat ileum set up to measure the tension developed in the longitudinal muscle and intraluminal pressure developed in the circular muscle in response to transmural electrical stimulation.

Vassilev et al. (1993) exposed Wistar rats to subtoxic doses of Co^{2+} or Ni^{2+}, receiving Co $(NO_3)_2$ or $NiSO_4$ with drinking water for 30 days, and measured the changes in the contractile responses to carbachol and in the inhibitory effects of verapamil and nitrendipine on isolated smooth muscle preparations of the ileum and the trachea.

Pencheva and Radomirov (1993) and Pencheva et al. (1999) studied the effects of GABA receptor agonists on the spontaneous activity of the circular layer in the terminal ileum of **cats**. Segments of the terminal ileum approximately 0.5 cm long were mounted in an organ bath along the axis of the circular layer through a cotton thread with a large knot situated at the inner part of the gut wall.

Similar preparations of the cat ileum were used by Kortezova et al. (1994) and Chernaeva and Mizhorkova (1995).

Vassilev and Radomirov (1992) used an isolated preparation of the **rat rectum**. The rectal region, 1–6 cm proximal to the anal sphincter, was removed and a 20-mm-long segment suspended in an organ bath. The influence of prostaglandins and antagonists on spontaneous mechanical activity and electrically stimulated responses was investigated.

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Cascade Superfusion Technique

Purpose and Rationale

The technique of isolated organ superfusion was developed by Gaddum (1953) for the assay of biologically active substances. Extension of the technique for multiple tissue superfusion with particular reference to the identification and the assay of prostaglandin-like activity was used by various authors (Vane 1964; Ferreira and Vane 1967; Ferreira and de Souza 1976; Gilmore et al. 1968; Hong 1974; Bult et al. 1977; Henman et al. 1978; Elliott and Adolfs 1984; Fournau et al. 1984).

Procedure

The apparatus consists of a double-wall glass container (height 20–25 cm, inner diameter 7–8 cm) with an outlet at the bottom. A constant temperature of 38 °C is maintained by circulation of warm water through the outer jacket. Inside the glass container can be suspended up to five pieces of tissue of various origins. The multiple-preparation tissue holder consists of a vertical rectangular rod and plastic platforms for attachment of the tissues and for accurate deflection of the superfusate onto the lower tissue. The rod is grooved at 10-mm intervals with 1-mm deep slots set at an angle of 20° to the horizontal. To the upper surface of the nonwettable platform, a small plastic hook is cemented at such a distance from the rod that when the tissue is in position, its attachment thread passes between the V-shaped notches cut into the margin of the upper tissue platform. The individual platforms are inserted onto the vertical rod by slotting into the requisite grooves appropriate to the tissue length.

Thus, the superfusate passes at a uniform flow rate down the tissues of the cascade, and the tension recording threads are separated from each other by about a 5-mm gap so that the responses can be conveniently recorded. The threads from the organs are connected over isotonic levers to isometric tension transducers. The lever is used for preloads according to the individual organ. Tension exerted by each tissue is recorded on a polygraph. Various media can be used for superfusion, e.g., Krebs–Henseleit solution gassed with 95 % O_2 and 5 % CO_2.

Many tissue preparations can be used for the cascade, such as rat fundic strip, rat duodenum, rat colon, rat bladder strip, guinea pig ileum, guinea pig proximal colon, rabbit stomach strip, rabbit celiac/mesenteric artery, or rabbit aorta strip. Moreover, donor tissue can be superfused and its effluent be tested in the organs of the cascade.

Evaluation

Many agonists and antagonists can be tested by appropriate selection of organs. Threshold doses and ED_{50} or ID_{50} values can be determined.

Modifications of the Method

The superfusion technique has been used for several purposes, e.g., for the assay of catecholamines (Armitage and Vane 1964) and for detecting active substances in the circulating blood (Vane 1964). A simple and inexpensive piece of apparatus for cascade superfusion procedures has been described by Naylor (1977).

Mombouli et al. (1996) described a bioassay of endothelium-derived hyperpolarizing factor (EDHF) using a perfusion–superfusion cascade where canine carotid arteries were used as donors of vasoactive substances and rings of coronary arteries without endothelium as detectors.

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In Vivo Evaluation of Spasmolytic Activity in Rats

Purpose and Rationale

Maggi and Meli (1982) described an in vivo procedure for estimating spasmolytic activity in the rat by measuring smooth muscle contractions to topically applied acetylcholine.

Procedure

Male rats weighing 350–400 g are anesthetized appropriately (e.g., ketamine/pentobarbital). The left jugular vein is cannulated for administration of test compounds. After laparotomy, occluding silk ligatures are applied at a distance of 2 cm from each other in the gut (colon or rectum). Through a small incision, the flanged tip of a polyethylene tubing (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen of this pocket-like space and secured in place by a purse-string ligature. The free end of the tubing is connected to a pressure transducer and the whole system filled with saline. The same procedure is performed with the urinary bladder. The organs are filled with warm saline (37 °C) to obtain a resting pressure of 4–12 mmHg. Warm saline-soaked cotton wool swabs are laid around the exteriorized organs which are maintained warm and moist with warm (37 °C) saline dropping from a reservoir at a rate of 10–15 drops/min.

After a 15-min stabilization period, saline flow is stopped and a dose–response curve to acetylcholine determined. A volume of 0.5 ml (an amount sufficient to put the whole outer surface of the organ into contact with the bathing solution) of acetylcholine at the desired concentration is applied within 2–3 s from a syringe to the outer surface of the target organ. To construct a dose–response curve of acetylcholine, increasing concentrations are applied to the target organ until maximal contraction is obtained. After at least 3 or more control curves have been obtained at 10-min intervals, the antagonist is administered.

Evaluation

The quantitative analysis of the data is carried out by plotting the results of each experiment as log

(acetylcholine dose ratio-1) against log dose antagonist (Arunlakshana and Schild 1959). The regression line is calculated according to the method of least squares and ED_{50} values and 95 % confidence limits according to Litchfield and Wilcoxon (1949). From ED_{50} values, the dose of the antagonist (mg/kg i.v.) to produce an acetylcholine dose ratio of 10 is calculated according to Daly et al. (1975). Parallel displacement to the right of the agonist dose–response curve and a slope of unity for the regression line indicate competitive antagonism. The DR_{10} values from different organs are compared by means of Student's *t*-test for unpaired data.

Modifications of the Method

In vivo registration of gut motility in guinea pigs was already described by Straub and Viaud (1933). A 4-cm-long part of the gut was ligated and filled with Tyrode solution. Gut motility was measured at variable intraluminal pressure.

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Colon Motility in Anesthetized Rats

Purpose and Rationale

The influence of spasmolytic drugs on carbachol-induced increase of colonic motility can be measured in anesthetized rats. The method has also been used to study the stimulation of colonic motility by an enkephalin analogue pentapeptide (Bickel 1983).

Procedure

Male Sprague Dawley rats weighing 350–500 g are anesthetized with pentobarbital i.v. A pressure-sensitive tip catheter is inserted into the colon ascendens, and the signals of the intraluminal pressure changes are recorded. The colonic contractions are stimulated by i.v. injection of 3 mg/kg carbachol. The height and the duration of the contractions are recorded. Then, the test compound is injected intravenously. The decrease of contractions is measured, and the duration of the spasmolytic activity determined by repeated administration of carbachol at 15-min intervals, until the contractions are not significantly different from the response obtained with carbachol alone.

Evaluation

Significant differences are calculated using Student's unpaired *t*-test.

Modifications of the Method

Maggi and Meli (1984) used eserine-induced hypertonus of **guinea pig** distal colon in vivo as a pharmacological procedure for testing smooth muscle relaxants. Male albino guinea pigs weighing 240–300 g are anesthetized with 1.5 g/kg urethane s.c. Through a midline abdominal incision, the proximal part of the hypogastric loop of the distal colon is exposed and occluding silk ligatures are applied at a distance of 2 cm from each other, taking great care to avoid any lesion to the vascular and nervous supply. Through a small incision, the flanged tip of a polyethylene tube (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen and secured by means of a purse-string ligature. The free end of the tube is connected to a pressure transducer and the whole system is filled with saline. Intraluminal pressure and its

variations are recorded on a polygraph. The effect of drugs is assessed as inhibition of eserine-induced hypertonus.

Théodorou et al. (1991) studied the absorptive and motor components of the antidiarrheal action of loperamide in **pigs**. Motility was recorded by implantation of intraparietal electrodes into various parts of the gut.

Raffa et al. (1987) used a method utilizing the insertion of a 3-mm glass bead into the distal colon in **mice** to evaluate the activity of intracerebroventricularly administered μ- and *δ*-opioid agonists on colonic bead expulsion time.

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Continuous Recording of Electrical and Mechanical Activity in the Gut of the Conscious Rat

Purpose and Rationale

Bueno et al. (1981) described a method for continuous electrical and mechanical activity recording in the gut of the conscious rat. In this study, two methods for the continuous recording of motor events – microtransducers and the electromyogram – are compared for the rat stomach and intestine.

Procedure

Male Wistar rats weighing 200–300 g are housed singly in wire-bottomed cages and allowed lab chow and water ad libitum during the training period of 30 days. Under halothane anesthesia, pairs of electrodes are implanted along the greater curvature of the pyloric antrum 5 and 2 cm from the pylorus, on the antimesenteric border of the duodenum 2 and 5 cm beyond the pylorus, and along the jejunum, 4 cm from the pylorus using a procedure described by Ruckebusch and Fioramonti (1975).

The contractions of the circular muscle layer are recorded with strain-gauge microtransducers sutured onto the serosa at less than 1 cm from each set of electrodes (Pascaud et al. 1978). The free ends of the electrodes and strain-gauge wires are carried subcutaneously and exteriorized on the back of the neck. The wires are inserted into a glass tube (15 and 0.5-cm external diameter) to prevent twisting and any contact with the metallic lids of the cage.

Evaluation

Both electrical and mechanical activities are continuously recorded starting 5–7 days after surgery on a multichannel recorder (e.g., 8-channel Dynograph, Beckman, USA) using lead selector couplers (type 9856, Beckman) at a constant time of 0.1 s for the electrical spiking activity and strain-gauge couplers (type 9863, Beckman) for the contractile force minitransducers. Simultaneously low-frequency signals (frequency < 3 Hz) of the electromyogram are eliminated through filters, and the spiking activity, integrated for each 20 s, is recorded on a potentiometric recorder. The electrical and mechanical activities are also recorded simultaneously on a magnetic tape recorder.

The index of motility, expressed as mcoul/min, is calculated from the integrated records of electrical activity. The index of mechanical motility is calculated as the area under all contraction waves

occurring during 1 min and is expressed as gs/min. Mechanical and electrical activities during a test period of 30 min after administration of stimulant drugs, e.g., the gastrointestinal hormones gastrin and cholecystokinin, or relaxing drugs, like anticholinergic agents, are compared with the values of a 30-min pretest period.

Modifications of the Method

Wright et al. (1981) described a similar method for long-term recording of intestinal mechanical and electrical activity in the unrestrained rat. Mechanical activity is detected using miniaturized half-bridge metal foil strain-gauge force transducers. The electrical activity is monitored by silver–silver chloride bipolar electrodes. The lead wires from the recording units are encased in a metal compression spring and are permanently joined to a ball connector positioned on the top of the cage, thus allowing the animal free access to all parts of the cage.

Stam et al. (1995) described computer analysis of the migrating motility complex of the small intestine recorded in freely moving rats. Myoelectric activity of the small intestine was recorded digitally in fasted, freely moving rats with multiple pairs of electrodes in the antimesenterial smooth muscle. A computer program was developed to distinguish the three characteristic phases of the migrating motility complex.

Fändriks (1993) measured duodenal wall motility, mucosal fluid transport, and alkaline secretion in anesthetized **cats**. A triple-lumen tube supplied with two small balloons was positioned via the esophagus in the duodenal lumen, and its distal end was led through a small incision in the antimesenteric border of the distal duodenum at the level of the ligament of Treitz. The most oral of the balloons was positioned immediately to the pylorus. After filling with air, the balloons occluded the lumen and isolated a 2-cm segment of the proximal duodenum. A double-lumen tube was inserted into the stomach for luminal perfusion.

Martinez et al. (1993) and Jimenez et al. (1994) studied gastrointestinal motility and coordination in **chickens**. The animals were chronically implanted with electrodes in the stomach, duodenum, ileum, ceca, and rectum.

Nakajima et al. (1996) used a telemetric device (supplied by Data Sciences International, Inc., St. Paul, MN) which can be implanted in the abdominal cavity of small animals. Gastric motility of freely moving rats could be continuously recorded for up to 60 days.

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Propulsive Gut Motility in Mice

Purpose and Rationale

The passage of a charcoal meal through the gastrointestinal tract in mice is used as a parameter for intestinal motility and to study the effect of laxatives.

Procedure

Groups of 10 female mice (e.g., NMRI strain) weighing 15 g are fed an oat diet for 3 days. Eighteen hours prior to the experiment, food, but not water, is withdrawn. The animals are treated either subcutaneously 15 min or orally 60 min before administration of the charcoal meal (0.2 ml of a 4 % suspension of charcoal in 2 % carboxymethylcellulose solution). The mice are sacrificed after various time intervals, 20, 40, 60, and 120 min. Ten animals serve as controls for each time interval. The entire intestine is immediately removed and immersed in 5 % formalin to halt peristalsis and then washed in running water. The distance the meal has traveled through the intestine as indicated by the charcoal is measured and expressed as percent of the total distance from the pylorus to the cecum.

Evaluation

Student's *t*-test is used to compare the control and the drug-treated group.

Critical Assessment of the Method

The charcoal passage test can be used for evaluation of laxative activity as well as for inhibition of intestinal motility.

Modifications of the Method

Instead of charcoal, unsubstituted Hostapermblau (CuPcB) suspended in gummi arabicum mucilage can be used.

Carmine red (15) suspended in a 1 % tragacanth solution was used for measurement of small intestine transit in **rats** (Leng-Peschlow 1986).

Miller et al. (1981) measured the intestinal transit in the rat by the use of radiochromium (^{51}Cr). Female Sprague Dawley rats weighing approximately 200 g were implanted with indwelling Silastic cannulae in the proximal duodenum. Following a 3-day recovery period, the animals were fasted for 18 h and then treated with the test compounds. Thirty min later, 0.2 ml of radiochromium (0.5 mCi $Na^{51}CrO_4$) was instilled into the small intestine via the indwelling Silastic cannula. Twenty-five min after chromium instillation, the animals were sacrificed. The small intestine was carefully removed and divided into 10 equal segments. The radioactivity was determined with an automatic gamma counting system. The effect of drugs could be quantified by determining the geometric center of the distribution of chromium through the small intestine.

Shook et al. (1989) used radiolabeled chromium to measure gastrointestinal transit in mice.

Megens et al. (1989) used the charcoal test to study the in vivo dissociation between the antipropulsive and antidiarrheal properties of opioids in rats.

Lish and Peters (1957) recommended an intestinal antipropulsive test in intact insulin-treated rats providing certain advantages over the commonly used charcoal meal test for screening of synthetic antispasmodic and antipropulsive agents.

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Nerve-Jejunum Preparation of the Rabbit

Purpose and Rationale

Perivascular nerve stimulation induces cessation of peristalsis of the rabbit jejunum (Finkelman 1930). Effects of test compounds on this phenomenon can be tested.

Procedure

Albino rabbits are sacrificed and the jejunal part of the gut prepared. The nerve lies in the mesentery along with the arterial blood supply. Nerve-jejunum preparations are suspended in an organ bath. The preparation is stimulated with pulses at a frequency of 20 Hz with 0.5 ms and about 5 V for 10 s at 3-min intervals. Test compounds are applied to the organ bath in a cumulative manner at 6-min intervals. Peristalsis movements for each period of 3 min between drug applications or of the period of cessation of peristalsis induced by nerve stimulation of the rabbit jejunum preparation are recorded. The effect of drugs on spontaneous peristalsis movement and on the cessation of peristalsis movement exerted by perivascular nerve stimulation is tested.

Evaluation

The areas of peristalsis movement for each period of 3 min are measured using a planimeter. The effects are expressed as percentage of change between controls and test compound treated preparations.

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Motility of Gastrointestinal Tract in Dogs

Purpose and Rationale

Intraluminal pressure and motility of the small intestine can be measured in unanesthetized dogs with balloon-catheter systems via a duodenal Mann and Bollman (1931) fistula according to Tasaka and Farrar (1976) or in the loop of a Thiry–Vella fistula (see below).

Procedure

Fistulas of the small intestine are created in male beagle dogs weighing 15–20 kg. The animals are anesthetized appropriately (e.g., isoflurane inhalation) and are fixed on an operation table. After shaving and careful disinfection of the skin, a midline incision is made. A 10–15-cm length of the ileum, approximately 15 cm proximal to the cecum, is excised. The remaining ileum is anastomosed end to end. The excised ileum is anastomosed, end to side, to the proximal or middle jejunum. Radiopaque tantalum markers are sutured to the serosa distal to this anastomosis in order to guide the direction for subsequent intubation. The other end is sutured to the skin. To create a skin ileostomy which does not shrink rapidly, a small amount of muscle, fascia, and subcutaneous tissue are excised from the abdominal wall.

For the measurement of the pressure inside the intestine, an air-filled system is employed. Air-filled latex balloons (Cementex), 5 mm in diameter, are attached to air-filled PE 190 polyethylene catheters (ID 1.19 mm) with a length of 120 cm. Three balloon-catheter pressure assemblies are tied together with the balloons 5 cm apart. The catheters are connected to Statham p23 db pressure transducers and to a polygraph. The transducers are rendered airtight by repeated applications of latex to all the connections.

The dogs are withheld from food but not from water 18 h prior to the experiment. The balloon-catheter assemblies are introduced through the fistula and secured in an appropriate position. The system is filled with air to a pressure of 10 mmHg. Similarly, balloon-catheter assemblies can be introduced into a Thiry–Vella fistula.

Intraluminal pressure is measured continuously; frequency and amplitude of pressure waves are recorded. After a period of 1 h, the test drug is administered orally or subcutaneously and the abovementioned parameters recorded for 10-min intervals.

Evaluation

For 10-min intervals, amplitude frequency (f_A), average degree and duration of amplitudes (A), and average pressure performance (PL_i) are calculated. Postdrug values are compared with predrug readings.

Modifications of the Method

Goldenberg and Burns (1973) reported on a technique using rubber balloon catheters inserted in the duodenum, ileum, or colon of dogs, secured by purse-string sutures and filled with water to record intraluminal pressure monitored by Statham pressure transducers connected to a polygraph. Furthermore, an antispasmodic agent can be tested for relaxation of morphine sulfate (0.3 mg/kg i.v.) induced spasms of the intestinal tract.

Fox et al. (1985) implanted chronically extraluminal strain-gauge force transducers on the serosal surface of the gastrointestinal tract of dogs.

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Thiry–Vella Fistula

Purpose and Rationale

As described first by Thiry (1864) and improved by Vella, a part of the jejunum is isolated, and the ends are exteriorized through the abdominal wall allowing to measure in vivo motility and function of the intestines in dogs and other species (Boldyreff 1925).

Procedure

Male beagle dogs weighing 15–20 kg are used. They are fasted 24 h preoperatively. General anesthesia is performed (e.g., isoflurane inhalation, ketamine/xylazine, etc.). The abdominal part is shaved with electric clippers and then with a razor. The skin is disinfected, e.g., with 70 % alcohol. Sterile drapes are applied to cover the whole operative field. A midline linea alba incision is made. A loop of the jejunum, about 70 cm in length, is separated leaving the blood supply through the mesenterium intact. Both distal and proximal ends are exteriorized through the abdominal wall and provided with stomata. An end-to-end jejuno-jejunal anastomosis is performed.

Evaluation

The preparation can be used to evaluate intestinal motility. A latex balloon connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB) is introduced through the proximal ostium. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of intestinal motor activity. Secretin inhibits motility of the small intestine dose-dependently.

Modifications of the Method

Sarr et al. (1981) and Bastidas et al. (1990) used **dogs** with Thiry–Vella loops to study jejunal absorption.

Bilchik et al. (1993) examined the effects of physiological doses of peptide YY in dogs with jejunal and ileal exteriorized, neurovascularly intact Thiry–Vella fistulas.

Ashton et al. (1996) created Thiry–Vella fistulas using a 20-cm segment of distal colon under general anesthesia in dogs. Colonic absorption of water and electrolytes was evaluated in awake, conscious animals.

Liu et al. (1996a) studied the effects of intravenous peptide YY on colonic water and electrolyte transport in awake dogs which had 20-cm neurovascularly intact colon Thiry–Vella fistulas.

In another study, Liu et al. (1996b) evaluated the effects of cholecystokinin and peptide YY on intestinal absorption of water and electrolytes using colonic, ileal, or jejunal fistulas in dogs.

These authors (Liu et al. 1995) also examined the effects of intraluminal administration of a new substituted peptide YY analogue on intestinal absorption of water and electrolytes in dog with jejunal, ileal, and colonic Thiry–Vella fistulas.

Barry et al. (1995) investigated the effect of luminally administered dopamine, the D_1-receptor agonist SKF 38393, the α_1-receptor antagonist terazosin, and the α_2-receptor antagonist yohimbine on ileal water and electrolyte transport in dogs with Thiry–Vella fistulas.

Walters et al. (1994) described the effect of a model of canine jejunoileal orthotopic autotransplantation on jejunal and ileal transport of water and electrolytes. For neurally intact jejunal loops, myoneural continuity between the loop and the proximal duodenum and jejunum was maintained by preserving a bridge of tunica muscularis devoid of mucosa between the proximal jejunum and the loop. All intrinsic neural and lymphatic continuity to the loop was carefully maintained by not transecting any of the mesentery to the loop.

For the autotransplanted jejunal loop, the dogs underwent construction of an identical loop with the muscular bridge after preparation of a jejunoileal autotransplantation. For this purpose, all neural, lymphatic, myogenous, and connective tissue connections with the jejunoileum were transected except for the fully isolated and stripped superior mesentery artery and vein at the base of the small bowel mesentery. The perivascular and adventitial tissues of these two vessels were carefully dissected away and transected under optical magnification. From here, the mesenteries to the distal duodenum and to the distal ileum were transected in a radial fashion, and the distal duodenum and distal ileum 5 cm from the ileocolonic junction were also transected. At this point, the jejunoileum was completely isolated from any neural/lymphatic continuity with the dog except for any neural/lymphatic elements traveling within the media of the mesenteric artery and vein.

Neurally intact and autotransplanted ileal loops were prepared in a similar way.

Remie et al. (1990) described in detail the preparation of a Thiry–Vella loop in the **rat**. After laparotomy the segment to be isolated has to be located carefully. The segment should be vascularized by two or more tributary arteries. For ligation of the arcade, two ligatures are placed around the blood vessels 2–3 mm from each other. The two ligatures are tightened, the gut wall is disinfected with iodine solution, and both the vessels of the arcade and the gut wall are cut. The same procedure is performed at the other end of the segment to be isolated. This segment is laid on gauze moistened with warm saline avoiding torsion of the vessels. For end-to-end anastomosis, the remaining parts of the gut are approximated and two corner sutures are placed. A continuous suture with transfixing stitches is placed on the anterior and posterior wall. Using

a pair of sharp scissors, two holes are made in the abdominal wall and the skin. Subsequently, a standard end-to-side anastomosis technique is used to sew the gut to the internal abdominal muscle. The two stay sutures are at 180° and the posterior wall is sutured first. Following this, the skin is sutured to the abdominal muscle, using a running suture.

Chu et al. (1995) used Thiry–Vella fistulas of either the jejunum or ileum in rats in order to determine whether the trophic effects of bombesin on the small bowel mucosa are mediated by nonluminal factors or endogenous luminal secretion.

Bárdos and Nagy (1995) prepared double Thiry–Vella fistulas in rats. The first Thiry–Vella loop was created from the lower duodenum. After 1 month or more, a similar fistula was prepared from the upper part of the colon. The two loops were positioned along the midline and formed a line of four openings of the abdominal wall providing an easy access for inserting stimulatory devices to either of the loops in the same animal. A rubber balloon made of latex rubber was tied to a silicon rubber tube, inserted into the isolated loop via one of its orifices, and then fixed to the body by a tape. Stimulation was performed by injection of various volumes of water into the balloon from an attached syringe. Behavioral reactions of the animals were recorded as scores during 10-s stimulation periods.

In order to purify the putative luminal cholecystokinin-releasing factor, Spannagel et al. (1996) collected intestinal secretions by perfusing a modified Thiry–Vella fistula of jejunum in conscious rats.

Snoj et al. (1992) found that phosphatidylcholine inhibited postoperative adhesions after small bowel anastomosis in the rat.

Gianotti and Tchervenkov (1992) used a Thiry–Vella loop in **guinea pigs** to study the stimulatory effect of intraluminal nutriment on burned guinea pig intestinal mucosa.

Philpott et al. (1993) created Thiry–Vella loops in 21-week-old **rabbits** in order to study the influence of luminal factors on intestinal repair during refeeding of malnourished infant rabbits.

Silbart et al. (1996) examined the ability of several bacterial endotoxins and their subunits to act as adjuvants or carrier proteins in stimulating an intestinal secretory Ig4 response to 2-acetylaminofluorene using Thiry–Vella loops in rabbits.

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Continuous Recording of Mechanical and Electrical Activity in the Intestine of Conscious Dogs

Purpose and Rationale

Cyclic motor activity occurring in almost all parts of the gastrointestinal tract is due to a migrating electric complex (Szurszewski 1969; Fioramonti et al. 1980; Sarna and Condon 1984; Sarna et al. 1984; Sarna 1985). Smooth muscle cells of the gut show periodic oscillations of membrane potentials called electrically controlled activity; they are also named slow waves, basic electrical rhythm, or pacesetter potentials. If the membrane potential depolarizes beyond a certain threshold during such an oscillation, the smooth muscle contracts. This is usually associated with a rapid burst of electrical oscillations, called electrical response activities or spikes. Electrical response activity is, therefore, associated with contractions on a 1:1 basis.

Implantation of extraluminal force transducers allows the monitoring of contractile activity in

conscious animals (Bass and Wiley 1972; Ormsbee and Bass 1976; Ormsbee et al. 1981). The data can be analyzed by a computer (Ehrlein and Hiesinger 1982; Schemann et al. 1985). The effects of opiate agonists, morphine, natural enkephalins, endorphins, and synthetic enkephalins and opiate antagonists, like nalorphine, as well as other gastrointestinal hormones, such as motilin, can be studied (Bickel and Belz 1985, 1988; Bickel et al. 1985).

Procedure

Male beagle dogs weighing 15–20 kg are anesthetized after premedication appropriately (e.g., by isoflurane inhalation). Under aseptic conditions, several miniaturized strain-gauge force transducers are sutured onto the muscular layers of the gastrointestinal tract. Before implantation, the transducers are externally calibrated giving linear signals over a range from 10 to 300 mN. Each transducer has its recording axis perpendicular to the longitudinal axis of the intestine to record contractions of the circular smooth muscles. The proximal ends of the strain-gauge transducers are fitted to a plug embedded in a stainless-steel cannula implanted into the abdominal wall. The signals are continuously recorded and stored online in a computer, allowing later analysis of the data.

For measuring electrical activity, bipolar electrodes are implanted at several parts of the intestinal tract. The lead wires from the electrodes are externalized via a stainless-steel cannula. Electromyogram is recorded on an electroencephalograph.

The animals are kept in a standardized environment with one daily feeding at 9:00 a.m. The dogs are trained to stand in a Pavlov stand. Eighteen hours prior to the beginning of the experiment, the animals are fasted with access to water. Motility data and electrical activity are first recorded for fasting dogs without drug treatment. Then the drug is injected intravenously during the period of quiescence of the migrating motor complex.

Evaluation

Recordings are analyzed for duration of cycles (min) and motor complexes (min), mean height (mN), and frequency (*n*/min). The length of the cycles is measured from the beginning of one complex to the beginning of the next complex. Electromyogram is analyzed for slow waves (cycles/min), maximum amplitude of the spike bursts (mV), duration of the spike bursts (ms), and duration of the effect (min). The data before and after administration of various doses of the test compound are compared by statistical means (Student's *t*-test).

Modifications of the Method

Itoh et al. (1977) and Nagakura et al. (1996) used extraluminal force transducers for recording contractile motility of the gastrointestinal smooth muscle in conscious dogs.

Nakada (1995) recorded gastrointestinal and gallbladder contractions in conscious dogs by chronically implanted strain-gauge transducers and gallbladder volume changes by a chronically indwelling gallbladder catheter.

Orihata and Sarna (1994) investigated contractile mechanism of gastroprokinetic agents in conscious dogs. The spatial and temporal parameters of gastric, pyloric, and duodenal contractions during the entire period of gastroduodenal emptying, during a 60-min period of drug infusion, and during the post-drug-infusion period were analyzed by a computer method.

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Absorption

Everted Sac Technique

Purpose and Rationale

The everted sac technique is used to study the transport of substances from the mucosal to the serosal surface (Wilson and Wiseman 1954).

Procedure

Male Wistar rats weighing 150–220 g are euthanized by an appropriate method. The small intestine from the ligament of Treitz to the ileocecal junction with a length of 6–7 cm is rapidly removed and everted with a glass rod. The sac is securely ligated at both ends and filled with Krebs–Henseleit bicarbonate buffer solution (pH 7.4) containing 0.4 % glucose and pre-gassed with 95 % O_2/5 % CO_2. The sac is incubated at 37 °C in a glass vessel containing the same buffer solution and gassed with 95 % O_2/5 % CO_2.

After 5 min, the drug solution is added to the glass vessel and the preparation is further incubated. After given time intervals, the fluid from the everted sac is removed and the volume determined. The concentration of the drug transported from the mucosal to the serosal side is determined by appropriate analytical methods.

Evaluation

Determinations of the drug in the everted sac at different time intervals allow evaluation of pharmacokinetic parameters.

Modifications of the Method

Madar (1983) used the small intestine everted sac of **chicken** for demonstration of amino acid and glucose transport.

Harnett et al. (1989) used everted segments of the distal ileum of **rats** to study taurocholate absorption.

Turner et al. (1990) used everted sacs of the ileum in **sheep** to study selenate and selenite absorption.

Goerg et al. (1992) used the stripped descending colon of the **rat** as everted sac to study the inhibition of neuronally mediated secretion in the rat colonic mucosa by prostaglandin D_2.

Under ether anesthesia, the descending colon is removed and transferred into ice-cold bathing solution. The colon is placed on a plastic rod. After a circular incision with a blunt scalpel, the serosa and muscularis propria are stripped away, leaving the mucosal–submucosal preparation consisting of the mucosa, the muscularis mucosae, and a part of the submucosa with the completely preserved submucosal plexus. The tubelike mucosal–submucosal preparation is mounted as an everted sac in a holding apparatus (volume of the outer compartment 25 ml). The potential difference between the outer mucosal side and inner serosal side is measured by two agar bridges. The tissue is short-circuited by a voltage clamp after correcting for the offset potential and compensating for the solution resistance.

Moreover, transmural ion fluxes can be determined with the everted sac technique. Net fluxes of sodium, chloride, and potassium are studied by direct measurements of volume changes and changes of electrolyte concentrations in the inner volume of the everted sacs. Unidirectional fluxes can be determined simultaneously with the direct measurement of net fluxes by adding the isotopes $^{22}Na^+$ and $^{36}Cl^-$ to one side of the everted sac.

Schilling and Mitra (1990) used the everted gut technique in rats to study enteral insulin absorption.

Mizuma et al. (1993) studied the active absorption in the intestine and metabolism of the β- and α-anomers of the glucoside and galactoside of p-nitrophenol to find a more suitable prodrug for poorly absorbed drugs. The everted sac technique was used to investigate the intestinal absorption of these glycosides from the mucosal to the serosal side of the rat jejunum.

Kitagawa et al. (1996) investigated the influence of various factors, such as temperature, on the absorption of methochlorpromazine in the small intestinal everted sac of rats.

Tanaka et al. (1996) estimated the transport characteristics of thyrotropin-releasing hormone (TRH) and its chemically modified derivative with lauric acid (Lau-TRH) across the rat small or large intestine by means of an in vitro everted sac experiment.

Sasaki et al. (1995) studied the absorption characteristics of azetirelin, a new thyrotropin-releasing hormone analogue in rats by means of in situ closed-loop and in vitro everted sac experiments.

Tuskulkao et al. (1995) examined the effects of stevioside (a natural nonnutritive sweetening agent) and of steviol (a product of enzymatic hydrolysis of stevioside) on intestinal glucose absorption in the hamster jejunum using the everted sac technique.

Motozono et al. (1994) determined the effect of age on gastrointestinal absorption of tobramycin in suckling, weanling, and adult rats by an everted sac method.

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Stomach Emptying and Intestinal Absorption in Rats

Purpose and Rationale

Reynell and Spray (1956) described a method for the simultaneous measurements of gastric emptying, intestinal transit, and absorption of test substances in the rat using phenol red as marker.

Procedure

Adult male Wistar rats weighing 200–300 g are allowed water but are deprived of food 24 h before the experiment. They are treated orally or subcutaneously with the test compound 15 min prior to oral administration by gavage of 1.5 ml 0.07 % phenol red in 2 % carboxymethylcellulose solution. Fifteen min later, the animal is sacrificed and the stomach is immediately removed. The whole stomach including the stomach content is alkalized with 1 N NaOH and homogenized. The homogenate is filtered and after precipitation of the protein with 10 % trichloroacetic acid centrifuged for 15 min at 3,000 rpm. The concentration of phenol red in the supernatant is measured colorimetrically in a photometer at 546 nm.

Evaluation

Percentage of stomach emptying (S_e) is calculated according to the following formula:

$$S_e = 100 - P_s \times P_a^{-1} \times 100$$

where:

- P_s = concentration of phenol red in the stomach (μg/ml)
- P_a = concentration of phenol red in the initial solution after addition of equal volumes of 1 N NaOH and trichloroacetic acid (μg/ml)

Modifications of the Method

By selective ligations of the different parts of the gastrointestinal tract and analysis for phenol red contents and for drug substance, as well as by sacrificing the animals after various time intervals, the degree and time course of absorption can be studied.

Droppleman et al. (1980) described a simplified method for assessing drug effects on gastric emptying in **rats**. Three ml of a semisolid test meal, based on methylcellulose, is given to rats fasted 24 h prior to the experiment. At a specified time following the test meal, the rats are sacrificed

and laparotomized, and the stomachs removed. The full stomachs are weighed on an analytical balance; they are opened and rinsed. Excess moisture is removed and the empty stomach weighed again. The difference is subtracted from the weight of 3 ml of the test meal, indicating the quantity emptied from the stomach during the test period. Gastric motor stimulants, e.g., metoclopramide, increase and anticholinergic compounds decrease gastric emptying.

Megens et al. (1990) used phenol red as marker to measure gastrointestinal propulsion after castor oil or paraffin oil challenge in **rats**.

Hegde et al. (1995) studied 5-HT_4 receptor-mediated stimulation of gastric emptying in rats using a specially prepared semisolid test meal containing charcoal.

Bonnafous et al. (1995) investigated benzodiazepine withdrawal-induced gastric emptying disturbances in rats. Male Wistar rats, weighing 200–250 g, fasted for 16 h, received by gavage 2 ml of a test meal containing 1 μCi/ml of ^{51}Cr sodium chromate, 15 min after drug administration. Thirty min later, the animals were sacrificed by cervical dislocation. The stomach, the small intestine (10 segments), and the colon were excised and placed into tubes. Radioactivity was determined by placing the tubes in a gamma counter. Gastric emptying was calculated as the percentage of total counts found in the small intestine and the colon.

Varga et al. (1995) determined gastric emptying in rats 5 min after a 3-ml intragastric load of 0.9 % NaCl using phenol red as marker in order to define which bombesin receptors are involved in the delay of gastric emptying by bombesin-like peptides.

Lasheras et al. (1996) studied gastric emptying in rats. Sixty min after oral administration of vehicle or test compounds, the rats received by gavage 40 steel spheroids (1-mm diameter) in 2 ml 3 % carboxymethylcellulose. Sixty min later, the animals were sacrificed and the spheroids remaining inside the stomach counted.

Yegen et al. (1996) studied the inhibitory effects of gastrin-releasing peptide on gastric emptying in rats using methylcellulose and phenol red as nonabsorbable marker.

Haga et al. (1994) studied gastric emptying in **mice**. Male mice, weighing 18–22 g, had free access to food and water before the experiment. The test compounds were administered orally in 10 ml/kg 0.5 % methylcellulose solution. The mice were deprived of food and water and sacrificed 4 h later by cervical dislocation. The stomachs were removed and opened. The contents of the stomach were mixed with 10 % trichloroacetic acid and centrifuged at 3,000 rpm for 30 min. The weight of the sediment was taken as the food remaining in the stomach.

Ding and Håkanson (1996) examined the effect of drugs on a cholecystokinin-A receptor-mediated response by gastric emptying of a charcoal meal in mice.

Costall et al. (1987) used the **guinea pig** to study the influence of a 5-HT_3 antagonist on gastric emptying.

Brighton et al. (1987) used scintigraphy following indium-111-labeled meals in beagle **dogs** and **baboons**. Indium-111-labeled polystyrene beads (500 mCi per dog) were mixed into a meal consisting of 50 g of finely crushed commercial dog food and 50 ml of milk. Images of 1-min duration were taken every 5 min for a period of 1 h using a large field of view gamma camera (ON Sigma 410).

Gullikson et al. (1991, 1993) studied gastric emptying of a solid meal in dogs.

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Intestinal Drug Absorption

Purpose and Rationale

Doluisio et al. (1969) described an in situ rat gut technique to determine absorption rates of drugs.

Procedure

Male Sprague Dawley albino rats weighing 220–260 g are fasted 16–24 h prior to surgery allowing, however, drinking water ad libitum. They are kept in cages having wide mesh floors to prevent coprophagia. The animals are anesthetized by using an appropriate method. The small intestine is exposed by a midline abdominal incision, and two L-shaped glass cannulae are inserted through small slits at the duodenal and ileal ends. Care has to be taken to handle the small intestine gently in order to maintain an intact blood flow. The cannulae are secured by ligation with silk suture, and the intestine is returned to the abdominal cavity. Four-cm-long segments of tubings are attached to the exposed ends of both cannulae, and a 30-ml hypodermic syringe fitted with a three-way stopcock and containing perfusion fluid warmed to 37 °C is attached to the duodenal cannula. As a means of clearing the gut, perfusion fluid is passed slowly through it and out the ileal cannula to be discarded until the effluent is clear. The remaining perfusion solution is carefully expelled from the intestine by means of air pumped through from the syringe.

Immediately afterwards, 10 ml of drug solution are introduced into the intestine by means of a syringe. The stopwatch is started, and the ileal

cannula is connected to another 30-ml syringe fitted with a three-way stopcock. This arrangement enables the operator to pump the lumen solution into either the ileal or the duodenal syringe, remove 0.1-ml aliquots, and return the remaining solution to the intestine within 10–15 s. To assure uniform drug solution concentrations throughout the gut segment, aliquots are removed from the syringes alternatively. Samples are collected every 5 min. Depending on the drug to be studied, the concentration is determined by chemical methods.

Evaluation

The concentrations of the drug are plotted on a logarithmic scale on the ordinate versus time on the abscissa on a linear scale. Half-life values can be calculated.

Modifications of the Method

Ochsenfahrt (1979) described a more sophisticated method to measure the absorption of drugs in the vascularly perfused, isolated intestine of the rat. Male rats weighing about 350 g are anesthetized with urethane. The abdomen is opened by a midline incision. The mesentery of the ascending and transverse colon is gently pulled away from the mesentery of the small intestine. The vessels to the colon are ligated and cut. The superior mesenteric artery is freed from the surrounding mesentery. The duodenal vessels are ligated and cut. A suitable segment of the jejunum of about 7-cm length is selected. The proximal cannula is tied into the lumen of this segment, and the duodenum is cut. The lumen of the segment is washed with 3–4 ml warm Krebs–Henseleit solution. Then, the distal cannula is tied. After the mesenteric vessels distal to the experimental segment are ligated, the rest of the small intestine, ileum, and colon are excised. Temporarily, the intestine is covered with gauze soaked with saline solution.

The preparation of the rat is interrupted for 8–10 min, while a second rat (donor rat), which has been anesthetized with urethane, is prepared. The donor rat supplies the arterial blood; the spontaneous respiration is supported with a respiration pump through a tracheal cannula. The right jugular vein of the donor rat is cannulated for the blood infusion and 2 mg heparin dissolved in 0.1 saline solution is administered. A cannula is placed into the left carotid artery of the donor rat; this tube is later connected to the superior mesenteric artery of the test segment. Blood lost by the donor rat is replaced with heparinized blood taken from other rats immediately before the beginning of the experiment.

In the test animal, a thin silicon tube is inserted in the left carotid artery. Two mg heparin, dissolved in 0.1 ml saline, is injected. The superior mesenteric vein is ligated distal to the splenic vein and a plastic cannula is inserted for venous outflow. The cannula is then connected to a drop counter. The luminal cannulae of the segment are connected to a recirculation unit which is filled with Krebs–Henseleit solution at 37 °C, and the luminal perfusion of the segment (mucosal solution) is started. The mucosal solution is oxygenated and recirculated. The superior mesenteric artery is ligated with a thread at its origin at the aorta and then cannulated. This cannula is first connected to the carotid artery of the test animal and later to the carotid artery of the donor animal. The isolated vascularly perfused segment is suspended in a serosal bath containing Krebs–Henseleit solution at 37 °C. Venous blood outflow is collected in plastic vials at 15-min intervals. Samples are taken from the mucosal and serosal solutions at the beginning and the end of each period. By this way, the mucosal disappearance rate, the venous appearance rate, and the serosal appearance rate can be measured.

Schilling and Mitra (1992) measured insulin absorption from the distal duodenum/proximal jejunum and from the distal jejunum/proximal ileum in anesthetized rats by the closed-loop technique.

Schümann and Hunder (1996) described a modified device for the differentiated study of intestinal transfer in isolated intestinal segments from mice and suckling rats in vitro. A luminal perfusion system for small intestinal segments was adapted for the use in mice and rat pups to investigate longitudinal differences in drug and toxin transfer.

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Duodenal Ulcer Formation

Cysteamine-Induced Duodenal Ulcers in Rats

Purpose and Rationale

Duodenal ulcers can be induced in rats by repeated administration of cysteamine or propionitrile in rats (Dzan et al. 1975; Szabo 1978; Szabo et al. 1979).

Procedure

Male Sprague Dawley rats with an initial weight of 200 g are used. Cysteamine HCl is administered three times on day 1 in a dose of 280 mg/kg orally. Protective drugs, such as H_2 antagonists, are given 30 min prior to cysteamine treatment. The rats are sacrificed on the third day. For histological evaluation, the stomach and duodenum are fixed in 10 % aqueous buffered formaldehyde and paraffin-embedded sections are stained with hematoxylin and eosin. Duodenal ulcers develop in the anterior (antimesenteric) and posterior wall of the proximal duodenum, about 2–4 mm from the pylorus. The more severe ulcers, located on the anterior wall, frequently perforate, resulting in focal or generalized peritonitis, or penetrate into the liver. The opposite ulcer invariably penetrates into the pancreas.

Evaluation

The intensity of the duodenal ulcer is evaluated using scores from 0 to 3:

- 0 = no ulcer
- 1 = superficial mucosal erosion
- 2 = deep ulcer usually with transmural necrosis
- 3 = perforated or penetrated ulcer

Critical Assessment of the Method

In view of the development of modern gastric K^+/H^+-ATPase inhibitors, the predictive value of methods using experimental ulcers in the rat for clinical healing rates in man has been challenged (Herling and Weidmann 1994).

Modifications of the Method

The cysteamine-induced duodenal ulcer has been used for pharmacological studies by many authors, e.g., Evangelista et al. (1992), Krantis et al. (1993), Pascaud et al. (1993), Tanaka et al. (1993), Morimoto et al. (1994), Pendley et al. (1995), Sikiric et al. (1997), and Drago et al. (1999).

Okabe et al. (1971) and Satoh et al. (1989) described a method for experimental, penetrating gastric, and duodenal ulcers in rats. Rats were anesthetized with ether and an incision was made in the abdomen. A round metal mold, 6 mm in diameter, was placed in close contact with the serosal surface of the duodenal wall, about 7 mm distal to the pylorus. **Glacial acetic acid** (60 ml) was poured into the mold and was left in place for 20 s. After the acetic acid was removed, the treated surface was rinsed with 50 ml of 0.02 N NaOH and the abdomen was closed. A drug or the vehicle was given p.o. once a day for 14 consecutive days beginning 2 days after the operation. The animals were sacrificed on the 16th day after the operation and the ulcerated area (mm^2) was measured.

Mepirizole-induced duodenal ulcers were described by Okabe et al. (1982), Satoh et al. (1989), and Tanaka et al. (1989). A drug or

the vehicle was given p.o. 30 min before mepirizole (200 mg/kg) was administered s.c. Twenty-four hours later, 1 ml of 0.5 % Evans blue solution was injected via the tail vein of each rat and the rats were sacrificed by CO_2 asphyxiation. The gastroduodenal region was removed and examined for lesions.

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Models of Inflammatory Gut Disease

Experimental Ileitis

Purpose and Rationale

An experimental model of inflammatory bowel disease produced by the intraluminal administration of the cytotoxic plant lectin ricin into the rabbit ileum was developed by Sjogren et al. (1994), Goldhill et al. (1995, 1997). In vitro, electric field stimulation results in larger non-cholinergic excitatory junction potentials in ricin-treated circular muscles than in controls.

Procedure

Inflammation

Acute ileitis is induced with ricin in male New Zealand white rabbits. The animals are anesthetized with intramuscular xylazine (9 mg/kg) and ketamine (50 mg/kg) and maintained with intravenous pentobarbital (15 mg/kg). A midline incision is made, a ligated terminal ileal loop (~10 cm in length) is constructed in each animal, and 1 ml of ricin (1 mg/ml) or vehicle is injected into the lumen. The loop is removed after 5 h, a time period that allows the development of ileitis, abnormal myoelectric activity, and increased response to electric field stimulation. Animals are sacrificed with an overdose of pentobarbital. The loop is opened along its length, gently flushed of luminal contents with cold oxygenated Krebs bicarbonate-saline and prepared for contractility studies.

Contractility Studies

Muscle strips (~1 × 0.4 cm) with mucosa removed are cut in the axis of the circular muscle and attached to isometric tension transducers in 10-ml organ baths with modified oxygenated Krebs bicarbonate-saline at 37.5 ± 0.5 °C. Tissues are allowed to equilibrate at L_i (the length at which no tension can be measured) for 20 min. The strips are then stretched to L_0, which is determined as the length at which maximum force is generated in response to acetylcholine (0.5–1 mM). Strips are then allowed to equilibrate for an additional 20 min.

Effect of Inflammation on Response to Tachykinins

Concentration–response curves are constructed to substance P and neurokinin agonists (or analogues) on separate muscle strips in vehicle- and ricin-treated tissues. Studies are performed in the presence or absence of tetrodotoxin to distinguish between neural and nonneural effects of ricin treatment.

Effects of Ricin Treatment to Responses to Electrical Field Stimulation

Muscle strips are passed through a pair of ring electrodes (2-mm diameter) and stimulated for 10 s by square-wave pulses (0.5-ms duration, supramaximal voltage) at 1–10 Hz. Stimulation is performed in the presence of atropine (1 mM) and N^G-nitro-L-arginine methyl ester) (L-NAME) (0.1 mM).

Evaluation

Maximum increases in muscle tone in response to tachykinin addition of electrical field stimulation are obtained through visual analysis of chart recorder outputs. Responses to tachykinins are expressed as absolute tension development. Electrical field stimulation data are expressed as a percentage of the response to 1 μM acetylcholine added at L_0 to reduce the variation of the data. Values are given as mean ± SEM. Tachykinin concentration responses are fitted to sigmoid curves and EC_{50} values (with 95 % confidence intervals) are determined from these curves. Differences between frequency or concentration–response

curves are assessed statistically by multivariate analysis of variance, with adjustments made for multiple comparisons. In cases in which curves are significantly different to one another, maximal responses were compared statistically using Student's *t*-test.

Modifications of the Method

Miller et al. (1991) induced ileitis in **rabbits** by luminal perfusion with histamine monochloramine or with acetic acid.

Rachmilewitz et al. (1997) induced inflammation in the small intestine in **rats** by intrajejunal administration of 0.1 ml 2 % iodoacetamide.

Sukumar et al. (1997) induced ileitis in rats by two doses of indomethacin (7.5 mg/kg) administered subcutaneously 24 h apart.

Ileitis in **guinea pigs** was induced by intraluminal trinitrobenzene sulfonic acid (Miller et al. 1993; Izzo et al. 1998; Mazelin et al. 1998).

Likewise, ileitis in **hamsters** was induced by intraluminal injection of trinitrobenzene sulfonic acid (Boyd et al. 1995).

Shibata et al. (1993) induced ileitis in **dogs** by administration of 10 ml 100 % ethanol and 1 g trinitrobenzene sulfonic acid dissolved in 10 ml water through a tube inserted into the ileum.

Interleukin-10 (IL-10) is produced by T cells, B cells, and macrophages. It downregulates the function of T helper (Th)-1 cells, natural killer (NK) cells, and macrophages. In 1993, Kuhn et al. reported that, in IL-$10^{-/-}$ mice, inflammation occurred in the whole intestine. The lesions were observed mainly in the duodenum, proximal jejunum, and ascending colon. Pathological thickening of the intestinal wall, due to hyperplastic changes, was observed in the duodenum and jejunum. In the colon, goblet cell depletion, degeneration of the epithelium, infiltration of IgA-producing plasma cells, and an increase in major histocompatibility complex (MHC) class II expression were detected. As in the IL-$2^{-/-}$ mice, the activation of $CD4^{+}$ cells and the depletion of their inhibitor, the regulatory T cells, are presumed to be the cause of the inflammation. In 2000, Jijon et al. assessed the mechanism behind a poly(ADP-ribose) polymerase (PARP)-induced increase in epithelial permeability that is associated with chronic, non-resolving colitis, which develops spontaneously in the IL-10-gene-deficient mouse. They demonstrated that treatment of IL-10-deficient mice with the PARP inhibitor 3-aminobenzamide reversed the typical signs of Crohn's disease in these mice, namely, increased permeability, high levels of mucosal interferon-α (IFN-α) and tumor necrosis factor α (TNF-α), and increased nitric oxide (NO) production. In the same year, Cantorna et al. (2000) used the IL-10-deficient mouse model to investigate the role of vitamin D on the course of inflammatory bowel disease (IBD). The animals were divided into three groups: vitamin D deficient, vitamin D sufficient, and active vitamin D supplemented. In contrast with vitamin D-deficient IL-10 knockout (KO) mice, vitamin D-sufficient mice did not develop diarrhea, waste, or die prematurely. To the authors' surprise, supplementation with active vitamin D for only 2 weeks blocked the progression and significantly ameliorated the symptoms in the mice that had established spontaneously developed IBD. The importance of animal models for the evaluation of pharmacological strategies was further emphasized in a straightforward ministudy conducted by Gratz et al. (2002), who administered murine monoclonal anti-TNF-α antibodies intraperitoneally into IL-10 KO mice that had established IBD. Demonstration of significant histological improvement of inflammation that correlated well with a resolution of diarrhea and rectal bleeding accentuated the pivotal role that TNF-α appears to play in the pathogenesis of IBD and emphasized the importance of this model. Recently, a pioneer therapeutic approach to IBD was tested in the IL-10 KO model by Watanabe et al. (2003a, b). The group developed poly-DL-lactic acid microspheres containing dichloromethylene diphosphonate that, once administered rectally, were specifically taken up by macrophages, subsequently depleting them. The authors showed reduced numbers of resident macrophages in the intestinal lymphoid follicles in this model, associated with suppression of the development of chronic colitis.

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Experimental Colitis

Purpose and Rationale

Inflammatory bowel diseases, ulcerative colitis, and Crohn's disease represent chronic alteration of the gastrointestinal tract of unknown etiology perhaps involving immunological events. The immunological parameters have been described as secondary but may possibly be attributed to the chronicity of the disease. Several compounds have been described to elicit cell-mediated

immune responses in the gut, such as dinitrochlorobenzene (DNCB) (Rosenberg and Fischer 1964; Norris et al. 1982; Norris 1989) or 2,4,6,-trinitrobenzene sulfonic acid (TNBS) (Morris et al. 1989; Selve and Wöhrmann 1992).

Procedure

A three-step concept is realized to mimic the human disease, using 2,4,6-trinitrobenzene sulfonic acid (TNBS) as a defined hapten:

1. Specific hypersensitivity by active immunization
2. Local inflammation by local challenge
3. Chronicity by chronic application of the immunogen

Female Sprague Dawley rats weighing 150–200 g are sensitized by intradermal injection of 0.8 % TNBS (Fluka, Neu-Ulm, Germany) solution into a shaved area on the back once daily for three consecutive days. TNBS is dissolved in 0.05 ml Freund's incomplete adjuvant together with 1 mg/ml ovalbumin. After 18 days, the animals receive a further intradermal booster injection. Intradermal challenge of 0.08 % TNBS in 0.05 ml 0.9 % NaCl solution with or without ovalbumin, or ovalbumin solution without TNBS, is given 14 days later in order to determine the type and specificity of the immunological reaction.

Ten days after the intradermal challenge, a flexible polyethylene tube of 0.5-mm diameter is implanted under ketamine (100 mg/kg i.p.) anesthesia 15 cm proximal to the cecum and emerging at the neck for TNBS or drug administration. After a 10-day recovery phase, the animals are treated daily for 3 weeks with 0.08 % TNBS in saline (0.2 mg/rat) given through the catheter. Control groups receive only saline. Drugs are applied either by gavage twice a day, suspended in carboxymethylcellulose, or intraluminally once a day, suspended in saline. The animals are sacrificed by CO_2 inhalation 24 h after the last intraluminal application of TNBS. The distal 10 cm of the small intestine anterior to the ileo-ceco-colic junction (5-cm distance to the open end of the catheter) including Peyer's patches is dissected, cut open longitudinally, and rinsed with saline.

Immediately after dissection, the distal small intestine is visually assessed for inflammation according to the following scores:

Enteritis score	Gross morphology
0	No visible damage of the whole 10 cm of the small intestine
1	Slight inflammation, slight redness (hyperemia), villi visible under 15-fold magnification
2	Intermediate inflammation, discontinuous hyperemia, intermediate redness of villi
3	Intensive inflammation, intensive hyperemia, intensive redness of villi

The dissected gut segments, precisely 10 cm long, are weighed for measurement of edema formation. They are then incubated in Tris buffer for 30 min at 37 °C in a shaking water bath (1 ml/100 mg tissue, Tris 50 mM, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml glucose). Following incubation, aliquots of the solutions are centrifuged (13,000 *g*, 2 min, 20 °C) for determination of leukotriene B_4 (TB_4) by a commercial radioimmunoassay (e.g., Amersham Buchler, Brunswick).

Evaluation

Results are expressed as means ± SEM of (*n*) experiments. Differences between control and inflamed tissue and influence of drug treatment are compared. Statistical significance is calculated by Wilcoxon–Mann–Whitney *U*-test for unpaired data. The level of significance is taken as $p < 0.05$.

Modifications of the Method

Several authors used the trinitrobenzene sulfonic acid model in *rats* with slight modifications (Hogaboam et al. 1996; Kitano et al. 1996; Yue et al. 1996; Lora et al. 1997; Taniguchi et al. 1997; Cruz et al. 1998; Fries et al. 1998; Goldhill et al. 1998).

Zea-Ariarte et al. (1994) studied chronic colitis in Wistar rats after intracolonic instillation of 20 or 42 mg of trinitrobenzene sulfonic acid in 30 % and 40 % ethanol and compared the effect with the administration of ethanol alone.

Alternatives to the 2,4,6-trinitrobenzene sulfonic acid model in **rats** were proposed:

Wallace et al. (1995) and Hawkins et al. (1997) recommended dinitrobenzene sulfonic acid to produce experimental colitis in the rat.

Patterson and Colony (1983) tested sulfasalazine and 5-aminosalicylic acid in experimental colitis induced in **guinea pigs** by topical dinitrochlorobenzene.

Neurath et al. (1996) studied chronic intestinal inflammation induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in **mice**.

These authors (Neurath et al. 1997) investigated the role of tumor necrosis factor in this mouse model and showed that no significant TNBS-induced colitis could be induced in mice in which the TNF-alpha gene had been inactivated by homologous recombination.

Cuzzocrea et al. (2004) found that 5-aminoisoquinolinone reduces colon injury in experimental colitis induced by dinitrobenzene sulfonic acid in mice.

Antony et al. (1997) and Oyen et al. (1997) used trinitrobenzene sulfonic acid to induce experimental colitis in **rabbits**.

Damms et al. (1998) compared gamma-camera imaging in rabbits with colitis after retrograde instillation of trinitrobenzene sulfonic acid with technetium-99 m-labeled liposomes after various time intervals with macroscopically scored severity of inflammation.

A model of diffuse colitis in **rats** induced by intraluminal colonic instillation or serosal application of dilute **acetic acid** was described by MacPherson and Pfeiffer (1976, 1978). Ritzpatrick et al. (1990) tested the anti-inflammatory effects of various drugs on acetic acid-induced colitis in the rat.

For instillation into the colon of rats, a 2-cm length of soft polyethylene tubing is fitted to a Luer-Stub adapter. The open end of the tubing is sealed with glue. The whole length of the tubing is perforated with a needle at 0.5-mm length by four holes, 90° apart. A 1-ml syringe containing 10 % acetic acid in saline is fitted to the adapter. The tubing is inserted intrarectally into the colon of rats and 0.2 ml is injected into the lumen. After 10 s contact in situ, the remaining acid is withdrawn and the lumen washed with three successive 0.5-ml volumes of isotonic saline. Test drugs are administered daily during the following days. Diffuse colonic lesions appear after 3 days in control animals and the animals experience a bloody diarrhea. An initial mucosal inflammation develops into submucosal edema; petechial hemorrhages become enlarged with subsequent neutrophil invasion, and pseudopolyps become evident.

Terzioglu et al. (1997) studied the effect of prostaglandin E_1 on the experimental colitis induced by rectal instillation of 10 % acetic acid in rats.

Fabia et al. (1994) induced colitis in rats in an exteriorized colonic segment by administration of 4 % acetic acid for 15 s. Four days later, this colonic segment was examined using a morphological scoring system and measurements of myeloperoxidase activity and plasma exudation into the colonic segment.

Eliakim et al. (1995) demonstrated that ketotifen ameliorated capsaicin-augmented acetic acid-induced colitis. Rats were pretreated with subcutaneous injections of 20, 30, and 50 mg/kg capsaicin. Colitis was induced 2 weeks later by flushing 2 ml 5 % acetic acid into the proximal colon.

Higa et al. (1997) studied the role of neutrophils in the pathogenesis of acetic acid-induced colitis in mice.

Millar et al. (1996) evaluated the antioxidant potential of new treatments for inflammatory bowel disease using acetic acid-induced colitis in rats.

Myers et al. (1997) determined colonic transit in rats by calculating the geometric center of distribution of a radiolabeled marker (^{51}Cr) instilled into the proximal colon after induction of distal colitis by intracolonic administration of 4 % acetic acid.

Palmen et al. (1998) studied the effects of local budesonide treatment on cell-mediated immune response in acute and relapsing colitis in rats.

Several **other agents** can induce experimental colitis in animals (Kim and Berstadt 1992), such as:

- **Phorbol esters** (Fretland et al. 1990)
- **Carrageenan** (Marcus and Watt 1969; Benitz et al. 1973; Watt and Marcus 1973; Abraham

et al. 1974; Jensen et al. 1984; Kitano et al. 1994; Pricolo et al. 1996)
- **Amylopectin sulfate** (Watt and Marcus 1972)
- **Dextran sulfate** (Ohkusa 1985; Okayasu et al. 1990; Axelsson et al. 1996; Shintani et al. 1997; Kanauchi et al. 1998)
- The **chemotactic peptide FMLP** (Magnusson et al. 1985; von Ritter et al. 1988; LeDuc and Nast 1990)

Ekstrom (1998) described **oxazolone**-induced colitis in rats. Dark Agouti rats were skin-sensitized with oxazolone and further challenged intrarectally with oxazolone dissolved in carmellose sodium/peanut oil.

Suzui et al. (1997) developed a rat model for human ulcerative colitis by using **1-hydroxyanthraquinone** to cause severe inflammation of colonic mucosa.

Aiko et al. (1997) and Stadnicki et al. (1998) induced chronic granulomatous colitis in female Lewis rats via intramural (subserosal) injections of **peptidoglycan–polysaccharide** into the distal colon.

Surfactants being used to enhance drug absorption may cause intestinal damage. Oberle et al. (1995) evaluated mucosal damage by surfactants in a single-pass in situ perfusion model in the rat. The release of LDH and mucus into the lumen of jejunum and colon following perfusion of the nonionic surfactants Tween 80 and Triton X-100 was determined.

Axelsson and Ahlstedt (1993) reviewed the actions of sulfasalazine and analogues in various animal models of experimental colitis, in particular in the hapten, immune complex, and dextran models.

Bach et al. (1985) studied the inhibition of LTC synthetase and of rat liver glutathione *S*-transferases by sulfasalazine.

Stein et al. (1993) determined arachidonic acid oxidation and damage in the colon in rats stressed by the **cold-restraint** method.

Kirsner et al. (1959) and Kraft et al. (1963) induced severe colitis in **rabbits** which had previously been sensitized to egg albumin and had mild colonic inflammation induced by intrarectal instillation of a small amount of diluted **formalin**.

Meenan et al. (1996) induced immune complex colitis in rabbits by using various formalin concentrations (2 %, 0.75 %, and 0.5 %).

Hodgson et al. (1978) induced colitis in rabbits by a modified technique. Preformed immune complex of human serum albumin and anti-HSA with antigen excess was injected to non-sensitized rabbits after provocation of mild inflammation in the colon with diluted formalin.

Kuroe et al. (1996a, b) induced granulomatous enterocolitis in rabbits by repeated submucosal injection of **muramyl dipeptide** emulsion into the rectum and colon with a flexible endoscope. Extraintestinal manifestations, such as pericholangitis, were observed.

Walsh and Zeitlin (1987) studied the effects of salazopyrin, 5-aminosalicylic acid, and prednisolone on immune complex-mediated colitis in **mice**.

A survey on various genetic and immune manipulations which lead to inflammatory bowel disease in mice was given by Powrie and Leach (1995).

$CD4^+$ T lymphocytes injected into severe immunodeficient (SCID) mice lead to an inflammatory and lethal bowel disease (Claesson et al. 1996; Leach et al. 1996; Rudolphi et al. 1996; Bregenholt and Claesson 1998).

Hermiston and Gordon (1995) described an inflammatory bowel disease in mice expressing a dominant negative N-cadherin resembling Crohn's disease.

Watanabe et al. (1998) reported that interleukin-7 transgenic mice develop chronic colitis with decreased interleukin-7 protein accumulation in the colonic mucosa.

Mitchell and Turk (1990) described a model of **granulomatous bowel disease in guinea pigs**. Epithelioid cell granulomas and primary macrophage granulomas were induced by the inoculation of BCG (Pasteur) and irradiated *Mycobacterium leprae*, respectively, into the terminal ileum.

Wallace et al. (1998) induced colitis in guinea pigs and in rats by intracolonic administration of trinitrobenzene sulfonic acid.

Some **monkeys**, such as the cotton-top tamarin, *Saguinus oedipus* (Chalifoux and Bronson

1981; Madara et al. 1985; Lushbach et al. 1985; Podolsky et al. 1988; Hesterberg et al. 1996; Warren 1996), and juvenile rhesus monkeys (Adler et al. 1990), develop **spontaneous colitis**.

Critical Assessment of the Methods

The relevance of animal models for the pathogenesis and treatment of human inflammatory bowel disease was reviewed by Dieleman et al. (1997) and by Sartor (1997).

A critical review of in vitro models in inflammatory bowel disease was given by McKay et al. (1997).

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Emetic and Anti-Emetic Activity

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Contents

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Assessment of Emetic and Antiemetic Activity in Dogs

Purpose and Rationale

Emesis comparable to man occurs only in a few animal species. Among laboratory animals, the dog is a suitable species to test antiemetic drugs. Apomorphine-induced emesis is also used to evaluate neuroleptic drugs (see chapter "▶ Neuroleptic Activity"). Burkman (1982) described a technique relying upon the use of apomorphine either as a reference standard against which other emetics can be compared or as a challenging agent against which antiemetic compounds can be evaluated.

Procedure

Beagle dogs weighing between 15 and 20 kg are used. Each dog is given 200 g food 30 min prior to an assay session. The threshold emetic dose of apomorphine hydrochloride is established for each dog by administering single doses at 5-day intervals in gradually increasing amounts. The starting dose is 22 mg/kg body weight, i.m., and is subsequently increased (or decreased) as required. Injection sites alternate between contralateral gluteus muscles. After every third or fourth dose of the emetic, the animals receive an equivalent volume of vehicle under similar conditions in order to detect the presence of a conditioned emetic response.

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The threshold dose is defined as the concentration provoking an emetic episode and determined for each individual animal. The threshold emetic dose is relatively stable for a given group of dogs over a period of 2 months. Continued administration to the same dogs for longer periods of time is inadvisable as Pavlovian emetic conditioning becomes evident after 8–10 doses of apomorphine. Establishment of an emetic threshold for a test compound using a similar dosing schedule allows to quantitatively express the test compound's emetic potency as a ratio compared with the reference standard. Usually, four to six animals are sufficient to provide a reliable estimate of the test compound's emetic efficacy and potency.

In the antiemetic assay, dogs whose apomorphine threshold emetic dose has been determined receive various concentrations of the potential antiemetic drug at a given time interval prior to apomorphine. The dose initially selected for the antiemetic is a fraction of the acute LD_{50} of this drug in mice. A new threshold dose is estimated in the presence of the test antiemetic and compared to the threshold dose in the presence of the reference standard chlorpromazine.

Evaluation

Using the threshold doses, the relative potency of an emetic compared to apomorphine, or the relative potency of an antiemetic compared to chlorpromazine, is calculated.

Modifications of the Method

Cisplatin-induced emesis in the **dog**, as described by Gylys et al. (1979), was used by Turconi et al. (1991) to test the antiemetic properties of 5-HT_3 receptor antagonists.

Heaslip and Evans (1995) studied the emetic, central nervous system, and pulmonary activities of rolipram in the dog after i.v. and intragastral application.

Gupta and Sharma (1996) tested the activity of antioxidants against emesis induced by an intravenous dose of 3 mg/kg cisplatin in healthy mongrel dogs.

Szelenyi et al. (1994) described emesis in **domestic pigs** as a new experimental tool for detection of antiemetic drugs and for evaluation of the emetogenic potential of new anticancer agents. Healthy young, 12–15-week-old domestic pigs of either sex were lightly anesthetized with ketamine (10 mg/kg, i.v.) and xylazine (2 mg/kg, i.v.), and a cannula was inserted into a superficial vein of one of the extremities. The challenging agents, e.g., cisplatin, carboplatin, cyclophosphamide, or ifosfamide, were infused intravenously at different doses during a total administration time of 15 min. After removing of the cannula, the animals were placed in their boxes for observation of emesis over 24 h. Drugs with antiemetic potential were given intravenously 15 min prior to cisplatin infusion.

Göthert et al. (1995) found a dose-dependent inhibition of cisplatin-induced emesis in pigs by anpirtoline, a mixed 5-HT_1 receptor agonist/5-HT_3 receptor antagonist.

Gardner et al. (1995; Gardner and Perren 1998) used the **house musk shrew** (*Suncus murinus*) to test antagonism against cisplatin-induced emesis.

Kwiatkowska et al. (2004) performed a comparative analysis of the potential of cannabinoids and ondansetron to suppress cisplatin-induced emesis in *Suncus murinus* (house musk shrew).

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Antiemetic Activity in Ferrets

Purpose and Rationale

The ferret is a well-established animal model of emesis which responds to cancer chemotherapeutic agents in a manner similar to that observed in man (Florczyk et al. 1982). The animals react with vomiting and retching after challenge with central (apomorphine), peripheral ($CuSO_4$), or mixed central and peripheral (ipecacuanha, cisplatin) emetic stimuli. The model has been used to test the antiemetic properties of 5-HT_3 receptor antagonists and tachykinin NK_1 receptor antagonists.

Procedure

Adult male ferrets weighing 1–1.5 kg are randomly assigned to the different treatment groups. Each animal is anesthetized by inhalation with methoxyflurane. A jugular vein is cannulated and exteriorized from the outside of the neck. Following recovery from the anesthesia, the animals are dosed with the test drug or the standard or the vehicle 30 min prior i.v. administration of 10 mg/kg cisplatin. The numbers of retches and vomits occurring following the administration of the emetogen are recorded in each animal for 5 h. Retching is defined as rhythmic inspiratory movements against a closed glottis and vomiting as forced expulsion of upper gastrointestinal contents.

Evaluation

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls.

Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test.

Modifications of the Method

Fink-Jensen et al. (1992) reported that the excitatory amino acid receptor antagonists, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(f) quinoxaline (NBQX) and 6-cyano-7-nitroqinoxaline-2,3-dione (CNQX), which preferentially block non-NMDA subtypes of excitatory amino acid receptors, effectively inhibit cisplatin-induced emesis in ferrets.

Emesis in ferrets was induced by X-irradiation or oral doses of copper sulfate (Andrews and Bhandari 1993).

Watson et al. (1995) studied the antiemetic effects of a selective NK_1 receptor antagonist using the gag reflex in ferrets. The gag reflex is mediated by mechanoreceptors in the superior laryngeal nerve, which projects to the nucleus tractus solitarius (Mifflin 1993). The gag reflex was evoked in conscious ferrets by gentle tactile stimulation of the pharynx and larynx and was recorded as an all or none response before and after drug administration.

Furthermore, the authors induced retching in the ferret by electrical stimulation of the vagal afferents under urethane anesthesia (Andrews et al. 1990). The dorsal or ventral abdominal vagus was isolated and ligated and the central cut end stimulated before and after drug administration.

Duplantier et al. (1996) studied the emetic behavior in the ferret induced by inhibitors of phosphodiesterase type IV (PDE IV) and by rolipram comparing with the [^{3}H]rolipram binding activity. Robichaud et al. (1999, 2001) compared several PDE IV inhibitors and studied the mode of action inducing emesis in ferrets.

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Assessment of Emetic and Antiemetic Activity in Pigeons

Purpose and Rationale

Emesis in pigeons can be induced by various agents. Formerly, the phenomenon has been used for standardization of cardiac glycosides (Hanzlik 1929). More recently, dose–response curves of emesis have been determined for various agents and antiemetic effects were evaluated (Wolff and Leander 1994, 1995).

Procedure

Male White Carneaux pigeons are kept in individual stainless steel cages at constant temperature and humidity. They are maintained at 90 % of their free-feeding body weights by once-daily feeding of approximately 20 g Purina Pigeon Checkers.

All testing is conducted during the illuminate phase of the light–dark cycle. On test days, the birds are fed 5 min before the start of an emetic trial. If vomiting occurs, the pigeons are given an additional 20 g of feed before being returned to their home cages at the conclusion of the observation period. Individual subjects are allowed a recovery period of at least 3 days between each drug test.

For the following compounds, emetic doses are reported:

- Cisplatin 10 mg/kg, injected into a wing vein

- Ipecac syrup, 1–3 ml/kg, administered via a feeding needle passed through the crop to the opening of the proventriculus
- Emetine, 1–20 mg/kg, injected into the pectoralis muscle
- *m*-(Chlorophenyl)-biguanide (mCPBG), 0.32–5 mg/kg, injected intramuscularly
- Ditolyguanidine (DTG), 5.6 mg/kg, injected intramuscularly

Test substances with potential antiemetic activity are injected at various doses 15 min before the emetic challenge. The animals are observed for vomiting during 2 h.

Evaluation

ED_{50} values with 95 % confidence limits are calculated for the activity of emetic substances, as well as for the inhibition of emesis by antiemetic drugs after a high dose of the emetic compound.

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Activity Against Motion-Induced Emesis

Purpose and Rationale

The house musk shrew (*Suncus murinus*) is a small insectivore that has been shown to exhibit emesis when exposed to linear reciprocation motion (Ueno et al. 1988; Okada et al. 1994).

Procedure

Adult male (body weight range 55–90 g) and female (body weight range 35–50 g) are used. The animals receive a dose of the test drug or vehicle in a volume of 4 ml/kg 15 min before motion testing. The animals are placed in a Perspex chamber (11 cm wide × 22 cm long × 11 cm high) that is attached to the platform of a shaker set to execute a linear horizontal movement of 4 cm at a frequency of 1 Hz along the long axis of the chamber. The animals are allowed approximately 3 min to become accustomed to the chamber before exposure to motion for a period of 5 min, during which the number and timing of emetic episodes are recorded. An emetic episode usually consists of a short period of rapid retching followed by a vomit. A cross-over design is used for the experiment, with animals exposed to motion testing following treatment with vehicle control on one occasion and following treatment with test drug on another. An interval of 12 days is allowed between treatments.

Evaluation

Group results are expressed as mean ± SEM. Either Student's *t*-test or the Wilcoxon signed-rank test is used as a measure of significance.

Modifications of the Method

Gardner and Perren (1998) described a model of postanesthesia-induced emesis in *Suncus murinus*.

Parker et al. (2004) tested the effect of cannabinoids on lithium-induced vomiting in the *Suncus murinus* (house musk shrew).

Lucot (1989) used cats to test the activity of HT_3 antagonists against motion-induced sickness.

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Foot Tapping in Gerbils

Purpose and Rationale

Foot tapping in gerbils, a centrally mediated behavior (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Vassout et al. 1994), has been claimed to be highly predictive for NK_1 antagonists to prevent cisplatin-induced retching in ferrets and to be a simple in vivo assay for CNS penetration (Rupniak et al. 1997).

Procedure

Mongolian gerbils of either sex weighing 35–70 g are anesthetized by inhalation of an isoflurane/oxygen mixture to permit exposure of the jugular vein through a skin incision in the neck, using blunt dissection to clear surrounding salivary gland and connective tissues. Test compounds or vehicle are administered using an injection volume of 5 ml/kg i.v. The wound is closed and a second incision is made in the midline of the scalp to expose the skull. The highly selective, peptidase-resistant NK_1 receptor agonist GR73632 delta Ava[L-Pro^9,N-MeLeu^{10}]Substanc P(7–11) (Hagan et al. 1991) is infused directly into the cerebral ventricles (3 pmol in 5 µl i.c.v.) by vertical insertion of a cuffed 27-gauge needle to a depth of 4.5 mm below the bregma. The scalp incision is closed and the animal allowed to recover from anesthesia in a clear Perspex observation box (25 × 20 × 20 cm). The duration of hind foot stepping is then recorded continuously for 5 min using a stop clock. The time relapse from induction to recovery from anesthesia, with intervening i.v. and i.c.v. injections, is about 3–4 min.

Evaluation

Data are subjected to one-way analysis of variance (ANOVA), followed by Dunnett's or Newman–Keuls multiple comparison *t*-tests.

Critical Assessment of the Method

The specificity of the method to predict antiemetic activity has to be proven.

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Gall Bladder Function

Andreas W. Herling

Contents

Bile Secretion

Cholagogic Activity in Mice

Purpose and Rationale

A rapid method for standardization of cholagogues in mice by simple weighing the gallbladder filled with bile was published by Litvinchuk (1976).

Procedure

Groups of 10 female mice (e.g., NMRI strain) weighing 15–20 g are used. Food, but not water, is withdrawn 24 h prior to the experiment. The test compound or the control solution is administered subcutaneously or orally. After 1 h, the animals are sacrificed and bled from the carotid artery. Laparotomy is performed, the liver exposed, and a No. 75 silk ligature is tied around the cystic duct, which is detached from the bile ducts and removed from the peritoneal cavity. If a large volume of bile has been accumulated, the full gallbladder is removed together with the bile ducts. The isolated gallbladder is weighed on a suitable balance; after which the contents are removed, the gallbladder walls are washed with distilled water and dried on filter paper, and the organ is weighed again. The difference in weight of the full and the empty gallbladder indicates the quantity of bile secreted during a measured time. The concentration of cholates, bilirubin, and cholesterol in the bile can be determined.

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Evaluation

The average of secreted bile in groups of 10 treated mice is compared with the average value of the control group using Student's *t*-test.

Critical Assessment of the Method

The method has the clear advantage of simplicity but does not measure the true bile excretion since the outflow from the bile bladder during the test period is neglected.

Modifications of the Method

Sterczer et al. (1996) studied the effect of cholagogues on the volume of the gallbladder in healthy dogs fasted for 24 h by two-dimensional ultrasonography. The volume was measured immediately before the administration of each test substance and at 10-min intervals for 120 min thereafter.

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Choleretic Activity in Rats

Purpose and Rationale

In contrast to other animals, rats do not possess a bile bladder. Therefore, cannulation of the bile duct in rats can be used as a suitable model to measure choleresis, i.e., bile production.

Procedure

Male rats (e.g., Sprague-Dawley strain) weighing 300–500 g are used. Food, but not water, is withdrawn 18 h prior to the experiment. The animals are anesthetized by using an appropriate procedure (e.g., isoflurane anesthesia or pentobarbitone). The trachea is cannulated and the abdomen opened by a midline incision. The pylorus is ligated and the bile duct cannulated from the duodenum with a thin (0.05 mm diameter) polyethylene catheter which is pushed up to the liver. The secreted bile volume is measured for 30-min intervals, and then the bile is returned to the duodenum. After a preperiod of 60 min, the test substances are administered subcutaneously or intraduodenally. The bile volume is registered in 30-min intervals for 2 h.

Evaluation

The average values of the postdrug periods are compared with the predrug readings.

Modifications of the Method

Several authors tested the choleretic activity of plant extracts and essential oils (De la Puerta et al. 1993; Peana et al. 1994; Trabace et al. 1994) and of synthetic compounds (Grella et al. 1992; Paglietti et al. 1994) in rats.

Tripodi et al. (1993) investigated the anticholelithogenic and choleretic activities of taurohyodeoxycholic acid by measurement of biliary flow and biliary solid content in rats.

Bouchard et al. (1993) induced cholestasis in rats by treatment with 17-α-ethinyl estradiol and studied the influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids.

Vahlensieck et al. (1995) studied the effect of *Chelidonium majus* herb extract on choleresis in the isolated perfused rat liver.

Miki et al. (1993) investigated the metabolism and the choleretic activity of homochenodeoxycholic acid in **hamsters** with bile fistula.

Pesson et al. (1959) recommended the **guinea pig** as the best choice among the common laboratory animals to study choleretic agents.

Cohen et al. (1992) reported a study in male **black-tailed prairie dogs** (*Cynomys ludovicianus*) weighing 1.0 ± 0.2 kg anesthetized with 20 mg/kg xylazine i.m. and 20 min later with 100 mg/kg ketamine i.m. Through an abdominal incision, the cystic duct is ligated, and gallbladder bile is aspirated. A PE-50 polyethylene cannula is inserted into the common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula is externalized, the abdominal incision closed, and the prairie dog placed in a restraining cage with access to food and water.

Matsumura et al. (1996) analyzed hypercholeresis in **dogs** with pigment gallstones after cholate infusion.

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Chronic Bile Fistula in Rats

Purpose and Rationale

Most of the techniques for the collection of bile in rats use restrained or anesthetized animals. Such factors as well as the surgical intervention itself may profoundly influence the results. Therefore, Remie et al. (1990, 1991) developed a technique for a permanent double bile fistula in rats. The procedure is described in detail.

Procedure

Preparation of Cannulae

Cannulae are made of silicon rubber. The proximal bile cannula, which will be inserted into the

common bile duct in the direction to the liver, is 18 cm long (Silastic tubing, Dow Corning, no. 605–135; 0.51 i.d. and 0.94 o.d.) and has one square cut and one beveled end. Two silicon rings are wrapped around the cannula at 7 and 50 mm, respectively, from the beveled end.

The distal bile cannula, which will be inserted into the common bile duct in the direction of the gut, is made of the same material, is also 18 cm long, and has one square cut and one beveled end. This cannula, however, must have a smaller tip diameter (Silastic tubing, Dow Corning, no. 605–105: 0.31 i.d. and 0.64 o.d.). To serve this purpose, the square cut end of the cannula is immersed in ether, causing the tubing to dilate. When the tubing is wide enough, a 13-mm piece of small-diameter Silastic tubing is inserted. Subsequently, two silicon rings are wrapped around the cannula, one at the joint of the two tubes and the other 5 cm from the tip. The tip is then cut at a 45° angle, 7 mm from the first silicon ring.

The duodenal cannula (Silastic tubing, Dow Corning, no. 605–135) is also 18 cm long and has one square and one beveled end. An additional ring is placed 30 mm from the tip. Before the cannulae are fixed to the skull, they must be connected to a stainless steel needle bent in a 90° angle.

Anesthesia

The animal is anesthetized with halothane/N_2O/O_2.

Preparation of the Crown of the Head

The head of the animal is shaved and disinfected. An incision of about 1 cm is made and the bregma exposed. Three stainless steel screws (1.0 × 4.2 mm) are mounted in the crown, two in the left and one on the right side of the bregma. The screws are tightened that approximately 2 mm is left between the skull and the head of the screws.

Double Cannulation of the Bile Duct

The abdominal wall is shaved and disinfected and the animal secured on the operation board with adhesive tape. A midline incision is made from the level of the pubic bones to the xiphoid cartilage. The abdomen is then opened by making an incision over the linea alba toward the sternum up to the distal part of the fourth sternebra, thus exposing the xiphoid cartilage.

Then, the intestines are lifted out and are laid next to the animal on moistened gauze. Using jewelers' forceps, the bile duct is stripped off its surrounding tissue and ligated with a 7-0 suture. The duct is placed under tension with an artery forceps for cannulation. With the aid of a microscope, a V-shaped hole is made just cranial of the first ligature with iridectomy scissors. The sterile proximal cannula is inserted into the duct. The second ligature is tied and pulled tight ensuring that the cannula is not obstructed. The bile is now flowing into the cannula. The first ligature is released and the threads are tied behind the silicon ring. The rat is then turned and the ligature reclamped, thereby putting the distal part of the duct under tension. A third ligature is loosely introduced around the duct, distal to the first ligature. Another V-shaped aperture is made between the first and third ligature for insertion of the distal bile cannula. The third ligature is tied and pulled tight. The first ligature is released from the artery forceps and tied around the second cannula behind the silicon ring. All the loose threats are cut close to the knots. The sections of the cannulae which lie between the silicon rings are placed kink-free in the abdominal cavity. The cannulae are fixed using 7-0 silk suture to the abdominal muscle near the xiphoid cartilage.

Cannulation of the Duodenum

After location of the place where the bile duct enters the duodenum (sphincter of Oddi), a four fine-stitch purse-string suture (7-0) is made in the wall of the duodenum at the outer border at about 1 cm proximal to the sphincter. Using a 20G needle, an incision is made inside the purse string. The cannula is inserted into the duodenum until the first, smaller silicon ring has entered the lumen, and the purse string is tightened between the first and the second ring. This cannula together with the bile cannula is placed kink-free in the abdominal cavity and anchored to the internal muscle.

The abdomen is closed of resorbable sutures leaving 1 cm of the skin unclosed.

Subcutaneous Tunneling and Anchoring of the Cannulae

From the back of the neck, a slender needle holder is pushed subcutaneously through the connective tissue in caudal direction as near as possible to the skin down to the xiphoid cartilage. The cannulae are then grasped and pulled through to emerge at the crown of the head. The abdominal wall is closed completely.

With a 5 cm piece of polyethylene tubing (0.75 × 1.45 mm), the two long ends of the L-shaped stainless steel adapters are connected and the short ends inserted into the respective cannulae. The cannulae together with the tubing are fixed to the skull with acrylic glue flowing under the heads of the screws.

Postoperative Care

The animals are allowed to recover in a warm and quiet place. They reach usually preoperative weight within 2–3 days and display normal feeding and drinking behavior. Supplementation with saline besides the normal tap water may be necessary.

Collection of Bile

The animals are housed in individual metabolic cages. For bile collection, they are attached to long-swiveled PE cannulae (0.75 × 1.45 mm). A stainless steel coil is used to protect the rats from gnawing on the tubing. For continuous collection of bile, the cannula can be connected to a fraction collector.

Critical Assessment of the Method

Among other applications, the method is suited to study the enterohepatic circulation of compounds.

Modifications of the Method

Castilho et al. (1990) studied the intestinal mucosal cholesterol synthesis in rats using a chronic bile duct-ureter fistula model. Male Wistar rats weighing 300–350 g were anesthetized with 50 mg/kg pentobarbital i.p. and submitted to a bile duct-right ureter fistula utilizing a PE-50 catheter after a right-kidney nephrectomy.

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Chronic Bile Fistula in Dogs

Purpose and Rationale

Herrera et al. (1968) described a special cannula which can be used to obtain bile or pancreatic juice from a duodenal pouch after appropriate surgical procedures.

Procedure

Male Beagle dogs weighing 15–20 kg are used. The abdomen is opened through a midline epigastric incision under appropriate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the

common bile duct is isolated. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodenojejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of three parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch. When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows bile to enter the duodenum in the normal manner. For collection of bile this plug is removed and a long obturator is inserted. The latter effectively isolates the bile secretion from duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured by a further purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula. The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, and after which the animals are permitted to drink water. Daily checks of the cannula are advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 4 weeks to recover. Eighteen hours prior to the experiment food is withdrawn, but water allowed ad libitum. The long hollow obturator is inserted and bile collected for 15 min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

Evaluation

Secretion of bile is measured at 15-min intervals, and volume and bile contents are determined from 1-ml samples. The values are compared with pretest data. The remaining bile is reinfused into the duodenum via the hollow obturator.

Modifications of the Method

Boldyreff (1925) described several techniques for fistulae of the gallbladder and also for the fistula of the ductus choledochus in dogs.

An abdominal incision about 10 cm is made on the median line. The duodenum is pulled out, and the orifice of the large (first) pancreatic duct is found. The orifice of the ductus choledochus with the orifice of the small (second) pancreatic duct is situated on the other side of the intestine some 2 or 3 cm nearer the stomach. The ductus choledochus goes straight from the gallbladder to the duodenum; further it lies parallel to it, and at its end it is attached to the wall of the duodenum. The small pancreatic duct goes from the gland straight to the duodenum.

At the very beginning of the operation, it is useful to cut the ligamentum that goes from the liver to the duodenum, because this facilitates orientation and operating. It is necessary to cut out a piece of the intestinal wall with the orifice of the ductus choledochus. But before this one must prepare off a little bit the intestine from the pancreas so as to be able to close conveniently and securely the hole in the intestine and divide between double ligatures the second pancreatic duct.

On the duodenum around the orifice of the ductus choledochus, an incomplete oval figure is now marked with a knife, so that the duct enters this figure through the incomplete part of the oval and has its orifice in the middle of this figure. The length of the oval is about 1.5 cm and its width 1 cm. A suture is then made on the edge of this oval, which is cut out not completely but leaving a

small bridge about 0.5 cm wide between the intestine and the oval; through the bridge the duct enters the oval. The mucosa of this bridge must be completely destroyed with a knife.

The oval piece of the intestine is now turned with the mucosa up, and its serosa is sutured to the serosa of the intestine. The hole in the intestine is very carefully closed with two layers of sutures. Two heavy threads are then passed underneath the intestine on either side of the place of operation; they are laid through the abdominal wall and tied after the operation is over. They serve as temporary supporting sutures. The oval piece of the intestine is now sutured with the skin of the abdominal wound, and the wound is closed in the usual manner. The supporting sutures must be taken out one day or two days after the operation.

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Prevention of Experimental Cholelithiasis

Purpose and Rationale

Several animal species develop gallstones, either spontaneously or induced by diet, such as ***Tupaias*** (Schwaier 1979; Schwaier et al. 1979), **ground squirrels** (Fridhandler et al. 1983; MacPherson et al. 1987; MacPherson and Pemsingh 1997), **hamsters** (Holzbach 1984; Cohen et al. 1995), and **owl monkeys** (Pekow et al. 1995).

The **prairie dog** (*Cynomys ludovicianus*) is the animal model that has been used most extensively to study diet-induced cholesterol gallstone formation and the inhibition thereof (Holzbach 1984; Conter et al. 1986; Matoba et al. 1989; Cohen et al. 1990; Broughton et al. 1991; Afdhal et al. 1993; Kam et al. 1996).

Davis et al. (2003) and Saunders et al. (1991) studied the use of statins for the prevention of gallstones in cholesterol-fed prairie dogs.

Procedure

Wild-caught male prairie dogs weighing about 1 kg were kept in quarantine for 3 weeks and fed normal rodent chow (cave: legislation on the use of wild-caught animals for biomedical research). Then the diet was changed to a lithogenic diet consisting of a 1.2 % cholesterol formulation for the study period of 28 days. Medication (drug or placebo) was applied through a gastric tube under slight sedation twice a day. Blood chemistry analysis for total cholesterol and triglycerides was performed on study days 0, 14, and 28.

At the completion of the study period, all animals were placed under general anesthesia and underwent an open cholecystectomy. A subcostal incision was made in the right upper quadrant and the gallbladder removed using clips on the cystic duct. The gallbladder was then opened and visually inspected for gallstones. Bile from the gallbladder was aspirated for microscopic analysis.

A sample of bile of each animal was examined using light microscopy to determine the presence of cholesterol crystals.

Evaluation

Statistical analysis was performed using Student's *t*-test for cholesterol and triglyceride data. The Fisher exact test was utilized for analysis of gallstone and cholesterol crystal formation data.

Modifications of the Method

Chapman et al. (1998) established and characterized primary gallbladder epithelial cell cultures in the prairie dog.

Chen et al. (1999) studied biliary sludge and pigmented stone formation in bile duct-ligated guinea pigs.

Stone et al. (1987) developed a rat model to explain the cyclosporine-induced cholestasis observed in transplantation patients.

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Gallbladder Motility

Activity on Isolated Gallbladder Strips from Guinea Pigs

Purpose and Rationale

Effects on the smooth musculature of the gallbladder can be studied in isolated strips of gallbladder from guinea pigs or cats (Chowdhury et al. 1975; Fara and Erde 1978; Cabrini et al. 1995).

Procedure

Guinea pigs of either sex weighing approximately 400 g are anesthetized with 3 g/kg urethane i.p. The gallbladder is removed and cut into longitudinal strips 10 × 3 mm. The strips are suspended in a Krebs solution bath between a stationary hook and an isometric strain gauge. Tension is recorded on a polygraph. The bath is maintained at 37 °C and aerated with a gas mixture of 95 % O_2 and 5 % CO_2. After a half-hour stabilization period, test doses of acetylcholine (1 mg/ml) are added to determine viability of the preparation. Dose-response curves for the muscle strips are obtained by introducing one dose of the stimulating agent and waiting until the maximal response to that dose is reached (usually 5–15 min). The bath is then rinsed three times and 15 min allowed before a new dose is tested.

Evaluation

Concentrations for the maximal response and ED_{50} values are calculated.

Modifications of the Method

Eltze et al. (1997) found that contractions of the isolated guinea pig gallbladder elicited by muscarinic stimuli are mediated by functional muscarinic M_3 receptors.

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Gallbladder Motility in Dogs

Purpose and Rationale

Gallbladder motility can be measured in anesthetized dogs with intraluminal manometry.

Procedure

Beagle dogs weighing 15–18 kg are fasted for 16 h prior to the experiment. Median laparotomy is performed under appropriate anesthesia (e.g., inhalation with isoflurane, pentobarbitone 25 mg/kg IV). An incision is made in the bile bladder, and a 2-mm diameter polyethylene catheter is introduced. The catheter is advanced as far as possible to the neck of the gallbladder and tied. The pressure in the interior of the gallbladder is recorded on a pen recorder via a Statham pressure transducer (P 23 BB) and a frequency measuring bridge.

Evaluation

Cholecystokinin increases dose dependently the intraluminal pressure. The preparation can be used for evaluation of cholecystokinin or cholecystokinin-like activity.

Modifications of the Method

Ryan and Cohen (1976) used adult opossums (*Didelphis virginiana*) of either sex, weighing 2.5–3.0 kg.

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Cholecystokinin Activity (Isolated Gallbladder or Intestine)

Purpose and Rationale

A sensitive bioassay for cholecystokinin utilizing strips of rabbit gallbladder has been described by Amer and Becvar (1969). Segments of the ileum and colon have been used by Paton and Zar (1968) and by Zetler (1984).

Procedure

Male albino rabbits weighing 1.5–2.5 kg are fasted 18 h prior to the experiment. The animals are sacrificed by cervical dislocation, and the liver is quickly dissected out. The gallbladder is teased away from the liver and immediately placed in Locke-Ringer solution. The biliary contents are emptied, and the gallbladder is cut spirally (right-handed spiral starting from the bile duct) into a strip of muscle tissue 30–40 mm in length and about 5 mm wide. The muscle strip is placed in an organ bath containing Locke-Ringer solution at 37 °C continuously bubbled with carbogen. The initial tension of the muscle is adjusted to 0.5 g. The bath fluid is changed every 30 min. The test compound and the standard (range 5–20 mIDU (Ivy dog units)) are added alternatively. The maximum of contractions is usually reached after 5 min. Then, the bath fluid is changed. The next dose is applied when the tension has achieved again the starting value.

Evaluation

The maximum of contraction is taken as end point. Using at least two doses of test compound and standard, parallel line assays can be carried out, and potency ratios can be calculated.

Critical Assessment of the Method

This bioassay has the advantage to be sensitive and less time-consuming than in vivo assays, such as measuring gallbladder motility in the anesthetized dog (see "J.6.2.2"). However, the assay is not specific for cholecystokinin, since also gastrin and gastrin analogues cause contractions of the isolated bile bladder. This assay allows also to calculate potency ratios of different gastrin analogues.

Modifications of the Method

The whole gallbladder of the mouse or a strip of guinea pig gallbladder is used for evaluation of cholecystokinin-like peptides, ceruletide, ceruletide analogues, and cholecystokinin octapeptide by Zetler et al. (1979) and Zetler (1984).

Paton and Zar (1968), Zetler (1984), Chang and Lotti (1986), and Barthol and Holzer (1987) used isolated segments of the ileum and colon from guinea pigs for determination of CCK-like activity.

Makovec et al. (1986) and Tachibana et al. (1996) studied the in vivo activity of derivatives of CCK in emptying the gallbladder in mice.

Henke et al. (1997) tested CCK-A agonists in the isolated guinea pig gallbladder.

Singh et al. (1995) used gallbladder strips from guinea pigs to evaluate CCK receptor antagonists.

Fukamizu et al. (1998) tested the effect of a cholecystokinin-A receptor antagonist against CCK-8-induced contractions in isolated smooth muscle fibers of gallbladders and longitudinal fibers of the ileum from guinea pigs.

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Sphincter of Oddi Function

Relaxation of the Sphincter of Oddi In Vitro

Purpose and Rationale

The integrity of the relaxation function of the sphincter of Oddi is a prerequisite for normal delivery of bile into the duodenum. Sphincter of Oddi relaxation is mainly executed by non-adrenergic, non-cholinergic (NANC) nerves that are essentially nitrergic in several species including guinea pigs (Pauletzki et al. 1993) and rabbits (Lonovics et al. 1994).

Procedure

Biliary sphincter of Oddi muscle rings of approximately 6-mm length from adult male New Zealand white rabbits weighing 3,500–4,000 g pretreated with various drugs or diet are prepared. The papilla Vater is eliminated, and the ampullary part of the muscle rings of approximately 3-mm length is mounted horizontally on two small L-shaped glass hooks, one of which is connected to a force transducer attached to a polygraph for measurement and recording of isometric tension. The experiments are carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer which is maintained at 37 °C and aerated continuously with carbogen. The initial tension is set at 10 mN, and the rings are allowed to equilibrate over 1 h during which period the sphincters develop characteristic 14–19/min rhythmic contractions. Atropine (1 μM) and guanethidine (4 μM) are continuously present (NANC solution). Changes in isometric tension in response to two consecutive trains of impulses of electrical field stimulation (40 stimuli, 50 V, 0.1 ms, and 20 Hz) are then studied.

Evaluation

The data representing changes in isometric tension expressed as means ± standard deviation are evaluated by means of analysis of variance (ANOVA) followed by a modified Student's *t*-test for multiple comparisons according to Bonferroni's method. Changes are considered statistically significant at P-values smaller than 0.05.

Modifications of the Method

In addition to studies with the isolated sphincter of Oddi of rabbits (Slivka et al. 1994; Sari et al. 1998; Jia and Stamler 1999) and guinea pigs (Harrington et al. 1992; Gocer et al. 1995; Lu et al. 1997), several pharmacological studies were reported with the isolated sphincter of Oddi of **opossum** (Perodi et al. 1990; Allescher et al. 1993) and of the **Australian brush-tailed possum** (*Trichosurus vulpecula*) (Baker et al. 1992, 1996).

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Function of the Sphincter of Oddi In Vivo

Purpose and Rationale

Dogs were used for in vivo studies of the function of the sphincter of Oddi (Sarles 1986; Pozo et al. 1990; Matsumara et al. 1991; Kobayashi et al. 1994; Wang et al. 1998). Shima et al. (1998) recorded the spontaneous motility and the response to cerulein on the canine sphincter of Oddi using a constant perfusion technique.

Procedure

Dogs weighing 7–9 kg fasted overnight are anesthetized by using appropriate methods (e.g., 25 mg/kg intravenous pentobarbital sodium) and are maintained under adequate anesthesia with 12.5 mg/kg intravenous pentobarbital sodium as

required. Systemic blood pressure is monitored through a catheter placed into the femoral artery. A femoral vein is cannulated and used for systemic administration of Ringer's solution and drugs.

After an upper median laparotomy, a small longitudinal incision is made in the common bile duct. Two catheters (outer diameter 2.0 mm) are cannulated and tied in the bile duct to avoid any leaks and the occlusion of the orifice of the catheters. One is distally placed at 5 mm proximal to the choledochoduodenal junction and is used to perfuse the sphincter of Oddi with saline at a rate of 0.12 ml/min using an infusion pump. The other is proximally placed and used to siphon off the hepatic bile. Pressure changes are recorded on a polygraph through a pressure transducer which is placed between the infusion pump and the catheter.

Evaluation

To evaluate the effects of intravenously administered drugs on the sphincter of Oddi, a motility index is calculated by measuring the square between the zero line and the trace of the sphincter of Oddi pressure changes per minute.

Results are expressed as means ± SEM; n is the number of independent observations of different animals. The paired and unpaired t-tests are used for statistical analysis. $P < 0.05$ is considered significant.

Modifications of the Method

Thune et al. (1992, 1995) studied simultaneously the flow resistances in the common bile duct and main pancreatic duct sphincters in anesthetized **cats** using a perfusion technique.

Elbrønd et al. (1994) prepared the sphincter of Oddi and duodenum in anesthetized **rabbits** with perfused catheters and bipolar electrodes. Increasing, successive doses of cholecystokinin were administered intravenously every 15 minutes. The digitized recordings were scored on a computer in control and stimulatory cholecystokinin sequences.

Further studies in **rabbits** were performed by Nakamura (1996) and Chiu et al. (1998).

Opossums were used by Calabuig et al. (1990), Hanyu et al. (1990), Cullen et al. (1996), and Herrmann et al. (1999) to study the function of the sphincter of Oddi.

Several authors used the **Australian brush-tailed possum** (*Trichosurus vulpecula*) for studies of the function of the sphincter of Oddi in vivo (Baker et al. 1990; Saccone et al. 1992; Cox et al. 1998a, b; Huang et al. 1998).

The **prairie dog** was used by several authors to study the function of the sphincter of Oddi (Ahrendt et al. 1992; Kaufman et al. 1993; Thierney et al. 1994).

Pasricha et al. (1995) reported a model in **pigs** for endoscopic biliary manometry, similar in technique to the procedure in humans.

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Pancreatic Function

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Contents

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Acute Pancreatic Fistula in Rats

Purpose and Rationale

The effect of exogenous hormones, e.g., secretin, or other drugs on pancreas secretion can be measured in rats with acute pancreas fistula.

Procedure

Male Sprague–Dawley rats weighing 150–200 g are used. Eighteen hours prior to the experiment, food is withdrawn with free access to water. Groups of four to five rats are used for each dose of drug evaluation; the control group consists of at least seven rats. The animals are anesthetized appropriately (e.g., by isoflurane inhalation, ketamine–pentobarbital) and the trachea is cannulated. The abdomen is opened by a midline incision and the pylorus ligated. The proximal part of the bile duct is ligated near the hepatic porta. The bile is drained via a thin polyethylene tube into the duodenum. The distal part of the bile duct with the orifices of pancreatic ducts is cannulated with another thin polyethylene tube. The pancreatic juice is collected in graduated microvessels. After a pretest period of 60 min, the test compounds are applied intravenously or intraduodenally.

Evaluation

The secretion after injection of the test compound is compared with the pretest values. Secretin increases pancreatic secretion volume in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2 + 2 assays in comparison with the international standard.

Modifications of the Method

Guan et al. (1990) inserted two separate cannulae for bile and pancreatic juice to Wistar **rats** under methoxyflurane anesthesia. Both fluids were returned to the intestine. Placing the rats in

modified Bollman-type restraint cages, experiments could be performed after a few days in conscious animals.

Ito et al. (1994) studied the inhibition of CCK-8-induced pancreatic amylase secretion by a cholecystokinin type A receptor antagonist in rats.

Niederau et al. (1989) compared the effects of CCK receptor antagonists on rat pancreatic secretion in vivo. Output of amylase in pancreaticobiliary secretion was measured after various doses of cerulein. The effects of high cerulein doses were dose-dependent inhibited by CCK-antagonists.

Alvarez and Lopez (1989) studied the effect of alloxan diabetes on exocrine pancreatic secretion in the anesthetized **rabbit**. After a 14- to 15-h fasting period, but with free access to water, rabbits weighing about 2.0 kg are anesthetized by intravenous injection of 1.0 g/kg urethane. After tracheotomy, a median laparotomy is performed, and the main pancreatic duct is exposed and cannulated near its entrance to the duodenum following ligation of the pylorus and cannulation of the bile duct for deviation of bile to the exterior.

Kim et al. (1993) studied the effect of [(CH_2NH)4,5] secretin on pancreatic exocrine secretion in **guinea pigs** and rats using an acute pancreatic fistula preparation.

Niederau et al. (1990) and Tachibana et al. (1996) determined pancreatic exocrine secretion in **mice**. Because the cannulation of mouse pancreatic duct is not possible for technical reasons, the amount of amylase was determined in vivo. Five minutes after IV administration of test drugs, mice were sacrificed and a 5-cm duodenal loop was removed. The duodenal contents were washed out with 1.0 ml ice-cold saline and collected for amylase activity.

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Exocrine Secretion of Isolated Pancreas

Purpose and Rationale

Procedures for isolation and perfusion of rat pancreas in order to record membrane potential and effective membrane resistance during the

collection of perfusates and for simultaneous measuring of flow of pancreatic juice were described by Kanno (1972), Kanno and Saito (1976), Kanno et al. (1976), and Saito et al. (1980). Similar techniques to measure exocrine secretion of the isolated pancreas have been used by other groups (Penhos et al. 1969; Mann and Norman 1984; Trimble et al. 1985; Norman and Mann 1986, 1987; Norman et al. 1989; Park et al. 1993).

Procedure

Male Sprague–Dawley rats weighing about 250 g are fasted for 24 h before the experiments but are allowed to drink water. Under appropriate anesthesia and after cannulation, the vascular system and the common duct are separately perfused. The rectum and the transverse colon are separated from the pancreas following ligation and sectioning of their vascular supply. Perfusion is performed through the hepatic end of the common duct with the outlet at the duodenal end. The **flushing preparation** is used when continuously recording the transmembrane potential, and, in this preparation, the common duct is flushed at a constant pressure of 5-cm H_2O following cannulation of both ends with stainless steel tubes. The **draining preparation** is used during the electrical measurements are made from as many acinar cells as possible in every 10 min. In the draining preparation, the hepatic end of the duct is ligated and the pancreatic juice collected from the duodenal end following cannulation with a stainless steel tube. In both preparations, the inlets of the vascular perfusion are the superior mesenteric artery and the coeliac artery, and the outlet is the portal vein. The rate of vascular flow is kept constant at 1 ml/min with the aid of a roller pump. The animal is then killed by cutting the carotid arteries and the perfusion begins. The spleen is removed after section of its vascular supply close to the hilum, care being taken to avoid interference with the supply from the splenic artery. The blood supplying stomach and liver is stopped by tying the hepatic artery and gastric arteries. The superior mesenteric vein and the descending branch of the superior mesenteric artery are then ligated. The mesentery with its embedded whole pancreas and the attached duodenum is then removed and mounted on a paraffin block in a lucite chamber containing 20 ml of standard Krebs–Henseleit solution. The contents of the duodenum is then drained with a polyethylene tube. The level of the standard Krebs–Henseleit solution in the bath is kept constant with a siphon. The temperature of the preparation and perfusing Krebs–Henseleit solution containing 8 vol% erythrocytes is maintained at 37 °C. The perfusion solution in the reservoir is continuously bubbled with 5 % CO_2 in O_2.

The rate of flow of pancreatic juice is measured by attaching a calibrated tube made of silicone rubber to the free end of the pancreatic duct cannula. Every 10th min, the tube is replaced and the rate of flow of the pancreatic juice is noted. The sample is diluted with Krebs–Henseleit solution up to 100 ml and the amount of amylase assessed.

Intracellular recordings are made from the pancreatic acinar cells by manually advancing KCl-filled microelectrodes under direct visual control. The microelectrode is connected to the probe of a solid-state preamplifier. Resting potentials are observed at the screen of a cathode ray oscilloscope and simultaneously recorded on a direct visual oscillograph.

Measurements are made every 10 min for half an hour as base level, followed by measurements every 10 min during infusion of drug for 1 h.

Evaluation

All results are expressed as mean ± SEM. Statistical analysis is performed using Student's *t*-test.

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Chronic Pancreatic Fistula in Rats

Purpose and Rationale

Sugiyama et al. (1996) described the preparation of a chronic pancreatic fistula in the WBN/Kob rat, a strain which develops spontaneously chronic pancreatitis.

Procedure

Male WBN/Kob rats at an age of 2 months are used. Before surgery, the rats have free access to chow and water. After an 18-h fast, laparotomy is performed through a midline incision under general anesthesia (e.g., isoflurane inhalation). A polyethylene catheter (inside diameter 0.28 mm; outside diameter 0.61 mm, length 30 cm), as an external pancreatic fistula, is inserted into the common biliary pancreatic duct immediately proximal to the ampulla of Vater. The bile duct is ligated proximally to the pancreas. Another catheter is introduced into the bile duct distal to the confluence of the hepatic ducts, and the opposite end of the catheter is inserted into the duodenum near the ampulla through the stomach to drain the bile into the intestine. A third catheter is inserted in the duodenum through the stomach to return pancreatic juice. A fourth catheter is placed in the femoral vein, and 0.15 M sodium chloride is continuously administered at a flow rate of 1 ml/h with a syringe pump. All catheters are brought into the abdominal cavity or the femoral region through a subcutaneous tunnel starting in the middle portion of the tail. The rat is then placed prone in a cage with the proximal portion of the tail fixed to a side wall of the cage. When awakened, the rat is allowed access to food and water ad libitum. Pancreatic juice is collected and continually recirculated with a syringe pump every 6 h until the secretion test.

The secretion test is started 3 days after surgery. After a 12-h fast, pancreatic juice is collected every 30 min in a plastic syringe. The juice volume is determined by weighing. A 50-µl sample is taken for the analysis of juice composition, and the remaining juice is returned to the duodenum for the next 30-min collection period.

After a 30-min basal period, the test compound is administered in graded doses, each dose for 30 min.

The bicarbonate concentration of pancreatic juice is measured with a microgasometer (Natelson 1958) in a sample size of 10 µl. Protein concentration is determined by measuring optical density at 280 nm with purified bovine trypsinogen as standard.

Evaluation

All values are presented as mean ± SEM. Results are analyzed by means of the Wilcoxon test. Differences are considered significant at $p < 0.05$.

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Acute Pancreatic Fistula in Dogs

Purpose and Rationale

The effect of exogenous hormones, e.g., secretin or gastrin, or of vagal stimulation, on pancreatic secretion can be measured in dogs with acute pancreas fistulas.

Procedure

Beagle dogs of either sex weighing 12–20 kg are used. The animals are fasted for a 24-h period and then anesthetized with 25 mg/kg sodium pentobarbital IV. After opening the abdomen along the midline, the pyloric sphincter is ligated and the common bile duct cannulated to prevent the entry of acid chyme and bile into the duodenum. The bile is allowed to drain. The pancreas is gently exposed and the major pancreatic duct ligated. A polyethylene tube of 2-mm diameter is inserted into the minor pancreatic duct for collection of the secretion. The left femoral vein is cannulated for continuous infusion or IV injection. The pancreatic juice is collected in an ice bath in a special tapered tube with fine calibrations for measuring volumes of less than 1 ml.

At the end of each collection period, the volume is recorded and the bicarbonate content determined titrimetrically. Furthermore, pancreatic enzymes, such as amylase, are determined in the samples. Determination of protein concentrations in the pancreatic juice can be used as end point since the total protein concentration is proportional to the individual enzymes (Keller et al. 1958). In a pretest period of 10 min, samples are collected every 2 min. Then, the test compound is injected intravenously and the pancreatic juice is collected every 2 min.

Evaluation

The secretion after injection of the test compound is compared with the pretest values. Secretin increases pancreatic volume and bicarbonate secretion in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2 + 2 points assays in comparison with the international standard.

Moreover, the model can be used for standardization of gastrin analogues.

Modifications of the Method

Glad et al. (1996) tested the influence of gastrin-releasing peptide on acid-induced secretin release

and pancreatobiliary and duodenal bicarbonate secretion in Danish country strain **pigs** weighing between 22 and 30 kg. The animals, starved overnight with free access to water, were premedicated with 4 mg/kg i.m azaperone and with 5 mg/kg IP metomidate. After 20 min, a cannula was placed in an ear vein, and 5–10 mg/kg metomidate was given IV followed by intubation and artificial respiration with 50 % O_2 and 50 % N_2O. Anesthesia was maintained with an intravenous bolus infusion of 0.53 % α-chloralose.

Both external jugular veins were cannulated for infusion of saline or drugs. A femoral artery was cannulated for withdrawal of blood samples and recording of blood pressure. After laparotomy, the cystic duct was ligated, and the common hepatic duct and the pancreatic duct were catheterized. The duodenal segment was defined as extending from the pylorus to the ligament of Treitz. A Foley catheter was passed through the pylorus into the proximal part of the duodenum and inflated. Distal to the pylorus, the pancreaticoduodenal arteries, veins, and nerves were dissected, and a double ligature was passed under these structures and tied around the duodenum. At the ligament of Treitz, an inflated Foley catheter was placed in the distal part of the duodenum and tied with a suture around the duodenum. A catheter was placed through a splenic branch of the left gastroepiploic vein and advanced through the lienal vein to the portal vein.

The flow of pancreatic juice and bile was tested before and after the experiment by means of an intravenous bolus of 5 pmol/kg secretin. Before the experiment, the duodenum was continuously perfused at a rate of 2 ml/min for 435 min with isotonic saline containing phenol red (10 mg/l) as a marker. After drug treatment (intravenous infusion of gastrin-releasing peptide or duodenal HCl perfusion), pancreatic and hepatic secretions were collected in 15-min periods and the volumes determined by weighing. Duodenal effluents were collected in 15-min periods and phenol red concentrations determined spectrophotometrically. Blood sampled were withdrawn for determination of secretin by radioimmunoassay.

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Chronic Pancreatic Fistula in Dogs

Purpose and Rationale

Herrera et al. (1968) described a special cannula which can be used to obtain pancreatic juice or bile from a duodenal pouch after appropriate surgical procedures (Preshaw and Grossman 1965).

Procedure

The principle technique is demonstrated in Fig. 1. Male Beagle dogs weighing 15–20 kg are used. The abdominal surgery is performed during appropriate anesthesia (in former times by, e.g., 30 mg/kg pentobarbital sodium; nowadays more appropriate by inhalation with halothane or isoflurane). The abdomen is opened through a midline epigastric incision. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the ductus pancreaticus minor, which represents the main pancreatic duct in dogs, is isolated. The proximal

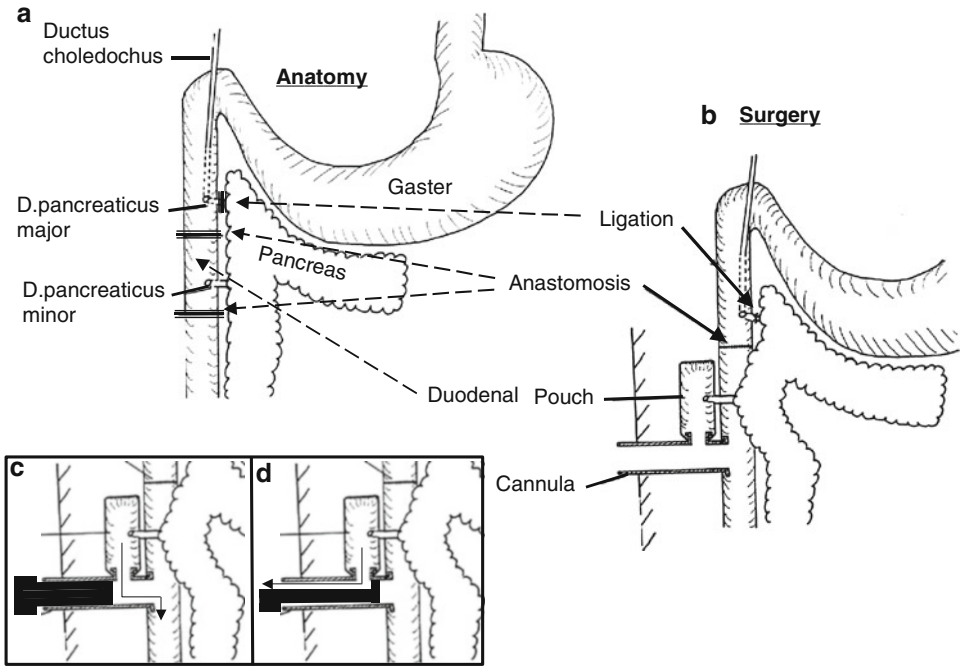

Fig. 1 Technique of chronic pancreatic fistula in dogs. (**a**) The normal anatomic situation. (**b**) Diagram of the duodenal pouch preparation. (**c**) Demonstration of the normal flow of exocrine pancreatic juice during non-assay condition. (**d**) Demonstration of the flow of exocrine pancreatic juice during study condition

level of the duodenal section lies immediately distal to the opening of the common bile duct, and the distal level of section lies 2.5 cm distal to the main pancreatic duct (ductus pancreaticus minor). The duodenum integrity is restored by end-to-end duodenojejunostomy. The duodenal pouch is closed at the proximal end.

The cannula to be inserted is made of stainless steel and consists of three parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point, there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the duodenal pouch.

When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows pancreatic juice to enter the duodenum in the normal manner (Fig. 1, part C). For collection of juice, this plug is removed, and a long obturator is inserted (Fig. 1, part D). The latter effectively isolates the pancreatic secretion from other duodenal contents.

The lateral limb of the cannula is inserted in the distal end of the isolated duodenal segment in which the main pancreatic duct opens. The remaining limb of the cannula inserted through a small duodenotomy and secured further by a purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula.

The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Checking the cannula daily is advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 2 weeks to recover. Eighteen hours prior to the experiment, food is withdrawn but water allowed ad libitum. The long obturator is inserted (Fig. 1, part D) and pancreatic juice collected for 15-min periods. After 1 h pretest time, the candidate compound is given either orally or intravenously.

Evaluation

Secretion of pancreas juice is measured at 15-min intervals and volume and enzyme content determined. The values are compared with pretest data.

Modifications of the Method

Boldyreff (1925) described details of the technique as recommended by Pavlov (1902) as well as his own modification.

Konturek et al. (1976, 1984) performed experiments with chronic gastric fistulas in **cats** as well as in **dogs** to compare the species-specific activities of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion.

Ninomiya et al. (1998) studied the effects of a cholecystokinin A receptor antagonist on pancreatic exocrine secretion stimulated by exogenously administered CCK-8 in conscious dogs with chronic pancreatic fistula.

Garvin et al. (1993) described distal pancreatectomy with autotransplantation and pancreaticocystostomy in dogs.

Kuroda et al. (1995) developed a new technique in dogs for pancreatico-gastrointestinal anastomosis that consists of pancreatectomy using the ultrasonic dissector and implantation of the pancreatic duct into the gastrointestinal tract without suturing the pancreatic parenchyma.

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Somatostatin Activity

Purpose and Rationale

Somatotropin is a neuropeptide, also called somatotropin release-inhibiting factor (SRIF or SRIF-14), which occurs not only in the brain but also in a variety of peripheral tissues. Two other related peptides, somatostatin-28 (SRIF-28) and cortistatin (Vasilaki et al. 1999), also occur naturally (Humphrey 1998). Somatostatin acts via G protein-coupled receptors of which five subtypes are cloned (Hoyer 1998).

Somatostatin analogues and antagonists have been tried for a variety of indications (Bass et al. 1996; Coy and Taylor 1996; Papageorgiou and Borer 1996; Rohrer et al. 1998; Yang et al. 1998; Hocart et al. 1999). The long-acting octapeptide somatostatin analog, octreotide, has been studied thoroughly (Ambler et al. 1996; Danesi and Del Tacca 1996; Hoffmann et al. 1996; Paran et al. 1996) and gained clinical use as gastric antisecretory agent.

Somatostatin inhibits basal pancreatic secretion as well as secretion stimulated by food, cholecystokinin, and secretin. Inhibition of pancreatic secretion can be used as bioassay to compare synthetic analogues with the original somatostatin.

Procedure

Male Wistar rats weighing 300–400 g are anesthetized with isoflurane and prepared with silastic cannulae. Two cannulae drain pancreatic juice and bile separately, one cannula is inserted into the duodenum for return of bile and pancreatic juice to the intestine and for intestinal infusion. Another cannula is located in the abdominal cavity and a fifth cannula into the right jugular vein for drug infusion. After surgery, the rats are placed into modified Bollman-type restrain cages. The rats have free access to food and water. During recovery and between the experiments, pancreatic juice and bile are collected and continuously returned to the intestine by a servo system consisting of a collection tube in a liquid-level photodetector coupled to a peristaltic pump.

Experiments are performed on the third to eighth days postoperatively. The rats are assigned to treatment groups on a random basis, and treatment days are equally divided among the third to eighth postoperative days. The rats are fasted overnight before each experiment. Bile and pancreatic juice are collected separately every 30 min; the volume of pancreatic juice is measured, and 10 ml is taken for protein assay. Different doses of the test drug and the standard are given as continuous IV infusion over a period of 2 h.

Evaluation

Inhibition of pancreatic fluid and protein output is calculated compared to control infusion and expressed as percentage. From dose–response curves, activity ratios can be calculated.

Modifications of the Method

Konturek et al. (1985) and Susini et al. (1980) studied the effect of somatostatin analogues on pancreas secretion in dogs.

Cai et al. (1986) reported on the biological activity of octapeptide analogs of somatostatin assaying GH concentrations in blood samples of anesthetized rats by RIA and studying the inhibition of gastric secretion on dogs.

Biological actions of prosomatostatin have been described by Meyers et al. (1980) using in vitro and in vivo GH bioassays.

Taylor et al. (1996) employed the technique of cytosensor microphysiometry for real-time evaluation of somatostatin subtype receptor activity in CHO-K_1 cells stably expressing the human sst_2 receptors.

Cytotoxic analogs of somatostatin have been synthesized and evaluated (Nagy et al. 1998).

Gilon et al. (1998) studied a synthetic receptor 5-selective somatostatin analogue in vivo in rats. The compound inhibited bombesin- and cerulein-induced amylase and lipase release from the pancreas without inhibiting growth hormone or glucagon release.

Jeandel et al. (1998) described the effects of two somatostatin variants on receptor binding, adenyl cyclase activity, and growth hormone release from the frog pituitary.

Hofland et al. (1994) determined relative potencies of somatostatin analogs on the inhibition of growth hormone release by cultured human endocrine tumor cells and normal rat anterior pituitary cells.

Rohrer et al. (1998) and Rohrer and Schaeffer (2000) described rapid identification of subtype-selective agonists of the somatostatin receptor through combinatorial chemistry.

Hoyer et al. (2004) characterized in vitro a non-peptide somatostatin sst_1 receptor antagonist.

Reubi et al. (2000) described SST_3-selective potent peptidic somatostatin receptor antagonists.

Nunn et al. (2003a, b) reported properties of putative small-molecule somatostatin sst_2 receptor-selective antagonists.

Using high-throughput parallel synthesis, Moinet et al. (2001) prepared non-peptide ligands for the somatostatin sst_3 receptor.

Gademann et al. (2001) reported that peptide folding induces high and selective affinity of a linear and small β-peptide to the human somatostatin receptor 4.

Nunn et al. (2003c) described β^2/β^3-di- and α/β^3-tetrapeptide derivatives as potent agonists at somatostatin sst_4 receptors.

Somatostatin analogs labeled with ^{99m}technetium, ^{111}indium, and ^{90}yttrium were developed for tumor diagnosis and therapy (O'Byrne and Carney 1996; Pearson et al. 1996; Stolz et al. 1996, 1998; Thakur et al. 1996).

Radioimmunoassays for somatostatin have been developed (Arimura et al. 1975; Gerich et al. 1979; Patel and Reichlin 1979; Patel 1984) and are available as commercial kits.

Siehler et al. (1998) reported that [^{125}I]Tyr^{10}-cortistatin$_{14}$ labels all five somatostatin receptors.

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Receptor Binding for Somatostatin

General Considerations

A family of five somatostatin receptor subtypes from various species including man has been described (Yamada et al. 1992; Reisine and Bell 1995; Hoyer et al. 1995; Bruns et al. 1996; Liapakis et al. 1996; Patel and Srikant 1994; Hoyer et al. 1995; Patel et al. 1996; Nilsson and

Folkesson 1997; Patel 1997; Pscherer et al. 1996; Shimon et al. 1997; Humphrey et al. 1998; Moller et al. 2003; Olias et al. 2004). There are several endogenous ligands, such as somatostatin-14, somatostatin-28, and the corticostatins (Meyerhof et al. 1992; De Lecea et al. 1996; Fukusumi et al. 1997). A number of synthetic peptide analogues have been developed, and their relative affinities at human recombinant receptors in radioligand receptor studies have been established (Raynor et al. 1992; Bass et al. 1996; Coy and Taylor 1996; Pearson et al. 1996; Piwko et al. 1996, 1997a, b).

Greenmman and Melmed (1994) evaluated the expression of three somatostatin receptor subtypes (SSTR3, SSTR4, and SSTR5) in pituitary tumor specimens. SSTR3 expression was studied by reverse transcription coupled to polymerase chain reaction, whereas SSTR4 and SSTR5 expression was determined by ribonuclease protection assay.

Somatostatin occurs not only in the hypothalamus and in the gut but also in several other organs. Somatostatin$_1$ receptors in the nucleus accumbens have been found to mediate the stimulatory effect of somatostatin on locomotor activity in rats (Raynor et al. 1993a). Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28 has been described by O'Carroll et al. (1992).

Cloning and expression of a mouse somatostatin receptor (SSTR 2B) has been reported by Vanetti et al. (1992). Pharmacological properties of two cloned somatostatin receptors have been described by Rens-Domiano et al. (1992).

Subtype-selective peptides have been identified by Raynor et al. (1993b). The somatostatin receptor, SSTR3, coupled to adenyl cyclase, has been cloned by Yasuda et al. (1992). Somatostatin receptors SSTR4 and SSTR5 have been cloned and characterized by Raynor et al. (1993c).

The somatostatin receptor SST_1 mediates inhibition of central neurons (Viollet et al. 1997).

The somatostatin receptor SST_2 inhibits growth hormone release (Raynor et al. 1993b), inhibits parietal cell function and ion secretion in rat distal colon (McKeen et al. 1996; Warhurst et al. 1996; Wyatt et al. 1996), and inhibits neurons in the rat locus coeruleus (Chessell et al. 1996). High basal gastric acid secretion has been found in somatostatin receptor subtype 2 knockout mice (Martinez et al. 1998). The cytoplasmatic tail of the somatostatin receptor SST_2 undergoes alternative splicing giving rise to two isoforms, SST_{2A} and SST_{2B} (Schulz et al. 1998).

The somatostatin receptor sst_3 mediates relaxation of isolated gastric smooth muscle cells (Gu et al. 1995).

The fourth human somatostatin receptor has been cloned and characterized by Rohrer et al. (1993).

The somatostatin receptor sst_5 may have a role in the inhibition of hormone release from the pituitary (Tallent et al. 1996) and pancreas (Rossowski and Coy (1994)) and mediate the antiproliferative effect of somatostatin in vascular smooth muscle (Lauder et al. 1997).

Procedure

Crude membrane preparations are obtained by homogenizing (Polytron, setting 6, 15 s) tumor cell cultures (e.g., human SCLC line NCI-H69, rat pancreatic tumor AR42J) or rat tissues (lung, pancreas, cerebral cortex) in ice-cold 50 mM Tris–HCl and centrifuging twice at 39,000 *g* (10 min), with an intermediate resuspension in fresh buffer. The final pellets are resuspended in 10 mM Tris–HCl for assay. Aliquots of the membrane preparation are incubated for 25 min at 30 °C with [^{125}I-Tyr^{11}]SRIF (2000 Ci/mmol, Amersham) in 50 mM HEPES (pH 7.4) containing bovine serum albumin (10 mg/ml; fraction V, Sigma), $MgCl_2$ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenylmethylsulfonyl fluoride (0.02 mg/ml). The final assay volume is 0.3 ml. The incubations are terminated by rapid filtration through Whatman GF/C filters (presoaked in 0.3 % polyethylenimine) under reduced pressure. Each tube and filter is then washed three times with 5 ml aliquots of ice-cold buffer. Specific binding is defined as the total [^{125}I-Tyr^{11}]SRIF bound

minus that bound in the presence of 200 nM unlabeled SRIF.

Evaluation

The binding parameters are calculated from the experimental data by nonlinear least-squares regression analysis using an appropriate computer program.

Critical Assessment of the Method

Somatostatin occurs in many organs and has more activities than anticipated at its discovery. Therefore, the occurrence of a family of receptors rather than a single receptor is not surprising.

Modifications of the Method

Receptor scintigraphy with a radioiodinated somatostatin analogue has been described by Bakker et al. (1990).

The tissue-selective binding of somatostatin-14 and somatostatin-28 in rat brain was studied by Srikant et al. (1990).

Feniuk et al. (1993) characterized somatostatin receptors in guinea pig isolated ileum, vas deferens, and right atrium.

Yang et al. (1998) reported synthesis and biological activities of potent peptidomimetics selective for somatostatin receptor subtype 2 by testing inhibition of forskolin-stimulated cAMP accumulation in a mouse cell line that contains a cAMP response element fused to the *E. coli* β-galactosidase gene and by measuring growth hormone release from primary cultures of rat anterior pituitary cells.

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Secretin Activity

Purpose and Rationale

Synthetic derivatives of secretin can be tested for activity against the standard using the bioassay of a chronic pancreatic fistula in dogs. Moreover, stability of secretin preparations has to be checked when using this bioassay.

Procedure

Dogs with a chronic pancreatic fistula prepared according to Preshaw and Grossman (1965), Herrera et al. (1968) are used. Eighteen hours prior to the experiment, food is withdrawn but water allowed ad libitum. Pancreatic juice is collected for 15-min periods. After 1-h pretest time, a test dose of 0.2 KE/kg Karolinska secretin is given intravenously. In 1-h intervals, the test preparation or the standard is given alternatively in logarithmic doses between 0.1 and 1.6 KE/kg intravenously. The volume of pancreas juice secreted during 30 min after injection is used as parameter.

Evaluation

Using at least two doses of test compound and standard, parallel line assays can be carried out and potencies can be calculated. Each of four animals receives two doses test compound and two doses standard, the application scheduled according to a Latin square. Potency ratios with confidence limits are calculated.

Critical Assessment of the Method

The method is time-consuming and needs dogs with chronic fistula. Nevertheless, for final evaluation of a test compound, this bioassay cannot be avoided.

Modifications of the Method

The standardization of secretin has been described as early as 1952 by Burn et al. using anesthetized **cats**. After laparotomy, the pylorus and first duodenal loop are brought outside and turned to the animal's left side. The bile duct is identified by gently squeezing the bile bladder. The pancreatic duct usually enters the duodenum about 2 mm below the entry of the bile duct. Starting about 2 cm below the bile duct, the pancreas is separated from the duodenum by blunt dissection, with the blood vessels being tied and divided where necessary. When the pancreatic duct is reached, two silk ligatures are placed around and a length of 5 mm is dissected. A dose of secretin is injected intravenously, and one of the ligatures is then tied as near the duodenum as possible. The duct is then cut and a flow of juice is usually visible. A small cannula is tied into the duct and attached to a rubber tube and L-piece whose position is adjusted so that the flow of juice continues. The cannula and the L-piece are clamped in position and the abdomen is closed.

The relation between the dose injected and the number of drops of pancreatic juice secreted is almost linear, but the slope differs in different cats. In making a comparison between two preparations, the dose–response relation is first determined for the standard preparation by the administration of three or more different doses. A dose of the test preparation is then injected and the effect compared with the curve of the standard. Further doses of the test preparation and comparison of the dose–response curves allow the calculation of the relative potency.

Izzo et al. (1989) studied the internalization of labeled secretin into isolated pancreatic acinar cells of **rats**.

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Receptor Binding for Secretin

Purpose and Rationale

Secretin receptor antagonists, such as reduced peptide bond pseudopeptide analogues of secretin, inhibit the binding of ^{125}I-secretin to secretin receptors in pancreatic acini (Haffar et al. 1991).

Radioimmunoassays for secretin have been developed (Boden and Wilson 1979; Chang and Chey 1980) and are available as commercial kits.

Procedure

Dispersed acini from guinea pig pancreas are prepared according to Peikin et al. (1978) and Jensen et al. (1982). After sacrifice, the pancreas of a guinea pig is immediately removed and trimmed of fat and mesentery. A suspension of dispersed acini is prepared by injecting the pancreas five times with 5 ml of digestion solution composed of standard incubation solution plus purified collagenase (0.12 mg/ml). The pancreas is incubated for four sequential 10-min periods at 37 °C in a Dubnoff incubator at 160 oscillations per min. The gas phase is 100 % O_2. The digestion solution is discarded, and the tissue is washed three times with 5 ml of standard incubation solution. The tissue is dispersed by passing it five times through each of a series of siliconized glass pipettes of decreasing bore (3, 1, and 0.5 mm). Large fragments and the duct system are discarded. The suspension of dispersed acini is layered over standard incubation solution containing 4 % albumin and centrifuged at 800 g. The supernatant is discarded, and the acini are washed twice with standard incubation solution containing 4 % albumin.

^{125}I-secretin and ^{125}I-labeled analogues are prepared using chloramine T and a method described by Chang and Chey (1980), Jensen et al. (1983), Zhou et al. (1989). They are separated from ^{125}I using a disposable C_{18} cartridge (Sep-Pak) and then separated from unlabeled peptide with reverse phase HPLC using a 4.6-mm × 25-cm column of mBondapak C_{18}. The column is eluated with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid (v/v) from 0 % to 54 % acetonitrile in 60 min using a flow rate of 1.0 ml/min.

For binding of ^{125}I-secretin, incubations are performed containing 0.25 ml of cell suspension of pancreatic acini (one pancreas in 10 ml of standard incubation buffer) and 50 pM ^{125}I-secretin with or without 1 mM secretin. Nonsaturable binding of ^{125}I-secretin is measured as the amount of radioactivity associated with acini when the incubation contains 50 pM ^{125}I-secretin plus 1 mM secretin. Secretin antagonists are added in various concentrations. Saturable binding of ^{125}I-secretin is expressed as percentage of radioactivity bound in the absence of the antagonists.

Evaluation

K_i values are calculated from percent of controls of bound ^{125}I-secretin using the equation:

$$K_i = [R/(R-1)][SB/(S+A)]$$

where R = the observed saturable binding of ^{125}I-secretin in the presence of the antagonist (B) expressed as a fraction of that obtained when B is not present. A is the concentration of ^{125}I-secretin (0.05 nM), and S is the k_d determined by Scatchard analysis.

Modifications of the Method

Bawab et al. (1991) characterized the downregulation of the ^{125}I-secretin binding sites and the associated desensitization of the secretion receptor-cAMP system in rat gastric glands.

Molecular cloning and expression of a cDNA encoding the secretin receptor in COS cells was reported by Ishihara et al. (1991).

Steiner et al. (1993) localized secretin receptors mediating rat stomach relaxation by autoradiography of frozen sections of the rat stomach with ^{125}I-labeled porcine secretin.

Ulrich et al. (1993) studied the intrinsic photoaffinity labeling of native and recombinant rat pancreatic secretin receptors.

Vilardaga et al. (1994) investigated the properties and regulation of the coupling to adenylate cyclase of secretin receptors stabile transfected in Chinese hamster ovary cells.

Molecular cloning, expression, and functional characterization of a human secretin receptor was reported by Chow (1995) and Patel et al. (1995) of the rabbit secretin receptor by Svoboda et al. (1998). The full-length human secretin receptor cDNA was subcloned into the mammalian expression vector pRc/CMV and expressed in cultured CHO cells (Ng et al. 1999). Intracellular cAMP accumulation of the stably transfected cells was measured by a radioimmunoassay, while the extracellular acidification rate was measured by the Cytosensor microphysiometer.

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Cholecystokinin Activity (Isolated Rat Pancreatic Acini)

Purpose and Rationale

Cholecystokinin (CCK) is one of the first discovered gastrointestinal hormones and one of the most abundant neuropeptides in the brain (Crawley and Corwin 1994). CCK is found in high concentrations in the mammalian brain and may be implicated in the neurobiology of anxiety and panic disorder (Bourin et al. 1996). Two types of CCK receptors have been identified (Wank 1995; Noble et al. 1999; Miyasaka and Funakoshi 2003): CCK-A receptors which are mainly located in the periphery, but are also found in some areas of the CNS, and CCK-B receptors (Noble and Roques 1999) which are widely distributed in the brain. Major biological actions of CCK are the reduction of food intake (Moran and Kinzig 2004) and the induction of anxiety-related behavior. Inhibition of feeding is mainly mediated by the A-type receptors, whereas anxiety-like behavior is induced by stimulating B-type receptors (Fink et al. 1998).

Isolated pancreatic acini of rats can be used for evaluation of cholecystokinin activity of synthetic derivatives and for plasma CCK-like activity (Liddle et al. 1984; Höcker et al. 1990; Schmidt et al. 1991).

Procedure

For plasma determinations, CCK or inhibitors are extracted from a plasma sample using PR-18 cartridges (Merck, Darmstadt, Germany). One ml plasma is diluted with 4 ml 0.1 % trifluoroacetic acid and applied to a cartridge. After a wash (15 ml), CCK and inhibitor are eluted with acetonitrile/water (80:20, v/v) and lyophilized. Pancreatic acini are prepared from female Sprague–Dawley rats weighing 180–200 g, 1–2 weeks post ovariectomy, by enzymatic digestion of pancreas with collagenase (Jensen et al. 1982). Test compounds, or extracted material reconstituted with Krebs–Ringer HEPES buffer, or CCK standard is incubated with acini (final volume 0.25 ml). Lipase is measured with an autoanalyzer (e.g., Hitachi type 705 or 805). Release is calculated as percent of the initial content determined in each incubation vial.

Evaluation

CCK bioactivity is determined by comparison with a CCK-8 standard curve. Results are expressed as CCK-like bioactivity.

Modifications of the Method

Amblard et al. (1998) evaluated cyclic cholecystokinin analogues for their ability to stimulate amylase secretion from isolated pancreatic acini.

Inhibition of CCK-8-induced release of amylase from pancreatic cells was used for

measurement of CCK_A antagonism (Yamazaki et al. 1995; Akiyama et al. 1996; Patel et al. 1996; Taniguchi et al. 1996; Ballaz et al. 1997; Martin-Martinez et al. 1997).

Deyer et al. (1993) reported on acetylcholine- and cholecystokinin-induced acid extrusion in mouse isolated pancreatic acinar cells as measured by the microphysiometer. The microphysiometer continually measures the pH of the medium bathing a cell sample. EC_{50} values for the acidification rate were determined for CCK and CCK analogues. Dunlop et al. (1997) used the Cytosensor microphysiometer to analyze the activity of cholecystokinin antagonists against the CCK-4-mediated response in hCCK-B CHO cells.

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Receptor Binding of Cholecystokinin

Purpose and Rationale

Receptor binding assays for CCK have been described by several authors, such as Innis and Snyder (1980) for rat pancreas; Gaisano et al. (1989), Doi et al. (1990), Blevins et al. (1996), and Maletínská et al. (1992) for isolated pancreatic acini; Saito et al. (1981) and Chang et al. (1983) for guinea pig cerebral cortex; Praissman et al. (1983b) for guinea pig mucosa; Van Dijk et al. (1984) for rat brain and pancreas; Steigerwalt et al. (1984) and Chang and Lotti (1986) for gallbladder membranes; and Kaufmann et al. (1993) for guinea pig cerebral cortex and rat pancreas.

Using transfected COS cells, Talkad et al. (1994) identified three different states of the pancreatic CCK receptor, with the very-low-affinity state being the most abundant.

DeTullio et al. (1999) and Herranz (2003) reviewed advances in the chemistry of cholecystokinin receptor agonists and antagonists.

Radioimmunoassays for cholecystokinin have been developed (Harvey 1979) and are available as commercial kits.

Procedure

Membranes of rat (Sprague–Dawley) pancreas, guinea pig (Hartley) cerebral cortex, and bovine gallbladder are prepared by homogenization in 50–100 vol of 50 mM Tris–HCl (pH 7.4 at 37 °C) using a Polytron (Brinkman, PT 10, setting 4 for 10 s for pancreas or brain and maximal speed for bovine gallbladder). Homogenates are centrifuged at 50,000 g for 10 min, and the pellets are resuspended in the same buffer and centrifuged as described above. The resulting pellets are resuspended in 4,000, 80, and 25 ml of binding assay buffer for each g of original tissue wet weight of the pancreas, brain, and gallbladder, respectively. ^{125}I-CCK-8 binding assay buffer contains 5 mM dithiothreitol, 50 mM Tris–HCl (pH 7.4 at 37 °C), 5 mM $MgCl_2$, 2 mg of bovine serum albumin, and bacitracin at 0.14 mg/ml for pancreas; 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, and 130 mM NaCl (pH adjusted to 6.5 with NaOH) for brain; and 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, soybean trypsin inhibitors at 0.2 mg/ml, and 130 mM NaCl (pH 6.5) for bovine gallbladder.

^{125}I Bolton–Hunter-labeled CCK-8 (New England Nuclear) is used. Free and bound ^{125}I-CCK-8 are separated by filtration using Whatman

G/F B glass fiber filters that are presoaked with 50 mM Tris–HCl (pH 7.4) containing bovine serum albumin (1 mg/ml). Immediately after the filtration, the filters are washed rapidly three times with 4 ml of Tris–HCl containing bovine serum albumin (0.1 mg/ml). Radioactivity is counted with a gamma counter, e.g., Beckman Instruments. Specific ^{125}I-CCK-8 binding is defined as the difference between total binding and nonspecific binding in the presence of 1 mM CCK.

Evaluation

IC_{50} values are determined by regression analysis of displacement curves. Inhibitor constants (K_i) are calculated from the formula

$$K_i = I/(K_{d'}/K_{d-1})$$

where I is the concentration of the inhibitor, and K_d and $K_{d'}$ are the dissociation constants of ^{125}I-CCK-8 in the absence and presence of the inhibitor, respectively.

Modifications of the Method

Subtypes of the cholecystokinin receptor, forming a cholecystokinin receptor family, have been described, such as CCK_A from pancreas and other parts of the gastrointestinal tract and in a few discrete brain regions (Kachur et al. 1991; Poirot et al. 1992; Wank et al. 1992a).

According to the IUPHAR Compendium of Receptor Characterization and Classification 1998, CCK_A and CCK_B receptors are designated as CCK_1 and CCK_2, respectively.

A survey on ligands for cholecystokinin receptors was given by Trivedi (1994).

Van der Bent et al. (1994) described molecular modeling of **CCK-A receptors**.

For CCK_A receptor binding assays (Fossa et al. 1997), the pancreas from a male Hartley guinea pig is dissected and placed in saline. Fatty tissue and blood vessels are dissected away and the tissue placed in 20 vol of buffer (50 mM Tris–HCl, pH 7.4, 0.35 mg/ml bacitracin, and 0.5 mg/ml soybean trypsin inhibitor) at 4 °C and minced using scissors. The tissue is homogenized (Polytron, setting no. 9 for two 15-s bursts), strained through gauze, and centrifuged at 100,000 g for 15 min at 4 °C. The supernatant is discarded and the pellet resuspended in 20 vol of buffer and recentrifuged. The final pellet is diluted to a concentration of 1.25 mg/ml (original wet weight) in buffer and kept on ice until used. The incubation reaction is initiated by the addition of 100 µl of tissue to 96-well plates containing 150 µl of incubation buffer (50 mM Tris–HCl, pH 7.4, and a final concentration of 5 mM $MgCl_2$, 5 mM dithiothreitol, and 1 % DMSO) with 60 pM final concentration of ^{125}I-BH-CCK_{8S} and drug or vehicle. After a 30-min incubation, the reaction is terminated by rapid filtration using a Skatron cell harvester (Skatron Instruments, Inc. VA) onto GF/B filters that were soaked for 2 h in 50 mM Tris–HCl and 0.1 mg/ml bovine serum albumin. The filters are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Povoski et al. (1994) reported cholecystokinin receptor characterization and cholecystokinin A receptor messenger RNA expression in transgenic mouse pancreatic carcinomas and dysplastic pancreas.

Yule et al. (1993)and Blevins et al. (1994) recorded intracellular Ca^{2+} concentration signaling stimulated by cholecystokinin or by a partial CCK agonist in Chinese hamster ovary-CCK-A cells or in isolated pancreatic acini.

Ghanekar et al. (1997) established a Chinese hamster ovary cell line bearing the mouse type A cholecystokinin receptor.

CCK-A agonists were evaluated as anorectic agents (Pierson et al. 1997; Simmons et al. 1998).

CCKB receptors occur predominantly in the central nervous system indicating involvement in behavioral functions (Moran et al. 1986; Wank et al. 1992b; Derrien et al. 1994; Schäfer et al. 1994) but also in pancreatic cancer cells (Smith et al. 1993; Zhou et al. 1992) and small lung cell cancer lines (Sethi et al. 1993).

For CCK_B receptor binding assays (Fossa et al. 1997), guinea pig cortex is homogenized

with a Teflon homogenizer in 20 vol of 50 mM Tris–HCl (pH 7.4) containing 5 mM $MgCl_2$ at 4 °C and centrifuged at 100,000 g for 30 min. The supernatant is discarded and the pellet resuspended and spun again. The pellet is diluted to a concentration of 10 mg/ml (original wet weight) with assay buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 130 mM NaCl, and 0.2 mg/ml bacitracin, pH 6.5) before use. The incubation reaction is initiated by the addition of 50 µl of tissue to 96-well plates containing 150 µl of assay buffer with 1 % DMSO final concentration, 50 pM final concentration of ^{125}I-BH-CCK_{8S}, and the appropriate concentration of drug or vehicle. Nonspecific binding is estimated using 1 µM CCK_{8S}. The reaction is terminated by spinning the plates using a H1000B rotor at 3,000 rpm for 5 min at 4 °C. The pellet is washed with 200 µl of 50 mM Tris–HCl and respun. The supernatant is again discarded, the pellet resuspended, and the tissues harvested onto Betaplate filters soaked in 0.2 % polyethylenimine for 2 h using a Skatron cell harvester (Skatron Instruments, Inc., VA). The filtermats are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Lee et al. (1993) described cloning and characterization of the human brain cholecystokinin-B/gastrin receptor.

Kaufmann et al. (1993) studied the binding of a series of succinylated cholecystokinin tetrapeptide derivatives to different tissues and their effects on intracellular calcium mobilization ($[Ca^{2+}]_i$) in the human T-cell line Jurkat and rat pituitary (GH3) cells.

Durieux et al. (1989) described [^{3}H]pBC 264 as the first highly potent and very selective radioligand for CCK_B receptors.

Slaninova et al. (1995) recommended the radioiodinated CCK_8 analogue, SNF 8702, as a selective radioligand for CCK_B receptors.

Knapp et al. (1990) found CCK-B receptor heterogeneity in various brain areas of the guinea pig using a highly selective CCK-B receptor radioligand.

Dunlop et al. (1996) described the functional characterization of a Chinese hamster ovary cell line transfected with the human CCK-B receptor gene. Functional coupling in these cells was demonstrated using agonist-stimulated mobilization of intracellular Ca^{2+}, measured with the FURA-2 technique.

Kaufmann et al. (1995) described the effects of guanyl nucleotides on CCK_B receptor binding in brain tissue and continuous cell lines, such as Jurkat T lymphocytes, rat pituitary GH3 cells, rat glioma C6 cells, and small cell lung cancer NCI-H69 cells.

Cuq et al. (1997) reported that mRNAs encoding CCK_B but not CCK_A receptors are expressed in human T lymphocytes and Jurkat lymphoblastoid cells.

CCKA receptor antagonists have been described by Chang and Lotti (1986), Makovec et al. (1986), Evans (1993), and Gully et al. (1993) as well as **CCKB receptor antagonists** (Hill and Woodruff 1990; Ohtsuka et al. 1993; Pendley et al. 1993).

Selective non-peptide **CCKB/gastrin receptor antagonists** have been described by (Bertrand et al. 1994; Makovec et al. 1999; Takeuchi et al. 1999). Harper et al. (1999) analyzed some of them in radioligand binding assays in mouse and rat cerebral cortex.

Cholecystokinin dipeptoid antagonists with anxiolytic properties which bind preferably to CCK_B receptors have been reported by Boden et al. (1993).

CCKA receptor antagonists (Ballaz et al. 1997) and **CCKB receptor antagonists** (Revel et al. 1998) with anxiolytic-like activity in animal models were described.

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Acute Experimental Pancreatitis

Purpose and Rationale

Acute interstitial pancreatitis can be induced in the rat by excessive doses of a pancreatic secretagogue (Lampel and Kern 1987) such as cerulein (Renner et al. 1986; Ito et al. 1991; Yazu et al. 1991).

Procedure

Induction of Pancreatitis

Male Wistar rats weighing 200 g are used. Pancreatitis is induced by cerulein given as five intraperitoneal injections of 40 μg/kg each at hourly intervals on day 1. Controls receive saline injections only. Treatment with potential drugs is started at day 1 and continued for 7 or 14 days. The rats are sacrificed after 1 or 2 weeks following cerulein injection.

Preparation of Pancreatic Acini

The rats are decapitated after overnight fasting and the pancreata are quickly removed. The pancreata are injected with digestion medium containing 170 U/ml collagenase and incubated for 15 min at 37 °C. The medium is changed for the second incubation for 60 min with 15 ml digestion medium containing 200 U/ml collagenase. Acini are dissociated by sequential passage through four pipettes of different diameters in a standard medium containing 0.1 % soybean inhibitor. The suspension is filtered through a single layer of gauze and layered over 15 ml of 4 % bovine albumin. After centrifugation for 2 min at 400 rpm, the pellet is washed three times with 20 ml of incubation medium containing 0.5 mM $CaCl_2$. Finally, the acini are resuspended in 10–20 ml of the incubation medium containing 10 mM HEPES, 145 mM NaCl, 4.7 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 16.5 mM glucose, 0.1 % bovine serum albumin, 0.01 % soybean trypsin inhibitor, and Eagles minimal essential amino acids and vitamins, adjusted to pH 7.4, and bubbled with 100 % oxygen.

Measurement of [^{3}H]-Thymidine Uptake Into Pancreatic Acini

Aliquots (5 ml) of the acini suspension are incubated at 37 °C for 30 min, and 6-[^{3}H]-thymidine (5 mCi/mmol) is added as a final concentration of 0.8 mCi/mol. After incubation for 60 min, 200 ml aliquots are filtered by vacuum through Whatman GF/B glass fiber filters. Filters which trapped the acini are washed with 15 ml ice-cold buffer. After adding 5 ml of Aquazol II to each vial, the radioactivity of the filters is counted with a scintillation spectrometer.

Measurement of Amylase Secretion from Pancreatic Acini

Acini are resuspended in 5 ml of the incubation medium and preincubated at 37 °C for 30 min. Carbachol at concentrations between 10^{-7} and 10^{-4} M or CCK-8 at concentrations between 10^{-11} and 10^{-8} M is added, and the acini suspension is incubated at 37 °C for an additional 30 min. Triplicate 300 ml aliquots are centrifuged for 5 s at 3,000 rpm. The amylase activity in the supernatant is measured by a commercial amylase test. Triplicate 500 ml aliquots are sampled before adding the secretagogue, and the amylase contents (total amylase) are measured after sonification.

Evaluation

Thymidine uptake is compared between control, cerulein-treated animals, and animals treated with

drugs. Dose–response curves of amylase secretion after carbachol and CCK-8 are established for each group. Moreover, amylase concentrations in the acini are compared.

Modifications of the Method

Griesbacher and Lembeck (1992) and Griesbacher et al. (1993) studied the prevention of **cerulein-induced** experimental acute pancreatitis in the rat by the bradykinin antagonist HOE 140.

Several authors (Ito et al. 1994; Ogden et al. 1994; Liu et al. 1995; Sledzinski et al. 1995; Weidenbach et al. 1995; Chen et al. 1996; Lembeck and Griesbacher 1996; Asano et al. 1997; Ito et al. 1997) used different dose regimens of cerulein administration to induce acute pancreatitis in rats.

Niederau et al. (1995) used cerulein to induce acute necrotizing pancreatitis in mice and evaluated the protective effects of secretin and of the cholecystokinin receptor antagonists proglumide and benzotript.

Huch et al. (1995) induced necrotizing pancreatitis by intraductal infusion of low-dose glycodeoxycholic acid (10 mmol/l) followed by intravenous cerulein (6 μg/kg/h) for 6 h.

Several other chemicals and drugs may induced acute pancreatitis (Vogel 1994).

Merkord et al. (1997) studied the pathogenesis and the time course of lesions of acute interstitial pancreatitis in rats induced by intravenous injection of 6 mg/kg **dibutyltin dichloride** (DBTC), an organotin compound used in chemical industry and in veterinary medicine. First, the cytotoxic effects on the biliopancreatic duct epithelium lead to epithelial necrosis with obstruction of the duct, subsequent cholestasis, and interstitial pancreatitis, and second, the hematogenic effects of DBTC cause direct injury of pancreatic cells followed by interstitial edema and inflammation. A chronic course is found when the obstruction of the duct and cholestasis persist.

Destruction of acinar cells has been found after the administration of **ethionine** in rats (Herman and Fitzgerald 1962).

Niederau et al. (1985), Neuschwander-Tetri et al. (1994), Norman et al. (1995), Van Laethem et al. (1995), and Taniguchi et al. (1996) described acute necrotizing pancreatitis induced by cerulein in **mice**.

Pancreatitis can be induced in mice by a **choline-deficient diet** (Lombardi et al. 1975; Niederau et al. 1990). Mice weighing 10–14 g are fed regular laboratory chow ad libitum before the experiment. A choline-deficient diet supplemented with 0.5 % ethionine is given for a period of 66 h, after which it is replaced by regular chow.

Lake-Bakaar and Lyubsky (1995), Hirano (1997), and Niederau et al. (1995) induced acute pancreatitis in female Swiss-Webster mice by feeding a choline- and methionine-deficient diet supplemented with 1 % ethionine.

Emanuelli et al. (1994) demonstrated that a single injection of endotoxin (lipopolysaccharides, *E. coli* 0111-B4) into the superior pancreaticoduodenal artery of **rabbits** induced a dose-dependent acute necrotizing pancreatitis.

Watanabe et al. (1993) induced acute hemorrhagic pancreatitis in rats by surgically **closing a 1-cm length of duodenal loop** at points proximal and distal to the orifice of the pancreatic duct for 6 h with bypassing the bile from the liver hilus distal to the closed loop. The effects of Hoe 140, a bradykinin antagonist, were studied.

Ha et al. (1994, 1996) used the closed duodenal loop technique to study the role of endogenous and exogenous cholecystokinin in experimental pancreatitis and the effect of a cholecystokinin receptor antagonist on the early stage of the healing process in acute pancreatitis.

Kimura et al. (1998) found beneficial effects of a cholecystokinin A receptor antagonist in three methods of acute experimental pancreatitis: pancreatitis in mice induced by six intraperitoneal injections of 50 μg/kg cerulein, necrotizing pancreatitis in rats induced by injection of sodium taurocholate into the common bile duct followed by four subcutaneous injections of 50 μg/kg cerulein, and in closed duodenal loop induced pancreatitis in rats.

Obermaier et al. (2001, 2004a, b) described **ischemia-/reperfusion-induced pancreatitis** in

rats. Male Wister rats weighing 290–330 g were anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital. They were placed on a heating pad and ventilated after tracheotomy with normal air (TSE, Bad Homburg, Germany). Polyethylene catheters were placed in the right carotid artery and the left jugular vein. The upper abdomen was opened by transverse laparotomy. The intestine was exteriorized to the right. The stomach was turned up cranially and fixed on the skin by sutures. All exteriorized segments were moistened and covered with transparent foil to prevent drying. The pancreas was separated from the stomach and the short gastric vessels were ligated. The border to the caudal apart of the pancreas tail segment was the constant branch of the pancreaticoduodenal artery running to the caudal part of the spleen. Pancreatic and connective tissues in the area of the celiac axis were dissected. Thus the complete vascular isolation of the pancreatic tail pedunculated on the splenic vessels was accomplished.

The common hepatic artery was identified in order to allow PO_2 measurements, the pancreas was fixed on an oval plastic pad with sutures to the connective tissue, and splenectomy was performed. A PO_2-sensitive Clark-type probe was inserted into the pancreatic tissue. The probe was fixed to connective tissue on its distal end with a suture. For local intra-arterial access, a small polyethylene catheter was inserted in the retrograde direction into the left gastric artery and fixed. The pancreatic tail segment was covered with foil to prevent access of ambient air and drying. After a stabilization period of 10 min, the pancreatic tail segment was flushed with 1 ml of heparinized saline via the catheter in the left gastric artery, after clamping the celiac axis and the common hepatic artery. Paleness of the pancreatic tail segment indicated successful flushing.

For the induction of isolated pancreatic tail ischemia, two clamps were put on the splenic vessels, and then the clamps of the celiac axis and the common hepatic artery were removed. Continuously decreasing tissue PO_2 indicated successful ischemia. The clamps on the splenic vessels were removed after an ischemic period of 120 min, and disappearing paleness always indicated successful reperfusion. The animals were observed for a further 2 h. At the end of the experiment, the pancreas was excised for histological examination. Biopsy samples from the nonischemic head served as control tissue to exclude experimental side effects on the nonischemic pancreatic head.

Microcirculatory measurements were done by intravital fluorescence microscopy. Histological examinations of the perfused pancreas tail were performed after staining with hematoxylin and eosin.

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Taurocholate-Induced Pancreatitis in the Rat

Purpose and Rationale

Retrograde infusion of bile salts into the pancreatic duct induces severe necrotizing pancreatitis indicated by reduced amylase output and histological changes.

Procedure

Male Sprague–Dawley or Wistar rats weighing 200–300 g are anesthetized by IM injection of a mixture of 87 mg/kg ketamine and 13 mg/kg xylazine. After laparotomy, the pancreaticobiliary duct is cannulated through the duodenal papilla with a polyethylene catheter (P10, Clay Adams) which is introduced by means of a puncture in the duodenum. A precision pump is used to infuse 0.6 ml 5 % sodium taurocholate into the pancreaticobiliary duct during a 10-min period at an infusion rate of 6 ml/h. The catheter is then withdrawn and the abdominal cavity surgically closed.

After various time intervals (several hours up to 2 weeks), the animals are anesthetized and the pancreaticobiliary duct is cannulated again. The response to various doses of cerulein is measured.

Evaluation

The degree of amylase output (mg/h) is taken as parameter. Dose–response curves after various doses of cerulein are established.

Modifications of the Method

The effects of various agents on experimental pancreatitis induced by retrograde intraductal injection of **taurocholate** solution or bile acid in rats have been studied by various authors (Bielecki et al. (1994), Hietaranta et al. 1995; Nakae et al. 1995; Niederau et al. 1995; Kimura et al. 1996; Mithofer et al. 1996; Paran et al. 1996; Tachibana et al. 1996; Norman et al. 1997; Plusczyk et al. 1997; Manso et al. 1998).

Tanaka et al. (1995)and Sakai (1996) induced necrotizing pancreatitis by retrograde injection of **deoxycholate** solution into the biliopancreatic duct of rats.

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Chronic Pancreatitis

Purpose and Rationale

Chronic pancreatitis was induced by various means in rats (Zhou et al. 1990, 1994; Goto et al. 1995; Puig-Diví et al. 1996), golden hamsters (Rutishauser et al. 1991, 1995), cats (Reber et al. 1992, 1999; Zhao et al. 1996a, b), dogs (Hayakawa et al. 1993; Tanaka et al. 1994, 1998), and pigs (Vinter-Jensen et al. 1997).

Puig-Diví et al. (1996) induced chronic pancreatitis in rats by trinitrobenzene sulfonic acid infusion into the pancreatic ducts.

Procedure

Male Sprague–Dawley rats weighing 300–350 g are anesthetized with ketamine (100 mg/kg IP.) after an overnight fast. Access to the pancreas is gained through a ventral midline incision. The duodenum is opened and the biliopancreatic duct cannulated through the papilla using polyethylene tubing (PE 10). The biliopancreatic secretion is allowed to drain freely for 15 min. To prevent liver damage, the duct is tied close to the liver. Retrograde infusion is initiated by means of a controlled pressure device that uses the height of a liquid column (trinitrobenzene sulfonic acid or vehicle) in a vertical pipette connected to the infusion cannula to control the intraductal pressure and infusion volume. Ductal pressure is never allowed to exceed 20-cm H_2O. In the treated group, 0.4 ml of 2 % trinitrobenzene sulfonic acid in phosphate buffered saline +10 % ethanol (pH 8) is infused. Ethanol is employed as an epithelial "barrier breaker" to facilitate trinitrobenzene sulfonic acid penetration into the tissue. Rats in the control group undergo the same procedure, except that trinitrobenzene sulfonic acid is absent from the infusion medium.

The total exposure time of the gland to the instillate is 60 min, followed by a washout period of 30 min. Ligatures are then released, the duodenum and the abdominal wall are sutured, and the animals are kept under observation for 2 h after surgery. Rats are then transferred to individual cages, where they are fasted for 24 h. Thereafter, they receive standard chow and their weight gain is recorded weekly.

Blood is withdrawn after various time intervals by cardiac puncture under light ether anesthesia for the determination of glucose and serum α-amylase (EPS test, Boehringer Mannheim GmbH).

Groups of treated rats and controls are sacrificed at 3, 4, and 6 weeks after the surgical procedure. Pancreata are fixed in 10 % neutral buffered formaldehyde and embedded in paraffin. Several sections are cut and stained with hematoxylin–eosin for light microscopy evaluation. The degree of periductal and intralobular fibrosis, patchy acute and chronic inflammatory cell infiltrates, common duct stenosis, and segmentary gland atrophy is evaluated.

Evaluation

Data are expressed as means ± SEM. A two-tailed Student's *t*-test for unpaired values is used for statistical comparison of mean values of serum amylase, glucose, and rat weight.

Modifications of the Method

Chung and Richter (1971)and Zhou et al. (1990, 1994) induced chronic pancreatitis in rats by **ligation of the pancreatic duct**.

Injection of oleic acid (Goke et al. 1989; Goldstein et al. 1989; Andersen et al. 1994; Kakugawa et al. 1996; Seymour et al. 1995, 1998) or of a viscous solution of zein–oleic acid–linoleic acid (Kataoka et al. 1998) into the pancreatic duct was used to induce chronic pancreatitis in rats.

Goto et al. (1995) described a chronic pancreatitis model with diabetes induced by intraperitoneal **cerulein** injection plus water immersion stress in rats.

Pancreatic blood flow was measured in **cats** with chronic pancreatitis induced by partial ligation of the pancreatic duct by Austin et al. (1980), Reber et al. (1992, 1999), and Widdison et al. (1992).

Zhao et al. (1996a, b) induced progressing lesions of chronic pancreatitis in cats by intraductal injection of alcohol or by a combination of intraductal and intraparenchymal injection of ethanol together with partial obstruction of the main pancreatic duct to 70 % of its original lumen by fixation of a small catheter in the papilla.

Hayakawa et al. (1993) induced pancreatolithiasis in **dogs** by partial obstruction of the major pancreatic duct.

Rats of the diabetic strain WBN/Kob (Tsuchitani et al. 1985; Nakama et al. 1985) develop **spontaneously chronic pancreatitis** (Ohashi et al. 1990; Sato et al. 1993; Sugiyama et al. 1996a; Sugiyama et al. 1996; Arai et al. 1998; Ito et al. 1998) with pancreatic fibrosis and parenchymal destruction and both endocrine and exocrine pancreatic dysfunction (see also K.2.0.2).

Transgenic mice overexpressing $TGF_{\beta 1}$ (Sanvito et al. 1995) develop tissue changes in the pancreas resembling changes found in chronic pancreatitis.

Shetzline et al. (1998) identified target tissues of pancreatic polypeptide using an in vivo radioreceptor assay in order to further elucidate the function of this hormone.

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Liver Function

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Contents

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Hepatocellular Function

Hepatitis in Long-Evans Cinnamon Rats

Purpose and Rationale

The Long-Evans Cinnamon strain of rats has been recommended as a useful model to study genetically transmitted fulminant hepatitis and chronic liver disease (Yoshida et al. 1987; Hawkins et al. 1995). The underlying cause is thought to be due to excessive copper accumulation in the liver of Long-Evans Cinnamon rats, thus making this animal a model for Wilson's disease in humans (Okayasu et al. 1992). Chelation therapy or feeding a copper-deficient diet can ameliorate the symptoms in Long-Evans Cinnamon rats and Wilson's disease (Togashi et al. 1992).

Procedure

Male Long-Evans Cinnamon rats obtained from a commercial breeder at an age of 5 weeks are housed in temperature- and humidity-controlled rooms at a 12:12 light/dark cycle. Groups of 6–10 rats are given different diets based on a 15 % purified egg protein diet and supplemented with vitamins or drugs. Drugs are applied via minipumps intraperitoneally implanted under ether anesthesia.

The occurrence of jaundice is easily observable as the time when the ears and tail turn yellow and the urine becomes bright orange, staining the fur in the lower abdominal region. Usually, the jaundice progressively worsens, ending in death of the

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animal within about a week. Incidence of jaundice and mortality versus time are used as parameters.

Evaluation

Statistics are performed using StatView II software package for Student's *t*-test, ANOVA, and the Scheffé F-test for comparison between means. All data are expressed as means. A p-value < 0.05 is used as the threshold of significance.

Modifications of the Method

Several drugs which are known to be effective or which are potentially effective in treatment of Wilson's disease were studied in this animal model, such as D-penicillamine (Togashi et al. 1992; Yokoi et al. 1994; Shimizu et al. 1997), trientine (= triethylenetetramine) (Iseki et al. 1992; Sone et al. 1996; Yamamoto et al. 1997), tetrathiomolybdate (Ogra et al. 1995; Suzuki 1997; Sugawara et al. 1999), or the investigative drug TJN-101 (Yokoi et al. 1995).

The interferon-γ transgenic mouse which carries the mouse INF-γ gene develops chronic hepatitis from the age of 6–10 weeks and was recommended by Okamoto et al. (1999) as a model for **chronic hepatitis**.

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Temporary Hepatic Ischemia

Purpose and Rationale

Hepatocellular function is altered by temporary hepatic ischemia as occurring during surgical management of acute hepatic trauma and being essential during hepatic transplantation. To study this, total hepatic ischemia in rats is produced by placing a ligature around the hepatic artery, the portal vein, and the common bile duct.

Procedure

Hepatic Ischemia Procedure

Male rats, weighing 300–350 g, are fasted for 16 h prior to the experiment but are allowed water ad libitum. The rats are anesthetized by an appropriate method (e.g., isoflurane inhalation, ketamine, etc.), and the abdominal cavity is opened through a midline incision. Splenectomy is performed following which a temporary extracorporeal splenofemoral shunt is established between the splenic vein and the right femoral vein using a PE-190 tubing. To insure total hepatic ischemia, the portal vein, the hepatic artery, and the bile duct are occluded by placing a tourniquet around the vessels. Blood pressure is measured via a catheter inserted into the right femoral artery. After heparinization (10 mg/kg), hepatic ischemia is produced for 60 min. During the ischemic period, 0.7 ml of saline is given i.v. at 20-min intervals for volume replacement. At the end of the 60-min ischemic period, the tourniquet around the portal vein, the hepatic artery, and the bile duct is removed in order to reestablish blood flow to the liver. The abdominal incision is then closed and the animals receive either saline (nontreated) or the drug. Following the administration of saline or the drug, the catheters are removed and the animals are returned to their home cages.

Sham-operated animals are prepared exactly in the same manner except that the tourniquet around the portal vein, the hepatic artery, and the bile duct is not placed.

Measurement of Indocyanine Green Clearance

Three hours following the end of ischemia, the experimental as well as the sham-operated rats are anesthetized and a femoral artery and vein of each animal cannulated. Sodium heparin (400 units) is given i.v. and the animals are allowed to be awake. Indocyanine green is given i.v. at 5 mg/kg (low) or 25 mg/kg (high) to the animals via the femoral vein, and 0.2 ml arterial blood samples are taken at 5, 6, 8, 10, 12, 15, 18, and 20 min later. The blood samples are diluted with 0.8 ml of 1 % bovine serum albumin in normal saline and centrifuged at 6,000 rpm for 20 min at 4 °C. The spectrophotometric absorbance of the supernatant is read at 800 nm and the indocyanine green concentration determined from a standard curve.

Evaluation

The $t_{1/2}$ of indocyanine green clearance is computed for each animal using a computer program which calculates the least square line of log indocyanine green versus time. Mean and standard errors for each group are compared using Student's *t*-test.

Modifications of the Method

Daemen et al. (1989) compared the electromagnetic versus the microsphere and the clearance method for liver blood flow measurement in the rat.

Kawaguchi et al. (2004) described the protective effect of a Rho kinase inhibitor against hepatic ischemia-reperfusion injury in rats.

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Model for Direct Transhepatic Studies in Dogs

Purpose and Rationale

A chronic conscious dog model for repeated transhepatic studies over a period of 6–8 weeks was developed by O'Brien et al. (1991). This model can be applied to the study of the hepatic effects of pancreatic hormone secretion and glucose metabolism, to studies of the hepatic mechanisms associated with high first-pass metabolism and food interactions of drugs (Semple et al. 1990), and to studies of insulin balance in dogs that have undergone previous pancreatectomy and islet cell autotransplantation.

Procedure

Four Silastic catheters, 0.062 in. ID × 0.125 in. OD (lengths: carotid 70 cm; jugular 70 cm; hepatic 80 cm; and portal 70 cm), and two ultrasonic transit time flow probes suitable for long-term implants (Burton and Gorewit 1984), 4 mm for the hepatic artery and 6 mm for the portal vein, are cleaned with chlorhexidine scrub and rinsed with distilled water. Double velour Dacron cuffs are placed 15 cm from the external ends of all the devices.

Male dogs weighing 20–25 kg are sedated and anesthetized with an appropriate method (e.g., isoflurane inhalation). Skin interface sites and subcutaneous pockets for placement of catheters are prepared. After skin closure the external ends of the catheters are sealed. Then the catheters and flow probes are placed into the abdomen by retrieving them from the subcutaneous pockets. First, the hepatic artery flow probe is placed, and then the portal venous flow probe inserted. To eliminate extrahepatic blood flow, the gastroduodenal artery is ligated. Then, the portal vein and the hepatic vein catheters are placed. After ensuring catheter patency, the abdomen is closed. Finally, a carotid artery catheter and a jugular venous catheter are placed.

Evaluation

Blood samples can be withdrawn from the catheters placed into the carotid artery, the right external

jugular vein, the portal vein, and the hepatic vein. Blood flow is measured by flow probes in the hepatic artery and the hepatic portal vein.

The following values are measured:

- Plasma flow in the portal vein and plasma flow in the hepatic artery
- Drug concentration in the portal vein, in the hepatic artery, in the hepatic vein, and in the right external jugular vein

From these data plasma flux in the portal vein, in the hepatic artery, and in the hepatic vein and the interval areas under the curve for these vessels are calculated.

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Liver Cirrhosis and Necrosis

General Considerations

Various factors induce liver cirrhosis in man, such as alcoholism, viral hepatitis, intoxications, bile duct disorders, inborn diseases, and others. In the process leading to cirrhosis, accumulation of connective tissue and parenchymal regeneration are competing events. Excessive formation of connective tissue with collagen overproduction in the liver reduces hepatic blood flow, impairs the metabolic functions of the liver, and increases portal vein pressure. These mechanisms result in hepatic failure, esophageal bleeding, portal hypertension, and ascites. Therefore, the search for agents to prevent liver cirrhosis is focused on inhibitors of excessive connective tissue formation in the liver (Kervar et al. 1976; Nolan et al. 1978). The main component of connective tissue formed as a response to chronic injury is collagen. The collagenous fibers consist of triple-helical molecules. Their formation depends on the presence of hydrogen bonds involving the posttranslationally hydroxylated amino acid hydroxyproline. If the number of hydrogen bonds is reduced due to a decrease of hydroxylated amino acids, the resulting collagen cannot form the triple helix and is degraded instead of being deposited in the extracellular matrix.

Insoluble collagen is responsible for most of the mechanical functions of connective tissue, e.g., the bone, tendon, and skin, being influenced by hormones; by desmotropic drugs, such as D-penicillamine; and by maturation and age (Vogel 1969, 1972, 1974a, b, 1976, 1978, 1980, 1989; Bickel et al. 1990, 1991). The aim of fibrosuppressive compounds is to reduce only the excessive formation of insoluble collagen in the liver leaving collagen synthesis and turnover in other tissues intact. Fibrosuppressive effects by inhibition of proline hydroxylation can be screened with in vitro methods; however, the desired organ specificity has to be tested in models of liver cirrhosis and fibrosis in vivo together with functional studies of the connective and supporting tissue.

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Inhibition of Proline Hydroxylation

Purpose and Rationale

The thermal stability of the triple helix of collagenous proteins is crucially dependent upon the intramolecular hydrogen bonds involving the 4-hydroxyproline residues synthesized by the enzyme prolyl 4-hydroxylase. This makes the enzyme a possible target for therapeutic antifibrotic agents.

Procedure

Enzyme activity is assayed in sealed test tubes. The reaction volume of 1 ml contains 50 mM Tris buffer, pH 7.5, 10–100 mM (60,000 d.p.m.) 2-oxo [1-^{14}C]glutarate, 1–50 mM $FeSO_4$, 0.1–1 mM ascorbate, 10–100 mg $(Pro–Pro–Gly)_{10}$, 0.1 mg catalase, 2 mg bovine serum albumin, 100 mM dithiothreitol, 0.05–0.2 mg enzyme, and inhibitors in various concentrations. After incubation at 37 °C for 30 min, the generated $^{14}CO_2$ is trapped and determined.

Evaluation

Inhibition modes are determined by plotting 1/v versus 1/concentration of the variable substrate (Lineweaver–Burk plot). The K_i values are derived from a secondary transformation (slopes or intercepts vs. inhibitor concentrations). The lines of best fit for primary plots and secondary transformations are calculated by using the method of least squares. The mean K_i value of 4–6 experiments is calculated.

Modifications of the Method

The collagen hydroxylases lysyl hydroxylase and prolyl 3-hydroxylase have similar reaction mechanisms as prolyl 4-hydroxylase, differing only in

the specificity for the amino acid sequence of the substrate (Kivirikko and Myllylä 1982). Instead of $(Pro–Pro–Gly)_{10}$, 50–500 mg Arg–Ala–Gly–Ile–Lys–Gly–Ile–Arg–Gly–Phe–Ser–Gly is used.

The activity of prolyl 3-hydroxylase is assayed in a reaction volume of 1.5 ml containing 50 mM Tris buffer, pH 7.5, and 1,000,000 d.p.m. of biologically obtained [2,3-^{3}H]proline-labeled protocollagen substrate in which all proline residues recognized by proline 4-hydroxylase are converted to hydroxyproline, 2 mM ascorbate, 0.2 mg/ml catalase, 2 mg/ml bovine serum albumin, 15 mM 2-oxoglutarate, 50 mM Fe^{2+}, 100 mM dithiothreitol, 0.2–2 mg enzyme, and inhibitors at various concentrations. After incubation at 37 °C for 30 min, the reaction is stopped by addition of 0.5 ml 10 % trichloroacetic acid (w/v). The reaction mixture is then distilled, and 1.6 ml of $^{3}H_2O$ is counted for radioactivity.

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Influence on Collagen Synthesis in Human Skin Fibroblasts

Purpose and Rationale

Secretion of collagen by fibroblasts and other cells capable of synthesizing extracellular matrix is dependent on the hydroxylation of proline residues by prolyl 4-hydroxylase. This enzyme is located in the cisternae of the endoplasmic reticulum. An agent aimed at inhibition of this enzyme must therefore pass both the external cell membrane and the endoplasmic reticular membrane. Organ specificity of a prolyl 4-hydroxylase inhibitor can be achieved by applying a prodrug which can be converted to the active agent only in cells of specialized tissues, e.g., in the liver, but not generally in fibroblasts.

Procedure

Confluent cultures of human skin fibroblasts are preincubated for 24 h at 37 °C without serum in glutamine-free Dulbecco's minimal essential medium supplemented with 50 mg/ml sodium ascorbate, 60 mg/ml 3-aminopropionitrile, and 100 U/ml penicillin G. The cells are then exposed to the potential inhibitor at various concentrations for 20 min, followed by the addition of 2 mCi [U-^{14}C]proline/ml. The incubation is continued for 5 h at 37 °C. Then, the cells are separated from the medium. After removal of non-incorporated [^{14}C]proline, the proteins from medium and cells are hydrolyzed, and the hydroxyproline content is determined by amino acid analysis. The total incorporation of radioactivity serves as marker for protein synthesis.

Evaluation

Two individual samples are taken for each concentration of the inhibitor and six samples for the controls. Proline incorporation is expressed as % of control radioactivity. Hydroxyproline synthesis is expressed as relative Hyp/Pro ratio according to the formula:

$$\left(\mathrm{Hyp/Pro_{sample}/Hyp/Pro_{control}}\right) \times 100.$$

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Influence on Collagen Synthesis in Chicken Calvaria

Purpose and Rationale

To find fibrosuppressive agents for therapeutic use, it is necessary to have prodrugs which cross cell membranes readily and are converted intracellularly to the active agent. Proinhibitors which are cleaved hydrolytically can suppress collagen synthesis in chicken calvaria.

Procedure

Calvaria are removed from chicken embryos, age 15 days, and washed for 3 min with DMEM at 37 °C. They are then transferred into Pyrex tubes (8–10 calvaria/tube) containing 3 ml medium supplemented with 2 mM glutamine, 6 mCi [U-^{14}C-]proline, and various concentrations of the inhibitor. The samples are incubated for 1.5–6 h at 37 °C. The experiment is terminated by placing the tubes in ice and separation of calvaria from the culture medium. The calvaria are washed once with 3 ml of fresh medium, which is then pooled with the incubation medium. Bovine serum albumin and phenyl methyl sulfonyl fluoride are added (final concentration 1 mg/ml and 6 mg/ml, respectively). The calvaria are extracted for 16 h with 25 ml of 0.5 M acetic acid.

The following procedure is identical for the medium and the calvaria extracts. The samples are extensively dialyzed against 0.5 M acetic acid at 4 °C. Aliquots are withdrawn for SDS-PAGE1 and the triple-helix stability assay is performed. The remaining material is lyophilized, resuspended in 2 ml 6 N HCl, and hydrolyzed at 105 °C for 24 h. After evaporation of the acid, the samples are dissolved in 2 ml H_2O, and the hydroxyproline content is determined according to Juva and Prockop (1966).

In order to study the degree of collagen hydroxylation and proportion of collagen biosynthesis, calvaria are incubated in the presence of 10 mCi [3,4-^{3}H]proline/ml and 2 mCi [U-^{14}C] proline/ml for 3 h under the conditions described above. After lyophilization, aliquots of media and calvaria samples are digested with collagenase according to Peterkofsky et al. (1982). The degree of hydroxylation is calculated from the ^{3}H/^{14}C ratio in the digested material; the amount of collagen as a proportion of total protein synthesis is determined by the relation of collagenase degradable versus collagenase-resistant radioactivity. The stability of the extracted collagenous material against digestion by a trypsin/chymotrypsin mixture is tested according to the procedure proposed by Bruckner and Prockop (1981): An aliquot of either culture medium or calvaria extract is incubated for 15 min at a temperature between 25 °C and 45 °C in a total volume of 800 µl of 0.04 M NaCl/0.1 Tris, pH 7.4. After quenching to 0 °C, the sample is digested with 200 µl of a mixture of each 1 mg trypsin and chymotrypsin/ml buffer for 15 min at room temperature. One hundred microliter of 1 mg BSA/ml buffer is added, and the

protease-resistant radioactivity, consisting of triple-helical collagen, is precipitated with 100 µl 100 % trichloroacetic acid (w/v). The sample is transferred in total to a Schleicher & Schuell OE 67 filter paper of 2.5 cm diameter. The digested material is removed by repeated washing with cold 5 % trichloroacetic acid and methanol. The filters are then dried and the radioactivity is determined. Unhydrolyzed samples are studied by SDS-polyacrylamide gel electrophoresis and autofluorography. The morphologic appearance of the control and treated cultures is studied by electron microscopy.

Evaluation

IC_{50} values of hydroxyproline synthesis are read graphically from concentration response curves. Total protein synthesis is estimated as the incorporation of proline; the mean ± standard deviation is calculated from four samples.

Modifications of the Method

Canalis et al. (1977) used cultured calvaria from 21-day fetal rats to study the effects of insulin and glucagon on bone collagen synthesis.

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Allyl Alcohol-Induced Liver Necrosis in Rats

Purpose and Rationale

Administration of allyl alcohol induces focal liver necrosis in rats which can be partially prevented by treatment with several drugs such as antibiotics.

Procedure

Female Wistar rats weighing 120–150 g are used. At 8:00 a.m. of the first day, food but not water is withdrawn. At 3:00 p.m. the compounds to be tested for protective activity are administered i.p. or orally. One hour later, the animals are dosed orally with 0.4 ml/kg of a 1.25 % solution of allyl alcohol in water. At 8:00 a.m. of the second day, the treatment with the potentially protective drugs is repeated. Food but not water is withheld until the third day. At 8:00 a.m. of the third day, the animals are sacrificed and the liver removed. The parietal sides of the liver (left, medium, and right lobe and lobus caudatus) are checked using a stereomicroscope with 25 times magnification. Focal necrosis is observed as white-green or yellowish hemorrhagic areas clearly separated from unaffected tissue. The diameter of the necrotic areas is determined using an ocular micrometer. These values are added for each animal to obtain an index for necrosis.

Evaluation

Using 10 animals for controls and for each treatment group, the mean of necrosis index is calculated and compared with Student's *t*-test. The protective effect is expressed as percentage decrease of the necrosis index versus controls.

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Carbon Tetrachloride-Induced Liver Fibrosis in Rats

Purpose and Rationale

Chronic administration of tetrachloride to rats induces severe disturbances of hepatic function together with histologically observable liver fibrosis.

Procedure

Groups of 20 female Wistar rats with a starting body weight of 100–150 g are used. The animals are treated orally twice a week with 1 mg/kg carbon tetrachloride, dissolved in olive oil 1:1, over a period of 8 weeks. The animals are kept under standard conditions (day/night rhythm 8:00 a.m. to 8:00 p.m., 22 °C room temperature, standard diet, and water ad libitum). Twenty animals serve as controls receiving olive oil only, 40–60 animals receive the carbon tetrachloride only, and groups of 20 rats receive in addition the compound under investigation in various doses by gavage twice daily (with the exception of the weekends, when only one dose is given) on the basis of the actual body weight. The animals are weighed weekly.

At the end of the experiment (8 weeks), the animals are anesthetized and exsanguinated through the caval vein.

In the *serum*, the following parameters are determined:

- Total bilirubin
- Total bile acids
- 7S fragment of type IV collagen
- Procollagen III N-peptide

The following organs are prepared for determination of hydroxyproline:

- Liver
- Kidney
- Aortic wall
- Tail tendons

The specimens of the organs are weighed and completely hydrolyzed in 6 N HCl. Hydroxyproline is measured by HPLC and expressed as mg/mg wet weight of the organs.

To measure **mechanical properties of connective tissue**, the following organs are prepared: the femur and tibia of both sides, tail tendons, and strips from the dorsal skin (Bickel et al. 1990, 1991a). Furthermore, the influence on the healing process of skin wounds is studied.

For **histological analysis**, three to five pieces of the liver weighing about 1 g are fixed in formalin and Carnoy solution. Three to five sections of each liver are embedded, cut, and stained with azocarmine aniline blue (AZAN) and evaluated for the development of fibrosis using a score of 0–IV.

Grade 0:	Normal liver histology
Grade I:	Tiny and short septa of connective tissue without influence on the structure of the hepatic lobules
Grade II:	Large septa of connective tissue flowing together and penetrating into the parenchyma. Tendency to develop nodules
Grade III:	Nodular transformation of the liver architecture with loss of the structure of the hepatic lobules
Grade IV:	Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scars

Evaluation

For detection of significant differences ($p < 0.05$), the unpaired *t*-test is used. For comparison of the scores in the histological evaluation, the χ^2-test is used.

Modifications of the Test

Instead of chronic intoxication with carbon tetrachloride resulting in liver fibrosis, acute hepatocellular damage can be achieved by short-term application of carbon tetrachloride.

Wistar rats with a starting body weight of 120–150 g are treated daily for 5 days with various oral doses of the compound under investigation. From day 2 to day 5 (four applications), the rats receive by gavage a dose of 1 mg/kg carbon tetrachloride dissolved in olive oil (1:1). Blood is withdrawn every day and the aminotransferases ALAT and ASAT, as well as total bilirubin, are determined in the serum.

Niederberger et al. (1995, 1998) induced liver cirrhosis and ascites in rats by treatment with carbon tetrachloride.

Sakamoto et al. (2005) induced liver cirrhosis in rats by intraperitoneal injection of 10 mg/kg dimethylnitrosamine three times a week for 3 weeks.

Kawaura et al. (1993) produced liver cirrhosis with ascites in **dogs** by administration of 2 ml carbon tetrachloride per kg body weight once a week for 4 weeks. Eight weeks afterwards, the supradiaphragmatic inferior vena cava was constricted to 50 % resulting in ascites formation of 500–1,000 ml. The dogs could be treated by ligation of the common hepatic artery and hepatocyte inoculation into the spleen.

Wirth et al. (1997) studied the effects of a bradykinin B_1 receptor antagonist in rats with CCl_4-induced liver cirrhosis.

In the model of CCl_4-induced liver fibrosis, the antifibrotic agents HOE077 and Safironil decreased collagen accumulation in the liver by inhibition of stellate cell activation, which was more pronounced in female than it was in male rats (Wang et al. 1998). In the same model Bickel et al. (1998) demonstrated powerful antifibrotic effects of a new prolyl 4-hydroxylase (P4-H) inhibitor, S4682, having a K_i of 155 nM at the purified enzyme P4-H. Inhibition of prolyl hydroxylation in stellate cells was inhibited with an IC_{50} of 39 μM by S4682. In drug-treated rats, ascites was significantly lower compared with controls. Histological examination proved a significantly lesser score of liver fibrosis after drug treatment.

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Bile Duct Ligation-Induced Liver Fibrosis in Rats

Purpose and Rationale

Bile duct ligation in rats induces liver fibrosis which can be evaluated by histological means and by determination of serum collagen parameters.

Procedure

Male Sprague Dawley rats weighing approximately 250 g are anesthetized with a mixture of ketamine and xylazine. Laparotomy is performed under antiseptic conditions. A midline incision in the abdomen is made from the xiphisternum to the pubis, exposing the muscle layers and the linea alba, which is then incised over a length corresponding to the skin incision. The edge of the liver is then raised and the duodenum pulled down to expose the common bile duct, which pursues an almost straight course of about 3 cm from the hilum of the liver to its opening into the duodenum. There is no gallbladder, and the duct is embedded for the greater part of its length in the pancreas, which opens into it by numerous small ducts. A blunt aneurysm needle is passed under the part of the duct selected, stripping the pancreas away with care, and the duct is divided between double ligatures of cotton thread. The peritoneum and the muscle layers as well as the skin wound are closed with cotton stitches.

The animals receive normal diet and water ad libitum throughout the experiment. Groups of 5–10 animals receive the test compound in various doses or the vehicle twice daily for 6 weeks. Then, they are sacrificed, and blood is harvested for determination of bile acids, 7S fragment of type IV collagen, and procollagen III N-peptide. The liver is used for histological studies and for hydroxyproline determinations. Control animals show excessive bile duct proliferation as well as formation of fibrous septa. The picture is consistent with complete biliary cirrhosis.

Evaluation

For detection of significant differences ($p < 0.05$), the unpaired *t*-test is used.

Modifications of the Method

Alpini et al. (1994) found an upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation.

Fiorucci et al. (2003) used 4-week bile-duct-ligated cirrhotic rats to study the effects of a nitric oxide-releasing derivative of ursodeoxycholic acid.

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Galactosamine-Induced Liver Necrosis

Purpose and Rationale

Single dose or a few repeated doses of D-galactosamine cause acute hepatic necrosis in rats (Decker and Keppler 1972). Prolonged administration leads to cirrhosis (Lesch et al. 1970).

Procedure

For induction of acute experimental hepatotoxicity, divided doses of 100–400 mg/kg D-galactosamine are injected to rats i.p. or i.v. during day one.

For induction of liver cirrhosis, male Wistar rats weighing 110–180 g are injected intraperitoneally three times weekly with 500 mg/kg D-galactosamine over a period of 1–3 months. Potential protective substances are administered orally with the food or by gavage every day. The rats are sacrificed at various time intervals and the livers obtained by autopsy.

Evaluation

The livers are evaluated by light microscopy and immunohistology using antibodies against macrophages, lymphocytes, and the extracellular matrix components, e.g., laminin, fibronectin, desmin, and collagen types I, III, and IV. The extent of liver cell necrosis and immunoreactivity for macrophages, lymphocytes, and the extracellular matrix components is graded semiquantitatively on a 0 to 4+ scale (0 = absent, 1 + = trace, 2 + = weak, 3 + = moderate, and 4 + = strong). Furthermore, serum enzyme activities, such as GOT and GPT, are determined.

Modifications of the Method

Other agents used to induce experimental cirrhosis are ethionine, thioacetamide (Dashti et al. 1996), dialkylnitrosamines, tannic acid, aflatoxins, pyrrolizidine alkaloids, and hepatotoxic components from mushrooms, such as amatoxins and phallotoxins (Zimmerman 1976).

Bruck et al. (1996) found an inhibition of thioacetamide-induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix-associated Arg–Gly–Asp epitope.

Intrahepatic cholestasis can be induced by alpha-naphthylisothiocyanate in rats (Krell et al. 1982).

Fulminant liver destruction can be induced with anti-Fas antibody.

Rodriguez et al. (1996) injected inbred female C57BL mice with anti-Fas antibody. The animals died within a few hours due to massive apoptosis of hepatocytes. This was accompanied by the sequential activation of cysteine proteases of the interleukin$_{1\beta}$-converting enzyme (ICE) and CPP32 (caspase-3) types in the cytosol of the hepatocytes. Systemic injection of the tripeptide *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone inhibited the intracellular activation of CPP32-like proteases in vivo and fully protected mice against Fas-mediated fulminant liver destruction and death.

Suzuki (1998) showed that the CPP32 subfamily, rather than the ICE subfamily, plays the dominant role in Fas antibody-induced hepatitis.

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Liver Fibrosis Induced by *Schistosoma cercariae*

CCl_4-induced liver fibrosis is the most commonly used model in liver fibrosis. The major shortcoming of this method is the high relative variability of the amount of collagen produced in the liver by treatment with the toxin. Determination of hydroxyproline, as a parameter of collagen deposition in the liver, showed a large relative variability of 75 % after 8 weeks of treatment with twice-weekly administration of CCl_4. To ensure adequate statistical interpretation, 50 rats/group are needed (Bickel et al. 1996).

Exposure of mice tails to *Schistosoma cercariae* causes infiltration of the cercariae into the circulation and subsequent deposition of the schistosome eggs in the liver. Thereafter liver fibrosis is produced in the liver in a time-dependent fashion. Hydroxyproline of the liver increases by threefold and sevenfold, 10 and 18 weeks after infection. The relative variabilities of the hydroxyproline content of the liver were 10–20 %, 10 and 18 weeks after infection, respectively (Phillips et al. 1977, 1987). The advantages of this model are the simplicity of fibrosis induction, the uniform generation of liver fibrosis with a very low variability with regard to the collagen produced in the liver, and, most of all, that it represents a disease model which accurately corresponds to the human disease. One should not forget that at least 200 million people suffer from schistosomiasis, most of them living in developing countries.

In this model the antifibrotic active compound Safironil caused a significant reduction of collagen type I and fibronectin in the liver. The beneficial effects of Safironil were at least in part due to a decrease in stellate cell activation by a mechanism sensitive to tumor growth factor $(TGF)_{\beta 1}$ (Phillips et al. 1997).

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Part XII

Antidiabetic Activity

Methods to Induce Experimental Diabetes Mellitus

Günter Müller

Contents

Pancreatectomy in Dogs

General Considerations

Dysfunction of the visceral tract has been considered for a long time to be the cause of diabetes mellitus. Bomskov in 1910 reported severe diabetic symptoms in dogs after cannulation of the ductus lymphaticus. This observation, however, could not be confirmed in later experiments (Vogel HG (1963), Unpublished data). The technique was similar to that described by Gryaznova (1962, 1963) for ligation of the thoracic duct in dogs.

Von Mehring and Minkowski in 1890 noted polyuria, polydipsia, polyphagia, and severe glucosuria following removal of the pancreas in dogs. The final proof for the existence of a hormone in the pancreas was furnished by Banting and Best (1922) who could reduce the elevated blood sugar levels in pancreatectomized dogs by injection of extracts of the pancreatic glands. The role of the pituitary gland in development of diabetes has first been elucidated by Houssay (1930, 1931) in pancreatectomized dogs (Survey by Beyer and Schöffling 1968).

Purpose and Rationale

The technique of complete pancreatectomy in the dog as described in detail by Foà (1971) and by Sirek (1968) has been used by many scientists as a

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relevant animal model for insulin-deficient diabetes mellitus in man. Some remarks of our own experiences are added to an abbreviated version of Sirek's description.

Procedure

Male Beagle dogs weighing 12–16 kg are used. After appropriate premedication the animal is placed on its back intubated with a tracheal tube, and anesthesia is achieved and maintained by inhalation anesthetics (e.g., fluroane). After removal of the fur and disinfection of the skin, a midline incision is made from the xiphoid process reaching well below the umbilicus. Bleeding vessels are ligated and the abdomen is entered through the linea alba. The falciform ligament is carefully removed and the vessels ligated. A self-retaining retractor is applied. By passing the right hand along the stomach to the pylorus, the duodenum with the head of the pancreas is brought into the operating field. First, the mesentery at the terminal process is cut, and the process itself is dissected free. The glandular tissue is peeled off from the inferior pancreaticoduodenal artery and vein. The vessels themselves are carefully preserved. Along a line of cleavage which exists between the pancreas, the pancreaticoduodenal vessels, and the duodenal wall, the pancreas is separated from the duodenum and from the carefully preserved pancreaticoduodenal vessels. The small vessels to the pancreas are ligated. The dissection is carried out from both sides of the duodenum. In the area of the accessory pancreatic duct, the glandular tissue being attached very firmly has to be carefully removed in order to leave no residual pancreatic tissue behind.

The pancreatic duct is cleaned, doubly ligated, and cut between the ligatures. The dissection proceeds until one encounters a small lobe containing the main pancreatic duct. The glandular tissue adheres here firmly to the duodenum. Blunt dissection and ligation of the vessels is followed by ligation of the pancreatic duct. By pulling on the pylorus and the stomach, the pyloric and the splenic parts of the pancreas are delivered into the wound. The duodenal part is placed back into the abdominal cavity. The mesentery of the body and tail of the pancreas is cut with scissors. The small vessels are doubly ligated and cut. The pancreatic tissue is bluntly dissected from the splenic vessels. The pancreatic branches of the splenic vessels are doubly ligated and cut. Working in direction from the spleen to the pylorus, the pyloric part of the pancreas is the last one to be dissected. Finally, all pancreatic tissue is removed.

The surgical field is checked once more for pancreatic remnants. The concavity of the duodenum and its mesentery is approximated by a few silk stitches, and the omentum is wrapped around the duodenum. Retroperitoneal injection of 5 ml 1 % procaine solution is given to prevent intussusception of the gut. 250,000 IU penicillin G in saline solution is instilled into the peritoneal cavity. The abdominal wall and the subcutaneous layer are closed by sutures, and finally the skin is sutured with continuous everting mattress stitches.

After the operation, the animal receives via a jugular vein catheter for 3–4 days the following treatment: 1,000 ml 10 % glucose solution with 10 IU human insulin regular, 3 ml 24 % Borgal (sulfadioxin/trimethoprim) solution, 2 ml 50 % metamizol, and 400 IU secretin. On the third day, the animal is offered milk. After the animal has passed the first milk feces, commercially available dry food together with a preparation of pancreatic enzymes for substitution is made available for the animal. Insulin is substituted with a single daily subcutaneous dose of a long-acting insulin (e.g., Lantus), and the insulin dose is individually adjusted according to the blood glucose levels achieved. Vitamin D3 is given every 3 months as an intramuscular injection of 1 ml Vigantol forte.

Modifications of the Method

Experiments performed by Houssay (1930, 1931) performing hypophysectomy in pancreatectomized dogs revealed amelioration of the diabetic state. These experiments contributed to the understanding of hormonal control in diabetes mellitus.

Rappaport and coworkers (1966) and Lau and coworkers (1976) used a pedunculated subcutaneous autotransplant of an isolated pancreas remnant for the temporary deprivation of internal secretion in the dog.

Subtotal pancreatectomy in rats was described by Scow (1957), Scow and coworkers (1957), Wagner and Cardeza (1957), Bonner-Weir and coworkers (1983), Noguchi and coworkers (1994), and Tanigawa and coworkers (1997). The pancreatic tissue between the common bile duct, duodenal loop, and portal vein in the duodenal segment, along the greater curvature in the gastric segment, and along the splenic vein in the splenic segment, was surgically excised in 3–4-week-old rats weighing 80–100 g.

Greeley (1937) proposed a 3-stage procedure with 3–4 weeks intervening between operations for pancreatectomy in rabbits.

Itoh and Maki (1996) reported surgical removal of 90 % of pancreatic tissue in 7- or 13-week-old mice. Under sodium pentobarbital anesthesia (65 mg/kg i.p.), the pancreas and the spleen were surgically removed with careful conservation of the common bile duct and major vessels surrounding the duodenum. Approximately 10 % (by weight and by insulin content) of the pancreas tissue was left intact adjacent to the lower duodenal loop.

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Alloxan-Induced Diabetes

Purpose and Rationale

Surveys on chemically induced diabetes in animals were given by Frerichs and Creutzfeldt (1968, 1971). This kind of diabetes predominantly based on insulin deficiency due to chemically (beta cell toxicity) destroyed pancreatic beta-cells.

Hyperglycemia and glucosuria after administration of alloxan have been described in several species, such as in dogs (Brunschwig et al. 1943; Tasaka et al. 1988), in rabbits (Baily and Baily 1943), in rats (Dunn and McLetchie 1943; Goldner and Gomori 1944), and in other species (Frerichs and Creutzfeldt 1968; 1971). Guinea pigs have been found to be resistant (Maske and Weinges 1957). Dosage and treatment regimen have been elaborated for the most frequently used species. In most species a triphasic time course is observed: an initial rise of glucose is followed by a decrease, probably due to depletion of islets from insulin, again followed by a sustained increase of blood glucose.

Procedure

Rabbits weighing 2.0–3.5 kg are infused via the ear vein with 150 mg/kg alloxan monohydrate (5.0 g/100 ml, pH 4.5) for 10 min resulting in 70 % of the animals to become hyperglycemic and uricosuric. The rest of the animals either die or are only temporarily hyperglycemic (Baily and Baily 1943; Pincus et al. 1954; Bänder et al. 1969).

Rats of Wistar or Sprague–Dawley strain weighing 150–200 g are injected subcutaneously with 100–175 mg/kg alloxan (Blum and Schmid 1954; Katsumata and Katsumata 1990; Katsumata et al. 1993).

Male Beagle dogs weighing 15–20 kg are injected intravenously with 60 mg/kg alloxan. Subsequently, the animals receive daily 1000 ml 5 % glucose solution with 10 IU regular insulin for 1 week and canned food ad libitum. Thereafter, a single daily dose of a long-acting insulin (e.g., Lantus) is administered subcutaneously, and the insulin dose is individually adjusted according to the blood glucose levels achieved. (Brunschwig et al. 1943; Geisen 1988).

Modifications of the Method

Kodoma and coworkers (1993) described a new diabetes model induced by neonatal alloxan treatment in rats. Male Sprague–Dawley rats 2, 4, or 6 days of age were injected intraperitoneally with 200 mg/kg of alloxan monohydrate after a 16 h fast. The most severe diabetic symptoms occurred in rats injected on day 6.

Heikkila and coworkers (1974) reported the prevention of alloxan-induced diabetes by ethanol administration in mice.

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Streptozotocin-Induced Diabetes

Purpose and Rationale

Rakieten and coworkers (1963) reported the diabetogenic activity of the antibiotic streptozotocin. The compound turned out to be specifically cytotoxic to beta-cells of the pancreas.

Procedure

Male Wistar rats weighing 150–220 g fed with a standard diet are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. As with alloxan, three phases of blood glucose changes are observed. Initially, blood glucose is increased, reaching values of 150–200 mg% after 3 h. 6–8 h after streptozotocin, the serum insulin values are increased up to four times, resulting in a hypoglycemic phase which is followed by persistent hyperglycemia. Severity and onset of diabetic symptoms depend on the dose of streptozotocin. After the dose of 60 mg/kg i.v., symptoms occur already after 24–48 h with hyperglycemia up to 800 mg%, glucosuria, and ketonemia. Histologically, the beta-cells are degranulated or even necrotic. A steady state is reached after 10–14 days allowing to use the animals for pharmacological tests.

Critical Assessment of the Method

Streptozotocin-induced diabetes in laboratory animals, mostly in rats, has become a valuable tool in diabetes research being used by many investigators.

Modifications of the Method

A survey on susceptibility of various species to streptozotocin was given by Frerichs and Creutzfeldt (1971). Multiple low doses of streptozotocin induce immune pancreatic insulitis in rats, thereby mimicking immune type 1 diabetes

in humans (Like and Rossini 1976; Rossini et al. 1977). Miller (1990) described the effect of streptozotocin on the golden Syrian hamster using a single i.p. injection of 50 mg/kg streptozotocin. Enhancement of streptozotocin-induced diabetes in CD-1 mice by cyclosporin A was reported by Iwakiri and coworkers (1987). Grussner and coworkers (1993) induced long-lasting diabetes mellitus in Yorkshire Landrace pigs with a dosage of 150 mg/kg streptozotocin.

Stosic-Grujicic and coworkers (1999) described protection of mice from multiple low dose streptozotocin-induced insulitis and diabetes by the immunosuppressive drug leflunomide. Bleich and coworkers (1999) found that elimination of leukocyte 12-lipoxygenase in mice ameliorates low dose streptozotocin-induced diabetes by increasing islet resistance to cytokines and decreasing macrophage production of nitric oxide. Masutani and coworkers (1998) studied the role of poly(ADP-ribose)-polymerase (Parp) in streptozotocin-induced diabetes. Parp-deficient (Parp−/−) mice were established by disrupting Parp exon 1 using the homologous recombination technique. These mice were almost resistant to streptozotocin-induced diabetes.

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Other Diabetogenic Compounds

Purpose and Rationale

Several other compounds have been found to induce symptoms of diabetes and/or obesity, such as dithizone (Maske and Weinges 1957; Frerichs and Creutzfeldt 1971;

Hansen et al. 1989; Goldberg et al. 1991) or gold thioglucose (Stauffacher et al. 1967; Caterson et al. 1988; Silva and Hernandez 1989; Heydrick et al. 1995) or monosodium glutamate (Sartin et al. 1985).

Procedure

Goldberg and coworkers (1991) injected various chelators, such as dithizone, 8-(p-toluenesulfonylamino)-quinoline (8-TSQ), and 8-(benzenesulfanylamino)-quinoline (8-BSQ) in a single i.v. dose of 40–100 mg/kg to cats, rabbits, golden hamsters, and mice. Dithizone injection causes a triphasic glycemic reaction in rabbits. A phase of initial hyperglycemia is detected after 2 h, followed by a normoglycemic phase after 8 h and a secondary permanent hyperglycemic phase after 24–72 h. Histologically, complete and partial degranulation of beta cells is observed.

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Growth Hormone-Induced Diabetes

Cotes and coworkers (1949) described the diabetogenic action of pure anterior pituitary growth hormone in cats. In intact adult dogs and cats the repeated administration of growth hormone induces an intensively diabetic condition with all symptoms of diabetes including severe ketonuria and ketonemia. Rats of any age subjected to a similar treatment do not become diabetic but grow faster (Young 1945) and show striking hypertrophy of the pancreatic islets.

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Corticosteroid-Induced Diabetes

Ingle (1941) described hyperglycemia and glucosuria in forced fed rats treated with cortisone. In the guinea pig and in the rabbit, experimental corticoid diabetes could be obtained without forced feeding (Hausberger and Ramsay 1953; Abelove and Paschkis 1954). In the rat, the adrenal cortex, stimulated by corticotrophin, has the capacity to secrete amounts of steroids which induce steroid diabetes (Ingle et al. 1946).

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Insulin Deficiency Due to Insulin Antibodies

Purpose and Rationale

A transient diabetic syndrome can be induced by injection of guinea pig anti-insulin serum in various species (Moloney and Coval 1955; Wright 1968).

Procedure

Bovine insulin, dissolved in acidified water (pH 3.0), is incorporated in a water–oil emulsion based on complete Freund's adjuvant or a mixture of paraffin oil and lanolin. A dose of 1 mg insulin is injected in divided doses subcutaneously to male guinea pigs weighing 300–400 g. Injections are given at monthly intervals, and the guinea pigs are bled by cardiac puncture 2 weeks after the second and subsequent doses of antigen. It is possible to get 10 ml blood from every animal once a month.

Intravenous injection of 0.25–1.0 ml guinea pig anti-insulin serum to rats induces a dose-dependent increase of blood glucose reaching values up to 300 mg%. This effect is unique to guinea pig anti-insulin serum and is due to neutralization by insulin antibodies of endogenous insulin secreted by the injected animal. In this way a state of insulin deficiency is induced. It persists as long as antibodies capable of reacting with insulin remain in the circulation. Slow rate intravenous infusion or intraperitoneal injection prolongs the effect for more than a few hours. However, large doses and prolonged administration accompanied by ketonemia, ketonuria, glucosuria, and acidosis are fatal to the animals. After lower doses, the diabetic syndrome is reversible after a few hours.

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Virus-Induced Diabetes

Juvenile-onset (type I) diabetes mellitus may be due to virus infections and β-cell-specific autoimmunity (Craighead 1978). The D-variant of encephalomyocarditis virus (EMC-D) selectively infects and destroys pancreatic β-cells in susceptible mouse strains similar to human insulin-dependent diabetes (Yoon et al. 1980; Giron and Patterson 1982; Giron et al. 1983; Vialettes et al. 1983). Adult, male ICR Swiss mice are susceptible to the diabetogenic effect of the D-variant of encephalomyocarditis virus in contrast to adult C3H/HeJ male mice which are relatively resistant. Pretreatment with cyclosporin A, a potent immunosuppressive drug, results in increased severity and incidence of diabetes in susceptible ICR Swiss mice and induction of diabetes in resistant C3H/HeJ mice (Gould et al. 1985).

Modifications of the Method

Hirasawa and coworkers (1997) studied the possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyelitis virus-induced diabetes in mice. The inactivation of macrophages prior to viral infection resulted in the prevention of diabetes. Utsugi and coworkers (1992) demonstrated that intraperitoneal inoculation with NDK25, a variant of encephalomyocarditis virus which has been cloned from the M variant of encephalomyocarditis virus, caused DBA/2 mice to develop non-insulin-dependent diabetes mellitus. See and Tilles (1995) challenged CD-1 mice with a diabetogenic strain (E2) of coxsackievirus B4. Islet cell destruction was associated with chronic islet cell inflammation, elevation of islet cell antibody, and prolonged presence of viral RNA in the pancreas. Stubbs and coworkers (1994) investigated the effect of Kilham rat virus (KRV) infection on GLUT2 expression in diabetes-resistant BB/Wor rats. Viral antibody-free diabetes-resistant rats did not develop spontaneous diabetes, but inoculation with Kilham rat virus induced autoimmune beta-cell destruction and hyperglycemia. Hayashi and coworkers (1995) investigated the role of adhesion molecules in the reovirus type2-induced diabetes-like syndrome in mice. Ellerman and coworkers (1996) studied Kilham rat virus-induced autoimmune diabetes in multiple strains of rat.

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Genetically Diabetic Animals

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General Considerations

Several animal species, mostly rodents, were described to exhibit spontaneously diabetes mellitus on a hereditary basis. These findings were highly appreciated with the expectation to get more insight into the pathogenesis of diabetes in humans. During the last few years since the discovery of leptin (Zhang et al. 1994) and its downstream signal transduction cascade (Friedman and Halaas 1998), tremendous new insight of the genetics of diabetic and obese animal disease models was derived. Up to now, at least six genetically diabetic animal models exhibit defects in the leptin pathway: the ob mutation in the mouse resulted in leptin deficiency. The db mutation in the mouse and the cp and fa mutations in the rat are different mutations of the leptin receptor gene. The fat mutation in the mouse results in a biologically inactive carboxypeptidase E, which processes the prohormone conversion of POMC into α-MSH, which activates the hypothalamic MC4 receptor. Finally the Agouti yellow (y) mouse exhibit a ubiquitous expression of the Agouti protein which represents an antagonist of the hypothalamic MC4 receptor.

Symptoms of diabetes and obesity are overlapping in many animal models (see also chapter on "► Genetically Obese Animals").

Critical Assessment

The pathophysiological mechanisms which finally lead to the diabetes phenotype (hyperglycemia, hyperinsulinemia, and insulin resistance) exhibited by the various animal disease models for non-insulin-dependent diabetes do not necessarily be identical to those in human disease. Therefore, detailed knowledge about the (patho-)physiology of these animal disease models is a prerequisite for interpretation of experimental results and their value for the human disease.

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Spontaneously Diabetic Rats

The occurrence of spontaneous diabetes has been reported in several strains of rats.

BB Rat

The BB rat (biobreeding (BB) rat) is a model of spontaneous diabetes associated with insulin deficiency and insulitis due to autoimmune destruction of pancreatic beta cells (Nakhooda et al. 1977, 1978; Like et al. 1982; Oschilewski et al. 1985; Lee et al. 1988; Solomon et al. 1989; Papaccio and Mezzogiorno 1989; Kolb et al. 1990; Velasquez et al. 1990; Lefkowith et al. 1990; Gottlieb et al. 1990; Ellerman et al. 1993). Diabetes is inherited as an autosomal

recessive trait and develops with equal frequency and severity among males and females. The onset of clinical diabetes is sudden and occurs at about 60–120 days of age. Within several days, diabetic animals are severely hyperglycemic, hypoinsulinemic, and ketotic unless insulin treatment is instituted. Pipeleers and coworkers (1991) described the transplantation of purified islet cells in diabetic BB rats. Hao and coworkers (1993) reported that the immunosuppressive agent mycophenolate mofetil can prevent the development of diabetes in BB rats. Klöting and Vogt (1991) characterized the features of a subline of diabetes-prone BB rats (BB/OK rats). The circadian variations in blood pressure and heart rate of this strain were compared with spontaneously hypertensive rats (Berg et al. 1997).

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WBN/Kob Rat

Spontaneous hyperglycemia, glucosuria, and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985; Tsuchitani et al. 1985; Koizumi et al. 1989; Shimoda et al. 1993). These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16-week-old males. These rats develop demyelinating, predominantly motor neuropathy, later accompanied by axonal changes (Yagihashi et al. 1993).

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Cohen Diabetic Rat

Diabetes in Cohen rats is characterized by hyperglycemia, glucosuria, and hyperinsulinemia, with late development of hypoinsulinemia, insulin resistance, and a decrease in the number and sensitivity of insulin receptors. The rats develop overt diabetes and diabetes-related complications when fed a diet rich in sucrose or other refined sugars and poor in copper content, but not when fed a starch or stock diet (Cohen et al. 1972; Velasquez et al. 1990).

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Goto-Kakizaki Rat

Nonobese, insulin-resistant Goto-Kakizaki (GK) rats are a highly inbred strain of Wistar rats that spontaneously developed type II diabetes. Defects in glucose-stimulated insulin secretion, peripheral insulin resistance, and hyperinsulinemia are seen as early as 2–4 weeks after birth. Impaired skeletal muscle glycogen synthase activation by insulin was observed, accompanied by chronic activation of diacylglycerol-sensitive protein kinase C.

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Zucker Fatty Rat

The Zucker fatty rat is a classic model of hyperinsulinemic obesity (Zucker 1965). Obesity is due to a simple autosomal recessive (fa) gene and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human type 2 diabetes. However, their blood sugar level is usually normal throughout life (Bray 1977; Clark et al. 1983; McCaleb and Sredy 1992; Abadie et al. 1993; Alamzadeh et al. 1993; Kasim et al. 1993; Galante et al. 1994).

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Zucker Diabetic Fatty Rat (Zdf/Drt-Fa)

The obese Zucker diabetic fatty rat is originally derived from the Zucker fatty rat (Peterson et al. 1990) and has the identical mutation (fa) in the leptin receptor. In addition a defect in the insulin promoter has been described (Griffen et al. 2001). Insulin promoter activity is reduced 30–50 % in homozygous ZDF fetal islets, and insulin mRNA levels are similarly reduced by 45 %. Only the males become diabetic at the age of around 10 weeks spontaneously with hyperglycemia of about 20 mmol/l; the females develop diabetes only when feeding a high-fat diet. Diabetes phenotype develops due to lipotoxicity to the β-cell (Lee et al. 1994). These rats are characterized besides hyperglycemia by insulin resistance, moderate hyperinsulinemia, and extreme hyperphagia due to the loss of calories by glucosuria and obesity.

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Wdf/Ta-Fa Rat

The WDF/Ta-fa rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty (fa) gene from the Zucker rat to the Wistar-Kyoto rat (Ikeda et al. 1981; Kava et al. 1989; Velasquez et al. 1990). The Wistar fatty rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet. Kobayashi and coworkers (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by pioglitazone in Wistar fatty rats (fa/fa). Sugiyama and coworkers (1990a, b) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

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OLETF Rat

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are: (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM. Administration of diazoxide (0.2 % in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4–12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995). Ishida and coworkers (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

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ESS Rat

The occurrence of spontaneous diabetes in a colony of rats (Stilman Salgado), called eSS rat, was reported by Tarrés and coworkers (1981). The animals show abnormal glucose tolerance tests from the age of 2 months onwards. The syndrome consists of a mild type of diabetes that does not diminish the longevity of the animals. Six-month-old rats show disruption of the islet architecture and fibrosis of the stroma (Dumm et al. 1990).

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Obese SHR Rat

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Wistar-Kyoto strain

with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets. From these rats, several substrains were developed, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia, and hyperinsulinemia with impaired glucose tolerance (Russell et al. 1994). Reduced insulin receptor signaling was found in the obese spontaneously hypertensive Koletsky rat (Friedman et al. 1997).

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SHR/N-cp Rat

The congenic SHR/N-cp rat strain, developed at the National Institutes of Health, USA (Hansen 1983, 1988; Michaelis et al. 1986; Adamo et al. 1989), was derived by mating a male Koletsky rat heterozygous for the corpulent gene (cp/+) to a female rat of the Okamoto strain. After a minimum of 12 backcrosses, homozygous (cp/cp) SHR/N-cp rats exhibit obesity, mild hypertension, hyperinsulinemia, and glucose intolerance.

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BHE Rat

The BHE rat colony was originally developed by breeding black and white hooded rats of the Pennsylvania State College strain and albino rats of the Yale (Osborne-Mendel) strain. The BHE rat is a model in which the diabetic state is manifested

only at maturity. BHE rats have hyperinsulinemia at 50 days of age associated with glucose intolerance and tissue resistance to insulin. Later on, BHE rats have less hyperinsulinemia with reduced pancreatic insulin stores and show mild hyperglycemia and hyperlipidemia.

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LEW.1AR1/Ztm-iddm Rat

Lenzen et al. (2001) and Jörns et al. (2004) described a type I (insulin-dependent) diabetes mellitus rat model (LEW.1AR1/Ztm-iddm), which arose through a spontaneous mutation in a congenic Lewis rat strain with a defined MHC haplotype (RT1.Aa B/Du Cu). Diabetes appeared in the rats with an incidence of 20 % without major sex preference at 58+/−2 days. The disease was characterized by hyperglycemia, glycosuria, ketonuria, and polyuria. In the peripheral blood, the proportion of T lymphocytes was in the normal range expressing the RT6.1 differentiation antigen. Islets were heavily infiltrated with B and T lymphocytes, macrophages and NK cells with beta cells rapidly destroyed through apoptosis in areas of insulitis.

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Spontaneously Diabetic Mice

KK Mouse

Nakamura (1962) and Nakamura and Yamada (1967) reported on a diabetic strain of the KK mouse. The animals were moderately obese and showed polyphagia and polyuria. Mice at the age of 7 months or older showed glucosuria and blood sugar levels up to 320 mg%. The pancreatic insulin content was increased, but histologically degranulation of the β-cells and hypertrophy of the islets were found. Sections of the liver showed a reduction of glycogen and an increase in lipid content.

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KK-Ay Mouse

Iwatsuka and coworkers (1970) reported on)yellow KK mice (also named KK-Ay mice),

carrying the yellow obese gene (Ay). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA1c levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early-onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

KK-Ay mice can be used to demonstrate the extrapancreatic action of antidiabetic drugs, such as glimepiride, a novel sulfonylurea (Müller et al. 1995; Takada et al. 1996). Sohda and coworkers (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones for hypoglycemic and hypolipemic activities in yellow KK mice. Hofmann and coworkers (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KK-Ay mice after treatment with the oral antidiabetic agent pioglitazone.

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NOD Mouse

The NOD mouse strain was established by inbreeding diabetic CTS mice derived originally from the JCLICR strain. Like the BB rat, the NOD mouse is a model of insulin-dependent diabetes mellitus and develops hypoinsulinemia secondary to autoimmune destruction of pancreatic β cells in association with insulitis and autoantibody production. Following insulitis, destruction of the insulin-producing pancreatic β cells ensues in many (but not all) mice, with physiologic manifestations of insulin depletion appearing between 3 and 7 months of age. The onset of diabetes can be prevented by an immunomodulating drug (Baeder et al. 1992) or by a soluble interleukin-1 receptor (Nicoletti et al. 1994). Hutchings and Cooke (1995) compared the protective effects afforded by intravenous administration of bovine or ovine insulin to young NOD mice. Bergerot and coworkers (1997) reported that feeding small amounts (2–20 μg) of human insulin conjugated to cholera toxin B subunit can effectively suppress β-cell destruction and clinical diabetes in adult NOD mice. Insulin-dependent diabetes mellitus

in NOD mice is the result of a CD^{4+} and CD^{8+} T cell-dependent autoimmune process directed against the pancreatic β-cells (Serreze and Leiter 1994; Verdaguer et al. 1997).

Elias et al. (1990) described induction and therapy of autoimmune diabetes in the nonobese diabetic (NOD/Lt) mouse by a 65-kDa heat-shock protein.

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Obese-Hyperglycemic Mice

Ingalls and coworkers (1950), Mayer and coworkers (1951), and Bleisch and coworkers (1952) observed hereditary diabetes in genetically obese mice. The obese-hyperglycemic mice were glycosuric; the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma were observed. One of the most interesting features was insulin resistance; doses as high as 400 IU/kg had little effect on blood sugar. The serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased, and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese-hyperglycemic mice is different from that of the human diabetic patient. The ob mutation was

identified as a mutation in the leptin gene in adipose tissue (Zhang et al. 1994), and the substitution of leptin reverses the obese and diabetic phenotype completely (Halaas et al. 1995). Other strains or substrains of mice with obesity and hyperglycemia have been described by Dickie (1962), Westman (1968), Stein and coworkers (1970), Coleman and Hummel (1973), and Herberg and Coleman (1977). Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide (amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-Avy/a).

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Diabetic db/db Mice

The diabetes db/db mouse strain is derived from an autosomal recessive mutation having occurred spontaneously in mice of the C57BL/KsJ strain which was identified as a mutation in the leptin receptor gene (Tartaglia et al. 1995). On this basis, the diabetes mouse (C57BL/6 J db/db) consistently develops a severe diabetic syndrome similar to that found in the C57BL/KsJ ob/ob mouse, characterized by early onset of hyperinsulinemia and hyperglycemia up to 20–25 mmol/l (Hummel et al. 1966; Coleman and Hummel 1967; Like et al. 1972). The db/db mouse, in contrast to the ob/ob mouse, develops significant nephropathy (Gardner 1978). Mutations on the leptin receptor result in an obese phenotype identical to that of ob mice (Li et al. 1998). C57BL/KsJ ob/ob mice are phenotypically the same as other strains of db mice. The leptin receptor (Ob-R) gene encodes 4 alternatively spliced forms: Ob-Ra, Ob-Rb, Ob-Rc,

and Ob-Rd (Lee et al. 1996). In the db/db mouse strain, the Ob-Rb transcript contains an insert with a premature stop codon as a result of abnormal splicing (Chen et al. 1996; Friedman and Halaas 1998).

Coleman and Hummel (1969) joined adult diabetic mice (db/db) of the C57BL/Ks strain in parabiosis with normal mice of the same sex. Little, if any, amelioration of the disease was observed in parabiont diabetics, and no symptoms of diabetes were observed in the normal parabiont. Instead, the normal partners lost weight, became hypoglycemic, and died of apparent starvation 50 days after surgery. In contrast, the diabetic partners gained weight rapidly and remained diabetic. Raizada and coworkers (1980) demonstrated a decrease of insulin receptors and impaired responses to insulin in fibroblastic cultures from the diabetic db/db mouse.

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Diabetes Obesity Syndrome in CBA/Ca Mice

A spontaneous maturity onset diabetes obesity syndrome occurs in a small proportion (10–20 %) of male CBA/Ca mice. Inbreeding can increase the incidence to 80 %. It occurs at 12–16 weeks

of age and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except for a slight increase in serum insulin. The male obese diabetic mice have a normal life expectancy.

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Wellesley Mouse

The Wellesley mouse, described first by Jones (1964), is a hybrid with predisposition to diabetes mellitus. The diabetic animals have elevated levels of immunoreactive insulin in serum, enlarged pancreatic islets, and reduced insulin responsiveness in peripheral tissues (Cahill et al. 1967; Gleason et al. 1967; Like and Jones 1967).

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Chinese Hamster

Meier and Yerganian (1959, 1961a, b) described the occurrence of hereditary diabetes mellitus in the Chinese hamster (*Cricetulus griseus*). Blood sugar levels of diabetic hamsters were elevated from a normal of 110 mg% up to 600 mg%. Severe polyuria, glucosuria, ketonuria, and proteinuria were observed. The diabetic condition could be improved by administration of insulin, and oral antidiabetic drugs were effective in mildly diabetic hamsters. Pathological changes were seen in histological sections of the pancreas, liver, and kidney. The number of pancreatic islets was decreased, and the cells of the remaining islets were abnormal. This animal model has been studied by several authors.

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Other Species with Inherited Diabetic Symptoms

Sand Rat

The sand rat (*Psammomys obesus*) lives in the desert regions of North Africa and the Near East. In the laboratory the animals develop diabetic symptoms when fed Purina laboratory chow instead of an all vegetable diet (Hackel et al. 1965a, b, 1967; Miki et al. 1967; de-Fronzo et al. 1967; Brodoff et al. 1967; Strasser 1968). The diabetic syndrome in the sand rat usually develops within 2–3 months with variations in severity between the animals. Severely hyperglycemic animals die prematurely from ketosis. Initially, the pancreatic islets appear normal. In the intermediate stage of the disease, degranulation of pancreatic β-cells is observed. This is followed by β-cell degeneration and necrosis with resultant insulinopenia and ketonuria. Histological studies by Dubault and coworkers (1995) showed insulitis in animals that became insulin dependent in later stages and recommended *Psammomys obesus* as a model of latent progression to insulin deficiency in type 2 diabetes patients.

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Spiny Mouse

The spiny mouse (*Acomys cahirinus*) is a small rodent living in the semi-desert areas of the Eastern Mediterranean (Pictet et al. 1967). Diabetes occurs in about 15 % of the animals under laboratory conditions accompanied by hyperplasia of the endocrine pancreas. Great variations in the appearance and severity of diabetes and obesity occur in this species. Some animals show obesity, mild hyperglycemia, and hyperinsulinemia. Others have frank hyperglycemia with glucosuria that leads to fatal ketosis. Regardless of the stage of the disease, all spiny mice characteristically have massive hyperplasia of pancreatic islets and increased pancreatic insulin content. Despite the large insulin stores, plasma insulin response to glucose is delayed or impaired suggesting an impairment of the insulin release mechanism.

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African Hamster (*Mystromys albicaudatus*)

Spontaneous diabetes mellitus was described in South African hamsters (*Mystromys albicaudatus*) by Packer and coworkers (1970), Stuhlman and coworkers (1972, 1974, 1975), and Schmidt and coworkers (1974). Characteristics established as part of the diabetic syndrome in this species include hyperglycemia, glucosuria, ketonuria, polyuria, polyphagia, and polydipsia. Pancreatic lesions include β-cell vacuolization, glycogen infiltration, nuclear pyknosis, margination of organelles, and β-cell death.

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Tuco-Tuco

The diabetic syndrome in tuco-tucos (*Ctenomys talarum*) is similar to that in sand rats and spiny mice (Wise et al. 1972). However, tuco-tucos tend to have less hyperglycemia and are less prone to ketosis. Many animals, mainly males, become hyperphagic and quite obese. Degranulation of β-cell is the usual lesion in the pancreas, but amyloid hyalinization of islets has been observed in a few animals.

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Macaca nigra

A high incidence of spontaneous diabetes mellitus was found in *Macaca nigra* (Celebes black apes) with analogies to human diabetes (Howard 1972, 1974a, b, 1975). Abnormal signs include hyperglycemia, decreased clearance of glucose, in intravenous tolerance tests, reduced insulin secretion, and increased serum lipids. Insulin secretory capacity is lost concomitant with amyloid infiltration into the islets of Langerhans. Secondary manifestations are atherosclerosis, thickened basement membranes of muscle capillaries, and cataracts. The genetic predisposition in these monkeys is exacerbated by changes in diet and environment.

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Recombinant Mice

Knockout Mouse Models

General Considerations

Insulin exerts its diverse biological effects by binding to and activating the membrane-bound insulin receptor (IR), thereby initiating the insulin signal transduction cascade (see chapter "▶ Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes"). IRs are not only present in the classical insulin target tissues, i.e., the adipose tissue, skeletal muscle, and liver, but are also widely expressed throughout the organism in tissues such as pancreas, central nervous system (CNS), lymphatic cells, and kidney. Signals initiated by the activated IR result in the diverse biological effects of insulin, such as the stimulation of glucose transport, the inhibition of gluconeogenesis, the stimulation of protein and lipid synthesis, and the regulation of gene transcription. Because the roles of many components of the signaling pathway were initially characterized in vitro, more recent research has focused on the analysis of insulin action in vivo by generating mice with targeted disruption of genes for these components. Since the pattern of inheritance of type II diabetes points to a disease of polygenic nature, not only single gene-deficient mice but also models with combined genetic disruptions have been created in an attempt to represent the polygenic nature of the disease.

Realization that metabolic diseases do not only involve many genes but also many organs necessitated engineering the mouse genome with spatial and temporal controls. This requires the generation of premutant mice whereby the alleles of interest is flanked by recognition sites for DNA recombinases such as Cre (loxP sites) or Flp (Frt sites; Branda and Dymecki 2004). When such premutant mice are bred with transgenic mice that express the corresponding DNA recombinase in a tissue-specific fashion, the gene of interest is inactivated only in that particular tissue. An added sophistication of spatially controlled mutagenesis is the inclusion of temporal control, which is achieved by using ligand-activated chimeric recombinases composed of fusion of the recombinase with the ligand-binding domain of a nuclear receptor (Metzger and Chambon 2001). Employing spatially and temporally controlled mutagenesis revealed that metabolic perturbation in one tissue encroaches on the metabolism of another. This tissue communication and crosstalk allows for metabolic flexibility, with respect to the use or production of metabolic fuel. Understanding this may ultimately reveal the genetic determinants that limit the degree of metabolic flexibility, thus promoting metabolic diseases in humans (Bickel 2004).

The first knockout models of insulin resistance aimed at the disruption of major molecules in IR signaling in each tissue. The successful generation of viable, heterozygous IR knockout mice showed that 50 % of IR expression is sufficient for the maintenance of physiological blood glucose concentrations. In contrast, homozygous IR-deficient mice rapidly develop diabetic ketoacidosis and die within 3–7 days after birth (Accili et al. 1996; Joshi et al. 1996), showing the indispensability of IR for the control of glucose metabolism. The new techniques for conditional gene inactivation in mice are particularly helpful if conventional knockout of a gene results in embryonic lethality, preventing the analysis of gene function in the adult. Conditional mutagenesis enables the inactivation of a gene of interest in a specific tissue or in a temporally controlled manner, thereby providing new insights into the contribution of a gene to a complex physiological phenotype such as insulin resistance.

Skeletal Muscle and Insulin Resistance

Because insulin resistance in skeletal muscle is one of the earliest detectable defects in type II diabetic patients, muscle-specific insulin receptor knockout (MIRKO) mice have been generated as a model for muscle-specific insulin resistance.

MIRKO mice displayed elevated fat mass, serum triglycerides, and free fatty acids, indicating that insulin resistance in muscle contributes to the altered fat metabolism associated with type II diabetes (Brüning et al. 1998). Additionally, Kim and coworkers (2001) demonstrated that IR deficiency in muscle promotes redistribution of substrates to adipose tissue, thereby contributing to increased adiposity in MIRKO mice. Surprisingly, blood glucose, serum insulin, and glucose tolerance were normal in these mice, initially leading to the interpretation that tissues other than muscle are more essential for insulin-regulated glucose disposal than previously assumed. However, Zisman and coworkers (2000) disrupted the gene for the glucose transporter 4 (GLUT4), which mediates glucose transport in response to insulin, selectively in mouse muscle tissue. They found that Glut4-mediated glucose uptake in muscle was indeed essential for the maintenance of normal glucose homeostasis. Consistently, mice with a combined muscle-specific functional inactivation of IR and the closely related insulin-like growth factor receptor (IGF-1R) also display a complete type II diabetic phenotype, implicating a compensatory role of IGF-1R for mediating insulin's stimulation of glucose transport via Glut4 in muscle (Fernandez et al. 2001).

Adipose Tissue and Insulin Resistance

The strong association between the worldwide epidemic of obesity and dramatically increasing prevalence of insulin resistance and type II diabetes has prompted recent research to focus on the mechanisms linking adipose tissue to whole-body insulin sensitivity, β-cell function, and overall glucose metabolism. Newborn mice with IR deficiency show a marked reduction in white adipose tissue mass, pointing to a role for IR in the regulation of adipocyte growth and differentiation (Accili et al. 1996). Mice with an adipose tissue-specific knockout of the IR (FIRKO) also display a reduced fat mass, loss of the normal correlation of plasma leptin and body weight, and protection against both obesity and obesity-related glucose intolerance. Interestingly, they also exhibit an 18 % extended lifespan (Bluher et al. 2003). Strikingly, whereas adipocytes of control mice exhibit a bell-shaped size distribution, adipocytes of FIRKO mice demerge into groups of small and large cells. Interestingly, these differently sized cells also show different expression of fatty acid synthase and the transcription factors CCAAT enhancer binding protein (C/EBP)-α as well as sterol regulatory element-binding protein 1 (SREBP1). Thus, inactivation of the IR revealed a previously unrecognized heterogeneity of adipose tissue. Consistent with the phenotype of GLUT4-null mice, which display a depletion of fat stores (Katz et al. 1995), the selective disruption of GLUT4 in adipose tissue caused an impaired glucose transport, followed by development of insulin resistance. The role of adipose tissue in muscle-specific IR deficiency was addressed in mice with a targeted inactivation of IR-mediated signaling in both muscle and adipose tissue (Lauro et al. 1998). These mice did not become diabetic despite peripheral insulin resistance and a mild impairment of β-cell function. Together, these data suggest that there may be a critical threshold in whole-body insulin resistance that finally leads to a diabetic phenotype. Moreover, this insulin resistance might result in excessive demands on the pancreatic β-cells to secrete insulin, leading to consecutive decompensation of these cells. These data clearly assign adipocytes an important role in lipid storage, development of obesity, and regulation of glucose homeostasis.

Role of the β-Cell in Impaired Glucose Tolerance

Mice with a specific disruption of the IR gene in β-cells show a selective loss of insulin secretion in response to glucose and a progressive impairment of glucose tolerance (Kulkarni et al. 1999), indicating that insulin stimulates its own secretion via IRs on β-cells, thus playing an important functional role in glucose sensing by the pancreatic β-cell. Therefore, defects in insulin signaling at the level of the β-cell may contribute to the observed alterations in insulin secretion in type 2 diabetes. Interestingly, a β-cell-specific IGF-1R knockout model also underscored a role for this receptor in the control of glucose-stimulated insulin secretion and glucose tolerance (Kulkarni

et al. 2002). The interplay between insulin resistance and insulin secretory defects has also been addressed by the generation of mice deficient for insulin receptor substrate 1 (IRS-1) and β-cell glucokinase (GK). The heterozygous β-cell GK knockout is characterized by decreased insulin secretion in response to glucose, whereas IRS-1-deficient mice are insulin resistant, but do not develop overt diabetes (Abe et al. 1998). In contrast to either individual mutation, the double-knockout mice developed a diabetic phenotype (Terauchi et al. 1997), demonstrating that the combination of individual minor defects in insulin action or insulin secretion can cause overt diabetes.

Insulin Resistance in the Brain

The liver-specific insulin receptor knockout (LIRKO) mouse, as expected, exhibits a dramatic phenotype with severe insulin resistance and progressive liver failure (Michael et al. 2000), mirroring the critical role of insulin signaling in liver for regulating glucose homeostasis and maintaining normal hepatic function. However, IRs and insulin signaling proteins are not exclusively expressed in classic insulin target tissues; they are also widely distributed throughout the CNS. CNS-specific disruption of the IR gene (NIRKO) revealed an important role of IRs in the regulation of energy disposal, fuel metabolism, and reproduction (Brüning et al. 2000). It has very recently been shown in conventional IR-deficient mice that combined restoration of IR function selectively in the brain, liver, and pancreatic β-cells rescues these mice from neonatal death, prevents diabetes in a majority of animals, and restores adipose tissue content, lifespan, and reproductive function. Interestingly, IR substitution either limited to the brain or liver and pancreatic β-cells was sufficient to prevent neonatal death but not lipoatrophic diabetes, leading to the surprising finding that IR signaling in nontypical insulin target tissues like the brain seems to be crucial to maintain fuel homeostasis and prevent diabetes.

Inactivation of IRS Proteins

Aside from IR knockouts, various molecules of the signal transduction pathway initiated by IR have been disrupted, such as IRS-1, which was believed to be the principal substrate for the IR and IGF-1R. Mice deficient for IRS-1 exhibit impaired glucose tolerance and a decrease in insulin/IGF-1-stimulated glucose uptake in vivo and in vitro, thereby providing a mouse model of genetically determined insulin resistance (Abe et al. 1998; Jenkins and Storlien 1997). Additionally, elevated blood pressure and plasma triglyceride levels were observed, as well as impaired endothelium-dependent vascular relaxation, indicating that insulin resistance plays an important role in the clustering of coronary risk factors leading to accelerated atherosclerosis. The residual insulin/IGF-1 action in IRS-1-deficient mice correlated with the existence of an alternative tyrosine-phosphorylated protein (IRS-2) (Araki et al. 1994), which is also capable of activating the signaling cascade. Surprisingly, disruption of IRS-2 impaired peripheral insulin signaling and pancreatic β-cell function, resulting in progressive deterioration of glucose homeostasis (Withers et al. 1998). This phenotype results from insulin resistance in the liver and skeletal muscle and β-cell dysfunction. Succeeding studies indicated tissue-specific functions for IRS-1 and IRS-2 in mediating the metabolic effects of insulin in vivo, IRS-1 having a major role in the skeletal muscle and IRS-2 in the liver, muscle, adipose tissue, pancreatic β-cells, and reproductive tissue. Interestingly, mice with a double knockout of IRS-1 and the subsequently identified IRS-3 (Lavan et al. 1997) displayed a phenotype of early-onset severe lipoatrophy associated with hyperglycemia, hyperinsulinemia, and decreased plasma levels of the anorexigenic hormone leptin. The IRS-1/IRS-3 double-knockout phenotype in mice (Laustsen et al. 2002) might mimic the situation in humans better than the single IRS-1 knockout.

The concept that several subclinical genetic alterations in insulin action can synergize to result in overt diabetes was tested in mice double heterozygous for IR and IRS-1 alleles with a ~50 % reduction in expression of either protein (Brüning et al. 1997). In these mice, the combined genetic defects led to aggravation of insulin resistance with five- to 50-fold elevated plasma insulin

levels and respective β-cell hyperplasia, and ~40 % of the animals developed overt diabetes at the age of 4–6 months. This mouse model of type II diabetes, in which diabetes arises in an age-dependent manner from the interaction between two genetically determined, subclinical defects in the insulin signaling cascade, demonstrates the important role of epistatic interactions in the pathogenesis of common diseases with non-Mendelian genetics. Moreover, this model has been used successfully to identify further modifying C57BL/6 loci regulating insulin sensitivity (Almind et al. 2003).

Inactivation of PPARs

It has been shown that the abnormal accumulation of lipids in tissues other than adipose adversely affects insulin sensitivity, indicating a complex system for the comprehensive control of lipid and glucose homeostasis. The key coordinators in this metabolic axis are members of the nuclear hormone receptor superfamily. Among those, PPARs (see ▶ Assays for Insulin and Insulin-Like Regulation of Gene and Protein Expression) respond to small lipid agents, e.g., dietary fatty acids, and contribute a key mechanism in the regulation of lipid and glucose metabolism (reviewed by Rangwala and Lazar 2004). The important physiological role of the PPARs, i.e., PPAR-α, PPAR-β/δ, and PPAR-γ, was deduced from findings identifying the PPARs as primary targets of two key classes of synthetic compounds that have been used in the successful treatment of diabetes and dyslipidemia. In particular, thiazolidinedione (TZD) insulin sensitizers are potent and specific PPAR-γ ligands and activators. Fibrates have predominant activity as PPAR-α agonists, favorably affecting serum lipid levels. Diverse knockout models have been created to study the function of the single PPAR isoforms in vivo.

Consistent with the concept that PPAR-α is the member of the PPAR family that mediates cellular lipid utilization, pharmacological inhibition of cellular fatty acid flux in mice lacking PPAR-α caused massive hepatic and cardiac lipid accumulation, hypoglycemia, and death in 100 % of male and 25 % of female animals, demonstrating a pivotal role for PPAR-α in lipid and glucose homeostasis in vivo. Nevertheless, there was no major phenotypic defect detectable in PPAR-α knockouts without pharmacological challenge (Djoudi et al. 1998). Muoio and coworkers (2002) showed that skeletal muscle of PPAR-α-deficient mice exhibited only minor changes in fatty acid homeostasis, and even mRNA expression of known PPAR-α target genes in muscle tissue was not significantly affected. They proposed that this finding might be explained by high levels of PPAR-β/δ compensating for the lack of PPAR-α, suggesting redundancy in the functions of PPARs as transcriptional regulators of fatty acid homeostasis.

In contrast to the embryonic lethality of homozygous PPAR-γ knockouts (Barak et al. 1999), mice deficient only for the PPAR-γ2 isoform survived, exhibiting an overall reduction in white adipose tissue, less lipid accumulation, and decreased expression of adipogenic genes in adipose tissue (Zhang et al. 2004). Consistently, embryonic fibroblasts of PPAR-γ2 knockouts showed a dramatically reduced capacity for adipogenesis in vitro. In addition, insulin sensitivity was impaired in these mice, with decreased expression of IRS-1 and Glut4 in skeletal muscle, but TZDs were able to normalize this insulin resistance (Zhang et al. 2004). In contrast, heterozygous PPAR-γ knockouts show reduced disposition to insulin resistance. Strikingly, this phenotype is blunted by treatment with a synthetic PPAR-γ ligand of the TSD class, indicating that optimal levels of PPAR-γ activity are crucial for its beneficial effects. To address these obvious incongruities, PPAR-γ has been selectively disrupted in the liver, adipose tissue, and muscle. Briefly, although adipose tissue appears to be the main site of TZD action, the conditional knockouts highlight important functions for the muscle and liver PPAR-γ in the control of body composition and insulin sensitivity. PPAR-γ deficiency in adipose tissue leads to progressive loss of fat, hyperlipidemia, fatty liver, and accompanying hepatic insulin resistance (He et al. 2003). The mice can maintain normal whole-body glucose homeostasis and normal insulin sensitivity only as long as some adipose tissue is present. These studies identify a molecular link between blood

glucose homeostasis and lipid metabolism, providing a genetic basis for the observed phenotypic correlation between obesity and type II diabetes mellitus.

Transgenic Animal Model for Type I Diabetes

Aichele and coworkers (1994) used a synthetic peptide corresponding to an immunodominant epitope of lymphocytic choriomeningitis virus glycoprotein (LCMV GP) to prime or to tolerize CD^{8+} T cells in vivo. Peptide-specific tolerance was then examined in transgenic mice expressing LCMV GP in the β islet cells of the pancreas. These mice developed CD^{8+} T cell-mediated diabetes within 8–14 days after LCMV infection. Specific peptide-induced tolerance prevented autoimmune destruction of β islet cells and diabetes in this transgenic mouse model. Oldstone and coworkers (1991) showed that virus infection triggers insulin-dependent diabetes mellitus in a transgenic mouse model. Ablation of tolerance and induction of diabetes by virus infection in viral antigen transgenic mice was reported by Ohashi and coworkers (1991). Von Herrath and coworkers (1994) investigated how virus induces a rapid or slow-onset insulin-dependent diabetes mellitus in two distinct transgenic mouse models. Von Herrath and coworkers (1995) evaluated the role of the costimulatory molecule B7-1 in overcoming peripheral ignorance in transgenic mice which expressed the glycoprotein or nucleoprotein of lymphocytic choriomeningitis virus as the self-antigen in pancreatic β-cells. Von Herrath and Holz (1997) reported that pathological changes in the islet milieu precede infiltration of islets and destruction in β-cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. RIP-LCMV transgenic mice that express the viral glycoprotein or nucleoprotein from lymphocytic choriomeningitis virus (LCMV) under control of the rat insulin promoter (RIP) in pancreatic β-cells develop autoimmune diabetes after infection with LCMV. Upregulation of MHC class II molecules associated with the attraction/activation of antigen-presenting cells to the islets occurs as soon as 2 days after LCMV inoculation of transgenic mice, clearly before CD^{4+} and CD^{8+} lymphocytes are found entering the cells. Possibilities of treatment of virus-induced autoimmune diabetes were discussed (von Herrath et al. 1997). Moritani and coworkers (1996) reported the prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes as a gene therapy model for autoimmune diabetes. Birk and coworkers (1996) generated transgenic NOD mice carrying a murine Hsp60 transgene driven by the H°2Eα class II promoter in order to examine the hypothesis of a pathogenic role for self-reactive cells against the stress protein Hsp60 in autoimmune destruction of pancreatic cells in the diabetes of NOD mice.

Future Developments

The employment of even more sophisticated strategies in conditional gene targeting may overcome these limitations in the future. Conditional inactivation of genes is mainly achieved by the Cre-loxP system (Sauer 1998). Moller (1994) and Plum and coworkers (2005) recently summarized our current knowledge about the use of knockout and transgenic animal models in diabetes research. Animal models of insulin resistance have recently been reviewed by Nandi and coworkers (2004).

Adipose Tissue-Specific Transgenic Mouse Models

Purpose and Rationale

There has been a paradigm shift from the notion of adipose tissue merely as a storage site for energy to one where adipose tissue plays an active role in energy homeostasis and various pathogenic processes, such as the development of type 2 diabetes (T2D) and cardiovascular diseases (Flier 2004). The predominant type of adipose tissue, commonly called “fat” in mammals, is white adipose tissue (WAT). WAT is composed mostly of adipocytes surrounded by loose connective tissue that is highly vascularized and innervated and contains macrophages, fibroblasts, adipocyte precursors, and various other cell types (Ahima 2006). The increase in WAT mass in obesity is associated with profound histological and biochemical changes

characteristic of inflammation (Weisberg et al. 2003; Xu et al. 2003). Obesity also results in an increase in fibrogen, plasminogen activator inhibitor-1, and various coagulation factors (Skurk and Hauner 2004). Visceral adiposity in the human being has been associated with cardiovascular diseases (Bealnger et al. 2002). Increasing WAT formation in the subcutaneous region was found associated with improvement in glucose and lipid metabolism and reduced incidence of atherosclerosis (Morton et al. 2004).

Many researchers (Shepherd et al. 1993; Kopecky et al. 1995; Horton et al. 2003; Jurczak et al. 2007) have established adipose tissue-specific transgenic mice to study the function of adipocyte-derived genes. A common feature of these approaches is that the fat-specific promoter/enhancer from the fatty acid-binding protein (aP2) is used. aP2 promoter/enhancer has also been used to prepare aP2-Cre recombinase transgenic mice, which can be utilized for adipose tissue-specific knockout mice preparation (Barlow et al. 1997, see also above). The strategy for the preparation of aP2-cholesteryl ester transfer protein (CETP) transgenic (Tg) mice is described here as an example for establishing adipose tissue-specific transgenic mice.

CETP is a hydrophobic plasma glycoprotein that mediates the transfer and exchange of cholesteryl esters (CEs) and triglycerides (TGs) between plasma lipoproteins and plays an important role in HDL metabolism (Tall 1993). Mice do not express CETP, and human CETP transgenic mice with predominant liver expression show reduced HDL-C levels (Agelion et al. 1991). However, the liver is not the major source of CETP in all mammalian species. In hamsters, for example, adipose tissue shows the highest levels of CETP expression, and CETP mRNA is almost undetectable in the liver (Jiang et al. 1991). Adipose tissue appears to be a highly conserved site of CETP expression across species. However, its function in adipose tissue is still unknown. It has been reported that plasma CETP concentrations are positively correlated between adipose CETP mRNA levels in hamster (Quinet et al. 1993). In monkeys, a correlation between adipose CETP mRNA and CETP levels is also observed (Quinet et al. 1991). Moreover, human adipose tissue maintained in organ culture synthesizes and secretes CETP (Radeau et al. 1995). Other studies have shown that plasma CETP activity in humans correlates with the degree of adiposity (Dullaart et al. 1994; Arai et al. 1994) and that weight reduction is associated with a decrease in plasma CETP activity (Dullaart et al. 1994). A hamster study also indicated that adipose tissue releases CETP activity during incubation in vitro and is subject to hormonal and nutritional regulation (Remillard et al. 2001). Furthermore, the release of CETP activity from cultured hamster adipose tissue increases following a period of fasting (Shen and Angel 1995). Despite these correlative findings, there is no direct evidence that adipose-derived CETP enters the circulation or that it influences plasma lipoprotein levels. In order to investigate these issues, an adipose tissue-specific CETP transgenic mouse line has been generated.

Procedure

Creation of aP2-CETP-pBK Vector

A 5.4-kb mouse aP2 enhancer/promoter region is digested using Kpn I and Not I from a aP2-pBluescript SK vector. The generated fragment is ligated to an upstream region of the CETP-pBK vector that contains a 6.8-Kb human CETP minigene.

Preparation of aP2-CETP Transgenic Mice

On day 1 at 1:00 pm, an intraperitoneal injection of pregnant mare's serum at 5 units/C57BL/6 J or FVB female mouse is performed to mimic follicle-stimulating hormone and induce superovulation. On day 3 at 1:00 pm, an intraperitoneal injection of human chorionic gonadotropin at 5 units/C57BL/6 J or FVB female mouse is performed to mimic LH and induce superovulation. Mating is set up of C57BL/6 J female (4–6 weeks) with C57BL/6 J male (8–20 weeks) or FVB female (4–6 weeks) with FVB male (8–20 weeks) for harvesting fertilized eggs and Swiss Webster female (7–15 weeks) with vasectomized (sterile) male (8–24 weeks) for preparing pseudopregnant female. On day 4 at 9:00 M, the plugged Swiss Webster females that will be used as

pseudopregnant egg recipient females are checked, and mated FVB or C57BL/6 J females are collected for egg harvesting. Thereafter, the abdominal cavity is opened. After detection, both sides of coils of oviducts are cut. The oviducts are broken in M2 medium (Sigma) to release the eggs. For removal of cumulus cells, the eggs are incubated with hyaluronidase (300 ug/ml) for 30 min at 37 °C. Thereafter, the eggs are washed by transferring the eggs to a dish containing fresh M2 medium three times. Subsequently, the clean eggs are transferred to M16 medium (Sigma) for cultivation for 2 h at 37 °C and then used for injection.

A aP2-CETP-pBK vector is digested by Kpn I. The linear DNA fragment is adjusted to a final concentration of 5 μg/ml in 10 mM Tris/HCl and 0.1 mM EDTA. Microinjection of the DNA into pronuclei is performed using a micromanipulator (Eppendorf) with appropriate holding pipette and injection needle and a injection chamber containing M2 medium covered with mineral oil. Ten to 20 eggs are transferred into the chamber each time. The optimal amount of DNA is injected into the (swollen) pronuclei of the eggs. The injected eggs are kept in M16 medium at 37 °C until transfer back to the oviduct of pseudopregnant females. The oviduct transfer can be performed right after the microinjection or the day after injection. For this, the pseudopregnant egg recipient mouse is weighed and then anesthetized by injecting intraperitoneally 0.016 ml/g of 2.5 % Avertin. A small incision of skin and underneath muscle is made on the lateral side of the mouse. Thereafter, the ovary, oviduct, and part of the uterus are pulled out. After detection of the infundibulum, i.e., the opening of the oviduct which is typically hidden within coils of the oviduct, the transfer pipet harboring the eggs is inserted into it and the eggs (20–30 eggs for each side of oviduct) are transferred into the oviduct by blowing on the pipet. The ovary, oviduct, and uterus are put back, and the incision is closed with a suture. The pubs are delivered 20–21 day after the oviduct transfer. The genotyping can be performed when the young mice reach 4–5 weeks age.

For the screening of aP2-CETP transgenic mice, 0.5 cm of the mouse tail tip is cut and then put into 0.5 ml of DNA digestion solution (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2.5 mM EDTA, and 0.5 % SDS containing 0.1 mg/ml proteinase K) and incubated at 58 °C under rotation overnight. After centrifugation of the tube, the supernatant is carefully transferred to a new tube and then supplemented with 0.5 ml of phenol for extraction of the DNA. After addition of 0.5 ml of chloroform, the DNA is extracted again. For precipitation of the DNA, 1 ml of ethanol is added. Following centrifugation (10,000 × g for 10 min), the pellet is washed once with 70 % ethanol and then dissolved in 60 μl of 10 mM Tris/HCl (pH 7.4) containing 1 mM EDTA by incubation at 56 °C for at least 2 h. The DNA solution can be used to do PCR and Southern blotting. CETP activity in serum of the mice can be measured by a fluorescence-based method (Roar Biomedical Inc., New York, NY), which is compatible with the radiolabeled method (Radeau et al. 1995). For this, 4 μl of donor, 4 μl of acceptor, 3 μl of plasma sample, and 89 μl of assay buffer (10 mM Tris/HCl, pH 8.0, 0.9 % NaCl, 2 mM EDTA) are mixed and then incubated at 30 °C for 1 h. Fluorescence emission is read at 460 nm under excitation of 530 nm using a fluorescence plate reader (7620 Microplate Fluorometer; Cambridge Technology, Watertown, MA).

Evaluation

It has been demonstrated that CETP mRNA is predominantly expressed in adipose tissue in two independently generated lines of aP2-CETPTg mice (Zhou et al. 2006; Jiang et al. 1992). There are also low levels of expression in the heart and muscle. This might be attributable to the contamination of adipose tissue in both organs. For instance, the epicardium contains substantial adipose tissue that could contribute to the total RNA prepared from heart. It has been reported that the aP2 promoter/enhancer also functions in macrophages (Yvan-Charvet et al. 2005; Fu et al. 2002). However, there is no detectable CETP mRNA in the macrophages from aP2-CETPTg mice. Although the expression levels are negligible compared with adipose tissue, it is still not known why the lung also can express low levels of aP2-CETP transgene.

To evaluate the impact of CETP expression on adipose tissue lipid levels, total lipids are extracted from both wild-type and aP2-CETPTg mouse adipose tissues and assayed for TGs, cholesterol, and phospholipids. Both TG and cholesterol levels are found to be significantly decreased (by 50 % and 40 %, respectively) compared with the wild type, whereas no significant change is found in phospholipid levels. It has been reported that when TG and cholesterol stores are depleted, adipocytes reduce their size (Chen and farese 2002; Le Lay et al. 2004). To see whether this is the case in the aP2-CETPTg adipocyte, adipose tissue is dissected and stained with hematoxylin and eosin. The images were analyzed to determine the cross-sectional surface area of each adipocyte. aP2-CETPTg mice have a greater number of small adipocytes than wild-type mice. This difference in frequency distribution is reflected in a 44 % decrease in mean surface area of adipocytes from aP2-CETPTg mice.

For practical advice, eggs from FVB mice have larger nuclei than that from C57BL/6 J, making it easier to inject the DNA fragment into FVB eggs rather than into C57BL/6 J eggs. Furthermore, it is better to use the gene or minigene for construction of the vector to create the transgenic mice rather than the cDNA (Farley et al. 2000; Jones et al. 2005).

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Metabolic Systems Biology

With the completion of the many genomes, genetics is positioned to meet physiology. Argmann and coworkers (2005) predicted the coming of “systems metabolism” in mammals

through the use of the mouse, as a model system to study metabolism. Building on mouse genetics with increasingly sophisticated clinical and molecular phenotyping strategies has enabled scientists to now tackle complex biomedical questions, such as those related to the pathogenesis of the common metabolic disorders. The ultimate goal of such strategies will be to mimic human metabolism with the click of a mouse.

Techniques in mouse genetics and phenomics have rapidly evolved over the past decade, which with the renaissance of integrative physiology positioned scientists well for the emergence of the discipline, systems biology. Such systems biology approaches hold great promise in the area of metabolism. The efficient conversion of metabolic parts to metabolic systems will depend on the integration of discovery-based and hypothesis-driven phenogenomic approaches, which rely on the study of mouse models derived from both reverse and forward genetic strategies. A stable marriage of in silico with wet biology that allows for reiterative testing of the computationally identified networks will be crucial for the success of systems biology approaches. Such integrated approaches will become indispensable future resources for drug target identification as well as drug discovery and evaluation. It is expected that such insight into complex metabolic networks will ultimately translate into better therapeutic strategies for metabolic diseases.

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Measurement of Blood Glucose-Lowering and Antidiabetic Activity

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Hypoglycemic Effects

Blood Glucose-Lowering Effect in Rabbits

Purpose and Rationale

The rabbit has been used since many years for standardization of insulin (see ▶ Measurement of Insulin and Other Glucose-Regulating Peptide Hormones). Therefore, it has been chosen as the primary screening model for screening of blood glucose-lowering compounds as well as for establishing time-response curves and relative activities (Bänder et al. 1969, Geisen 1988).

Procedure

Groups of 4–5 mixed breed rabbits (e.g., Hoe: BASK, SPFWiga) of either sex weighing 3.0–4.5 kg are used. For insulin evaluation, food is withheld overnight. For evaluation of sulfonylureas and other blood glucose-lowering agents, the animals are on a normal diet (e.g., Era mixed feed 8300) prior to the experiment. The animals are gently placed into special restraining boxes allowing free access to the rabbit's ears.

Oral blood glucose-lowering substances are applied by gavage in 1 ml/kg of 0.4 % starch suspension or intravenously in solution. Several doses are given to different groups. One control group receives the vehicle only. By puncture of the ear veins, blood is withdrawn immediately before and 1, 2, 3, 4, 5, 24, 48, and 72 h after treatment. For time-response curves, values are also measured after 8, 12, 16, and 20 h. Blood glucose is determined in 10 µl blood samples with the hexokinase enzyme method (Glucoquant test kit).

Evaluation

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects at a time interval are calculated using the trapezoidal rule. The values of the experimental group are compared statistically with the t-test or the Wilcoxon test for each time interval with those of the control group. Differences between several treated groups and the control group are tested using a simultaneous comparison according to Dunnett or Nemenyi/Dunnett. Dose dependencies and relative activities are determined by means of linear regression analysis after Fieller (1944) and Sidak (1967). All data for statistical comparisons have to be tested for homogeneity of variances according to Levene (1960) and for normal distribution according to Shapiro and Wilk (1965). In the case of regression analyses, the lines are additionally tested for parallelism according to Kurtz et al. (1966) and for linearity according to Scheffé (1959). The level of significance for all procedures is chosen as 5 %.

Modifications of the Method

For special purposes, the effect of blood sugar-lowering agents is studied in glucose-loaded animals. Rabbits of either sex weighing 3.0–4.5 kg are treated either once (0.5 h after test compound) or twice (0.5 and 2.5 h after test compound) orally with 2 g glucose/kg body weight in 50 % solution.

A biological assay of insulin preparations in comparison with a stable standard using the blood sugar-lowering effect in rabbits has been proposed by Harrison and coworkers (1925).

The biological assay of insulin using the blood sugar-lowering effect in rabbits has been until recently the official assay in several pharmacopeias, such as the European Pharmacopoeia, 2nd edn. 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988; United States Pharmacopeia 23; and the National Formulary 18, 1995.

The rabbit blood glucose bioassay as well as the mouse convulsion assay and the mouse glucose assay were used for establishing international standards for highly purified human, porcine, and bovine insulins (Bristow et al. 1988).

In several pharmacopeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd edn. 1997); but the rabbit blood sugar method is still valid in the United States Pharmacopeia USP 24, 2000.

Procedure

Four groups of at least 6 randomly distributed rabbits weighing at least 1.8 kg are kept in the

laboratory and maintained on a uniform diet for not less than one week before use in the assay. About 24 h before the test, each rabbit is provided with an amount of food that will be consumed within 6 h. The same feeding schedule is followed before each test day. During the test all food and water is withheld until the final blood sample has been taken. The rabbits are placed into comfortable restraining cages to avoid undue excitement.

Immediately before use two solutions of the standard preparation are made, containing 1 unit and 2 units of insulin per ml, respectively, and two dilutions of the preparation being examined which, if the assumption of potency is correct, contain amounts of insulin equivalent to those in the dilutions of the standard preparation. As diluent, a solution is used of 0.1–0.25 % w/v of either m-cresol or phenol and 1.4–1.8 w/v of glycerol being acidified with hydrochloric acid to a pH between 2.5 and 3.5.

Each of the prepared solutions is injected subcutaneously to one group of rabbits, using the same volume, which should usually be between 0.3 and 0.5 ml for each rabbit, the injections being carried out according to a randomized block design. Preferably on the following day, but in any case not more than 1 week later, each solution is administered to a second group of rabbits following a twin-crossover design. One hour and 2.5 h after each injection, a suitable blood sample is taken from the ear vein of each rabbit. Blood sugar is determined by a suitable method, preferably using glucose oxidase.

Evaluation

The results of the assay are calculated by standard analytical methods (e. g., USP 23, 1995).

Critical Assessment of the Method

The classical bioassay based on blood sugar-lowering activity in rabbits has been replaced by chemical methods in some pharmacopeias (Underhill et al. 1994) but is still included in USP 24 (2000) and will be still necessary for evaluation of synthetic insulin derivatives.

Modifications of the Method II

An assay of insulin activity after intraperitoneal injection in rats has been described by Rafaelsen and coworkers (1965) and Young (1967). Shults and coworkers (1994) reported as one of the first on an implantable potentiostat-radiotelemetry system for in vivo sensing of glucose, implanted into the paravertebral thoracic subcutaneous tissue of a dog. An enzyme electrode sensor measures the oxidation current of hydrogen peroxide formed by the stoichiometric conversion of the substrate glucose and oxygen as a cofactor in an immobilized glucose oxidase layer. Salehi and coworkers (1996) described the development of a compact, low-power, implantable system for in vivo monitoring of oxygen and glucose concentrations.

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Blood Glucose-Lowering Effect in Rats

Purpose and Rationale

Rats are used for screening as well as for quantitative evaluation of blood glucose-lowering agents.

Procedure

Male Wistar rats (e.g., Hoe:WISKf, SPF 71) weighing 180–240 g are kept on standard diet (e.g., Altromin 1324). Groups of 4–7 non-fasted animals are treated orally or intraperitoneally with various doses of the test compounds suspended in 0.4 % starch suspension. One control group receives the vehicle only. Blood is withdrawn from the tip of the tail immediately before and 1, 2, 3, 5, and 24 h after administration of the test compound. Blood glucose is determined in 10 µl blood samples with the hexokinase enzyme method (Glucoquant test kit).

Evaluation

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Statistical evaluation is performed as described for tests in rabbits.

Modifications of the Method

Studies in Glucose-Loaded Rats

For special purposes, the effect of blood sugar-lowering agents is studied in glucose-loaded animals. One g glucose/kg body weight is given in a 50 % solution either orally 5 min after oral administration or subcutaneously 5 min after intraperitoneal administration of the test compound.

Studies in Streptozotocin-Diabetic Rats

Male Wistar rats (e. g., Hoe:WISKf, SPF 71 strain) weighing 170–220 g are kept on standard diet

(e.g., Altromin 1324). Ten to fourteen days prior to the study, they are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. Blood sugar levels rise from 5.5 to 6.0 mmol/l up to 25.0–28.0 mmol/l and glucosuria occurs. Plasma insulin levels fall below 4 μU/ml. Compounds which release insulin from pancreatic islets as sole hypoglycemic activity are not effective in rats with severe streptozotocin-induced diabetes.

Blood Glucose-Lowering Effect in Mice

Purpose and Rationale

Eneroth and Ahlund (1968, 1970a and b) recommended a twin-crossover method for bioassay of insulin using blood glucose levels in mice instead of hypoglycemic seizures giving more precise results. This test was induced into the British Pharmacopoeia 1980 and continued up to 1988. Moreover, the test is included as alternative in the European Pharmacopoeia, 2nd edn. 1980, and in Deutsches Arzneibuch, 9. Ausgabe, 1986.

Procedure

Non-fasting mice of the same strain and sex are used having body masses such that the difference between the heaviest and lightest mouse is not more than 2 g. The mice are assigned at random to four equal groups of not less than 10 animals. Two dilutions of a solution of the substance or of the preparation to be examined and two dilutions of the reference solution are prepared using as diluent 0.9 % NaCl solution adjusted to pH 2.5 with 0.1 N hydrochloric acid and containing a suitable protein carrier. In a preliminary experiment, concentrations of 0.02 IU and 0.10 IU are tested. Each of the prepared solutions (0.1 ml/10 g body weight) is injected subcutaneously to one group of mice according to a randomized block design. Not less than 2.5 h later, each solution is administered to a second group of mice following a twin-crossover design. Exactly 30 min after each injection, a sample of 50 μl of blood is taken from the orbital venous sinus of each mouse. Blood glucose concentration is determined by a suitable method, such as described by Hoffman (1937).

Evaluation

The potency is calculated by the usual statistical methods for the twin-crossover assay.

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Blood Glucose-Lowering Effect in Dogs

Purpose and Rationale

Besides experiments in rats and rabbits, studies in dogs are necessary to predict the effect of a new compound in man due to differences in species-related metabolism.

Procedure

Male Beagle dogs weighing 15–20 kg are kept on standard diet (e.g., Erka mixed feed 8,500). Food is withdrawn 18 h prior to the administration of the test compound which is given either orally or intravenously in various doses. Control animals receive the vehicle only. Blood is collected at different time intervals up to 48 h. Blood glucose is determined with the hexokinase enzyme

method (Glucoquant test kit) and plasma insulin with an immunological method (Riagnost kit).

Evaluation

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Similarly, plasma insulin levels are plotted versus time and compared with control values. Statistical evaluation is performed as described for tests in rabbits.

Modifications of the Method

Studies in Pancreatectomized Dogs

The surgical technique of pancreatectomy in dogs is described in chapter ▶ Methods to Induce Experimental Diabetes Mellitus. The animals are pancreatectomized up to 2–3 years prior to the study. They are kept on dry feed (Vipromix) together with 2–3 g pancreatic enzymes (Viveral). Insulin is substituted with a single daily subcutaneous dose of 32 IU Insulin Ultratard HM. For substitution of vitamin D an intramuscular dose of 1 ml Vigantol is given every 3 months.

On the day before the study, the animals receive 32 IU of the shorter-acting Basal-H insulin. This insulin is administered at the same time when food and test compound are given in the morning. The test drug is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Studies in Alloxan-Diabetic Dogs

Chemical diabetes can be induced by a single intravenous dose of 60 mg/kg alloxan. Afterward, the animals receive infusions of 1,000 ml 5 % glucose together with 10 IU regular insulin via a jugular vein catheter daily during one week and canned food ad libitum. Thereafter, a single dose of 28 IU Insulin Ultratard HM is given daily and the animals are fed commercial diet (Altromin pellets). On the day before the study, the dogs receive 28 IU of the shorter-acting Basal-H insulin. This insulin is given at the same time as food and test compound in the morning. The test drug is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Continuous Blood Glucose Monitoring

A device for continuous blood glucose monitoring and infusion in freely mobile dogs was described by Geisen and coworkers (1981), Geisen (1988) and Bander and coworkers (1969).

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Blood Glucose-Lowering Effect in Other Species

Instead of rats, male guinea pigs (e. g., Pirbright white, Hoe:DHPK, SPF Lac) weighing 250–380 g can be used. Blood is withdrawn by puncture of ear veins before and 1, 3, and 5 h after administration of the test compound or the vehicle. Blood sugar determinations and statistical evaluations are performed as described for rabbits and rats.

Genetically obese and diabetic yellow KK mice have been used by Sohda and coworkers (1990) for evaluation of hypoglycemic activity of potential antidiabetic drugs. Gill and Yen (1991) studied the effect of ciglitazone in obese-diabetic viable yellow mice (VY/Wfl-Avy/a).

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Euglycemic Clamp Technique

Purpose and Rationale

The euglycemic glucose clamp technique has provided a useful method of quantifying in vivo insulin sensitivity in humans (DeFronzo et al. 1979). In this technique, a variable glucose infusion is delivered to maintain euglycemia during insulin infusion. Whole-body tissue sensitivity to insulin, as determined by net glucose uptake, can be quantitated under conditions of near-steady-state glucose and insulin levels. Kraegen and coworkers (1983, 1985) developed the euglycemic glucose clamp technique for use in the intact conscious rat.

Procedure

Male Wistar rats weighing 150–200 g are fasted overnight and anesthetized with pentobarbital (40 mg/kg, i.p.). Catheters are inserted into a jugular vein and a femoral vein for blood collections and insulin and glucose infusion, respectively. To evaluate the insulin action under physiological hyperinsulinemia (steady-state plasma insulin concentration during the clamp test around 100 μU/dl) and maximal hyperinsulinemia (under which maximal insulin action may appear), two insulin infusion rates, 6 and 30 mU/kg/min, are used. The blood glucose concentrations are determined from samples collected at 5-min intervals during the 90-min clamp test. The glucose infusion rate is adjusted so as to maintain the blood glucose at its basal level during the clamp test. The final glucose infusion rate is calculated from the amount of glucose infused for the last 30 min (from 60 to 90 min after start of the clamp) in which the blood glucose levels are in a steady state. The glucose metabolic clearance rate is obtained by dividing the glucose infusion rate by the steady-state blood glucose concentration. The steady-state plasma insulin concentration is calculated from the insulin concentrations at 60 and 90 min after the start of the clamp. At the start and end of the euglycemic clamp test, free fatty acid concentration is also determined and the free fatty acid suppression rate is calculated.

Evaluation

All values are analyzed by one-way ANOVA. When the steady-state plasma insulin is maintained at submaximal concentration by the euglycemic clamp technique, the glucose infusion rate and glucose metabolic clearance rate value are considered to reflect the state of receptor binding levels in the peripheral tissue as an index for insulin sensitivity. Under maximal hyperinsulinemia, these values are thought to reflect the state of the enzymes and glucose transport system activated after the binding to receptors, indicating mainly insulin responsiveness.

Modifications of the Method

Burnol and coworkers (1983) used the euglycemic insulin clamp technique coupled with isotopic measurement of glucose turnover to quantify insulin sensitivity in the anesthetized rat.

Bryer-Ash and coworkers (1995) used this technique to demonstrate reduction of insulin sensitivity by amylin corresponding to reduced insulin receptor kinase activity.

The effects of counterregulatory hormones on insulin-induced glucose utilization by individual tissues in rats, using the euglycemic-hyperinsulinemic clamp technique combined with an injection of 2-[1-3H]-deoxyglucose, were studied by Marfaing and coworkers (1991).

Lang (1992) determined the insulin-mediated glucose uptake in normal and streptozotocin-diabetic rats using the euglycemic-hyperinsulinemic clamp technique.

Hirshman and Horton (1990) reported increased insulin sensitivity and responsiveness in peripheral tissues of the rat after glyburide as determined by the glucose clamp technique.

Lee and coworkers (1994) studied the metabolic effects of troglitazone on fructose-induced insulin resistance with the euglycemic-hyperinsulinemic clamp technique in rats.

Tominaga and coworkers (1992, 1993) studied the influence of insulin antibodies in anesthetized rats and of thiazolidinediones on hepatic insulin resistance in streptozotocin-induced diabetic rats by the glucose clamp technique.

Gelardi and coworkers (1991) used the hyperinsulinemic-euglycemic clamp technique to evaluate the insulin sensitivity in the obese offspring of streptozotocin-induced mildly hyperglycemic rats.

Hulman and coworkers (1993) studied insulin resistance in the conscious spontaneously hypertensive rat with the euglycemic-hyperinsulinemic clamp technique.

Cheung and Bryer-Ash (1994) described a modified method for the performance of glucose insulin clamp studies in conscious rats under local anesthesia.

Burvin and coworkers (1994) developed a modification of the euglycemic insulin clamp technique and used it to repeatedly assess in vivo insulin effects in awake streptozotocin-induced diabetic rats.

Xie and coworkers (1996) described a modified euglycemic clamp technique in cats.

Finegood and coworkers (1987) estimated endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps using unlabeled and labeled glucose infusates in dogs.

Xie and coworkers (1996) described an insulin sensitivity test using a modified euglycemic clamp in cats and rats. This test uses the amount of glucose required to be infused to maintain euglycemia over a 30-min period in rats and 60 min in cats following a bolus administration of insulin as the index of insulin sensitivity. Glucose levels are determined as short intervals and variable glucose infusion is used to hold glucose levels within a few percentage points of the basal pretest glucose level. A new blood sampling technique is described that allows each insulin sensitivity test to be carried out using a total of only 0.5 ml of blood.

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Hypoglycemic Seizures in Mice

Purpose and Rationale

The biological assay of insulin using hypoglycemic seizures in mice has been suggested already in 1923 by Fraser. The biological standardization of insulin using the mouse convulsion method has been published in detail by the Health Organisation of the League of Nations in 1926 (Trevan and Boock, Hemmingsen and Krogh) and has been until recently the official assay in several pharmacopeias, such as the European Pharmacopoeia, 2nd edn. 1980; Deutsches Arzneibuch 1986; and British Pharmacopoeia 1988. In most pharmacopeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd edn. 1997).

Procedure

Ninety-six mice of either sex (but not of mixed sexes) weighing 20 ± 5 g are randomly distributed into 4 groups. The mice are deprived of food 2–20 h immediately preceding the test. Solutions of the insulin standard and of the test preparation containing 30 and 60 milliunits/ml are prepared by diluting the original solution with 0.9 % NaCl solution, pH 2.5. 0.5 ml/20 g mouse of these solutions is injected subcutaneously. The mice are kept at a uniform temperature, between 29 °C and 35 °C, in transparent containers within an air incubator with a transparent front. The mice are observed for 1.5 h and the number of mice that are dead, convulse, or lie still for more than 2 or 3 s when placed on their backs is recorded.

Evaluation

The percentage of mice of each group showing the aforementioned symptoms is calculated and the relative potency of the test solution calculated using a 2 + 2 point assay.

Critical Assessment of the Method

Attempts to replace the tests in mice and rabbits by in vitro tests, such as the rat diaphragm test, the rat epididymal fat pad test, or even the radioimmunoassay, failed due to several reasons (Trethewey 1989). Nevertheless, for industrial production and for stability studies, the classical bioassays based on hypoglycemic seizures in mice or hypoglycemia in rabbits have been replaced by chemical methods (Stewart 1974, Underhill et al. 1994).

Modifications of the Method

A modification of the mice seizure method using rotating hollow cylinders has been proposed by Young and Lewis (1947). A similar technique which increases the sensitivity of the mice seizure method has been used by Vogel (1964, unpublished data). The equipment consisted of seven perforated metal drums with a diameter of 15 cm. The drums were rotated in oblique position at 10 rotations per minute. Female mice weighing 16–20 g were deprived of feed at the afternoon before the test. On the test day, groups of 6 mice received doses of 0.5, 1.0, or 1.5 IU/kg of test preparation or standard subcutaneously and were placed after 20 min into the rotating drums. Controls received saline only. Mice with insulin-induced seizures dropped out from the rotating drum. The number of animals dropping out was counted after 15, 30, 45, and 60 min. Mice dropping out due to hypoglycemic seizures received 0.5 ml of 10 % glucose solution intraperitoneally. ED50 values and activity ratios were calculated according to Litchfield and Wilcoxon (1949).

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Effects of Insulin Sensitizer Drugs

Purpose and Rationale

Insulin sensitizer drugs are reported to improve symptoms in patients with established type 2 diabetes (NIDDM = non-insulin-dependent diabetes mellitus) (Colca 1995; Kuehnle 1996). In contrast to sulfonylureas, these compounds do not lower blood glucose in normal animals. Various animal models resembling type 2 diabetes are used for evaluation. Details of these models are described in the respective chapters. In vitro techniques showed an increased glucose uptake into muscle tissue and into adipocytes.

Procedure

In Vivo Studies

Chang et al. (1983) studied ciglitazone in ob/ob and db/db mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats.

Fujita et al. (1983) investigated the effects of ciglitazone in obese-diabetic yellow KK (KK-Ay) mice and obese Zucker-fatty rats.

Diani et al. (1984) treated C5BL/6 J-ob/ob and C57BL/KsJ-db/db mice for several weeks with ciglitazone and studied the morphological effects on pancreatic islets.

Fujiwara et al. (1988) performed studies in KK and ob/ob mice and Zucker-fatty rats.

Moreover, Fujiwara et al. (1991) studied the effects of CS-045 on glycemic control and pancreatic islet structure at a late stage of the diabetes syndrome in C57BL/KsJ-db/db mice.

Ikeda et al. (1990) and Sohda et al. (1990) used insulin-resistant animals (yellow KK mice, Zucker-fatty rats, and obese beagle dogs with moderate insulin resistance).

Gill and Yen (1991) studied the effects on endogenous plasma islet amyloid polypeptide and insulin sensitivity in obese-diabetic viable yellow mice.

Hofmann et al. (1991, 1992) treated insulin-resistant KK-Ay mice.

Stevenson et al. (1991) studied the effects of englitazone in nondiabetic rats and found no overt hypoglycemia but an enhancement of insulin action.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activating insulin receptor kinase in genetically obese Wistar fatty rats treated with various doses of pioglitazone.

Sugiyama et al. (1990) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

Tominaga et al. (1993) used the glucose clamp technique in streptozotocin-induced diabetic rats.

Yoshioka et al. (1993) found antihypertensive effects in obese Zucker rats.

Lee et al. (1994) studied the metabolic effects on fructose-induced insulin resistance in rats.

Apweiler et al. (1995) administered BM 13.09143 to lean and obese Zucker rats and performed hyperinsulinemic-euglycemic clamp studies in these animals.

Fujiwara et al. (1995) found a suppression of hepatic gluconeogenesis in long-term troglitazone-treated diabetic KK and C57BL/KsJ-db/db mice.

Lee and Olefsky (1995) studied the effects of troglitazone in normal rats with the euglycemic glucose clamp technique.

In Vitro Studies

Kirsch et al. (1984) found a reversal of cAMP-induced post-insulin receptor resistance in rat adipocytes in vitro.

Ciaraldi et al. (1990) performed in vitro studies using cultured hepatoma cells (Hep G2) and muscle cells (BC3H-1) and found an increased glycogen synthase I activity in both cell types.

Murano et al. (1994) found a stimulation of fructose-2,6-bisphosphate production in rat hepatocytes.

Kellerer et al. (1994) reported the prevention of glucose-induced insulin resistance of insulin receptor in rat-1 fibroblasts.

Bader et al. (1993) found an increased [32P] incorporation in the 95-kD β-subunit of the insulin receptor and an increased phosphorylation of the synthetic substrate poly[GluNa4:1Tyr] in receptors isolated from the skeletal muscle of obese Zucker rats treated with CS 045.

Teboul et al. (1995) found that thiazolidinediones convert myogenic cells (C2C12N myoblasts, a subclone of the C2C12 cell line) into adipose-like cells. Thiazolidinediones or fatty acids prevented the expression of myogenin, α-actin, and creatine kinase and abolished the formation of multinucleated myotubes. In parallel, these treatments induced the expression of a typical adipose differentiation program including acquisition of adipocyte morphology and activation of adipose-related genes.

Tafuri (1996) reported that troglitazone enhanced the rate and percent differentiation of fibroblasts to adipocytes. Basal glucose transport and synthesis of GLUT1 transporter messenger RNA were increased.

Stevenson et al. (1990) examined the effects of racemic englitazone (CP 68722) in adipocytes and soleus muscles from ob/ob mice and in 3 T3-L1 adipocytes. Administration of the drug in various doses lowered plasma glucose and insulin dose dependently without producing frank hypoglycemia in either diabetic or nondiabetic animals. Basal and insulin-stimulated lipogenesis were enhanced in adipocytes from ob/ob mice. Glycogenesis and basal glucose oxidation in isolated soleus muscles were stimulated.

Kreutter et al. (1990) used 3 T3-L1 adipocytes and found a stimulation of 2-deoxyglucose uptake.

Masuda et al. (1995) found an insulinotropic mechanism distinct from glibenclamide in isolated rat pancreatic islets and HIT cells.

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Effects of Thiazolidinediones on Peroxisome Proliferator-Activated Receptor-γ

Purpose and Rationale

Peroxisome proliferator-activated receptors (PPARs) compose a subfamily of the nuclear hormone receptors. Three distinct PPARs, termed α, δ, and γ, each encoded by a separate gene and showing a distinct tissue distribution pattern, have been described (Keller and Wahli 1993; Green 1995; Devchand et al. 1996; Lemberger et al. 1996; Schoonjans et al. 1996a, b, 1997, Willson et al. 2000, Walczak and Tontonoz 2002). Ligands

that induce the transcriptional activity of PPARα and PPARγ have been identified (Forman et al. 1995; Devchand et al. 1996; Lehmann et al. 1995). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ (Kliewer et al. 1997). PPARγ is a central regulator of adipocyte gene expression and differentiation (Tortonoz et al. 1995; Brun et al. 1996; Wu et al. 1998, Lowell 1999). Thiazolidinedione derivatives which are antidiabetic agents are potent and selective activators of PPARγ (Young et al. 1998; Henke et al. 1998, Murakami et al. 1998; Reginato et al. 1998; Ribon et al. 1998, Vázquez et al. 2002). Berger et al. (1996) found a correlation of antidiabetic actions of thiazolidinediones in db/db mice with the conformational change in peroxisomal proliferator-activated receptor-γ; Murphy and Holder (2000) suggested a therapeutic potential of PPARγ agonists in the treatment of inflammatory diseases and certain cancers.

Steppan et al. (2001) and Berger (2001) showed that adipocytes secrete a signaling molecule which they called resistin (for resistance to insulin). Circulating resistin levels in mice are decreased by thiazolidinediones and are increased in diet-induced and genetic forms of obesity. On the other hand, adipocytes secrete another adipocytokine called adiponectin. Serum levels of adiponectin correlate with insulin sensitivity and are increased by the treatment with thiazolidinediones (Choi et al. 2005, Do et al. 2006).

Procedure

Plasmids

The pSG5-haPPARγ 1 expression construct is generated by inserting the hamster PPARγ complementary DNA into the EcoRI site of the pSG5 expression vector (Stratagene, La Jolla, CA). As a reporter construct, pPPRE-chloramphenicol acetyltransferase (CAT) is used containing two copies of the peroxisomal proliferator response element from the enhancer region of the murine acyl coenzyme A-oxidase gene adjacent to the glutathione-S-transferase minimal promotor and the CAT gene.

Cell Culture and Transfections

COS-1 cells are seeded at 2.1 × 105 cells per dish in 35-mm dishes (for transactivation assays) and 3 × 106 cells/dish in 150-mm dishes (for binding assays) in DMEM (high glucose) containing 10 % charcoal-stripped fetal calf serum, nonessential amino acids, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 10 % CO2. After 24 h, transfections are performed with Lipofectamine (Life Technologies, Gaithersburg, MD). For transactivation experiments, transfection mixes are used containing 1 µg receptor expression vector and 1 µg pCH110 (Pharmacia, Piscataway, NJ) as an internal control and, for binding studies, 20 µg receptor expression vector.

Binding Assay

Transfected cells are grown for 48 h after transfection with the receptor expression vector. Receptor preparation is performed according to Tilley et al. (1989). Cell lysates containing receptor are prepared in TEGM (10 mM Tris–HCl, pH 7.2; 1 mM EDTA; 10 % glycerol; 7 µl/100 ml β-mercaptoethanol; 10 mM Na molybdate; 1 mM dithiothreitol; 5 µg/ml aprotinin; 2 µg/ml leupeptin; 2 mg/ml benzamide; and 0.5 mM phenylmethylsulfonylfluoride). Plates are placed on ice, rinsed with TEG (10 mM Tris–HCl, pH 7.2; 50 mM EDTA; and 10 % glycerol), and scraped into 0.5 ml TEGM. The material is pooled, frozen in liquid nitrogen to lyse the cells, and thawed on ice. The lysate is centrifuged at 22,000 g for 20 min at 4 °C to remove the debris and stored frozen (−80 °C) until use. For each assay, an aliquot of receptor-containing lysate (0.1–0.25 mg protein) is incubated with 10 nM ditritiated AD-5075 (21 Ci/mmol) with or without test compound for ~16 h at 4 °C in TEGM (300-µl final volume). Unbound ligand is removed by incubation on ice for ~10 min after the addition of 200 µl dextran/gelatin-coated charcoal. After centrifugation at 3000 rpm for 10 min at 4 °C, 200-µl aliquots of supernate are counted in a liquid scintillation counter.

PPAR binding assays using gel filtration to separate the bound radioactivity ligand from the free ligand (Kliewer et al. 1997) have been replaced by the scintillation proximity assay (SPA),

which increases the throughput of PPAR competition assays (Nichols et al. 1998, Elbrecht et al. 1999). In this technique, the ligand binding domain is labeled with biotin and immobilized on streptavidin-modified scintillation proximity beads. When a potential radioligand of PPARγ binds to the PPARγ ligand binding domain, light, which leads to a detectable signal, is emitted. Competition with a non-labeled ligand avoids the binding of the radioligand to the PPARγ ligand binding domain and no signal is emitted. With this binding assay, there is no need to separate free radioligands from bound ligands, thus simplifying the process.

Su et al. (1999) described the use of a PPAR-γ-specific monoclonal antibody to demonstrate thiazolidinediones induced PPARγ receptor expression in vitro.

Transactivation Assay

After transfection, cells are incubated for 48 h in culture medium with or without increasing concentrations of test compounds. Cell lysates are produced using reporter lysis buffer (Promega Corp., Madison, WI). CAT activity is determined using radiolabeled butyryl CoA as substrate in a diffusion-based assay. β-Galactosidase activity is determined according to Hollons and Yoshimura (1989).

Lipogenesis Assay

PPARγ plays an important role in the regulation of cell differentiation (Lowell 1999). Adipogenesis requires combined high levels of PPARγ and other transcription factors such as CAAT/enhancer-binding protein (C/EBP) and adipocyte determination and differentiation-dependent factor 1/sterol regulatory-binding protein (ADD-1/SREBP-1). PPARγ plays an essential role in the molecular control of adipogenesis. Ectopic expression of PPARγ induces adipocyte differentiation (Tortonoz et al. 1994), whereas in the absence of PPARγ, adipocytes fail to develop (Lowell 1999). Adipocyte differentiation leads to expression of adipocyte-specific genes, such as aP2 (Tortonoz et al. 1994) or lipoprotein lipase (Schoonjans et al. 1996c), which are under the control of PPARγ. The lipogenesis assay, based on preadipocyte differentiation to adipocytes, where PPARγ plays an important role, has been used to select PPARγ agonists for in vivo studies. Henke et al. (1998) tested compounds for their ability to promote differentiation of C3H10T1/2 stem cells via measurement of glucose incorporation into total lipid of the cells.

Protease Digestion Assay

The protease digestion assay is performed according to the method of Allan et al. (1992) with minor modifications. The plasmid pSG5-haPPARγ1 is used to synthesize 35S-radiolabeled PPARγ1 in a coupled transcription/translation system. The transcription/translation reactions are subsequently aliquoted into 22.5-μl volumes, and 2.5 μl PBS with or without thiazolidinethione is added. The mixtures are incubated for 20 min at 25 °C, separated into 4.5-μl aliquots, and 0.5 μl distilled water or distilled water-solubilized trypsin is added. The protein digestions are further incubated for 10 min at 25°C, then terminated by the addition of 20 μl denaturing gel loading buffer, and boiled for 5 min. The products of the digestion are separated by electrophoresis through a 1.5-mm 12 % polyacrylamide-SDS gel. After electrophoresis, the gels are fixed in 10 % acetic acid and 40 % methanol for 30 min, treated in EN3HANCE for an additional 30 min, and dried under vacuum for 2 h at 80 °C. Autoradiography is then performed to visualize the radiolabeled digestion products.

Evaluation

Binding Assays

For binding assays, competition curves are generated by incubation of 10 nM [3H]AD-5075 with hamster PPARg1 produced by transient transfection of COS-1. The percentage of ligand bound after incubation in the presence of the indicated concentration of each unlabeled compound for 16 h is plotted.

Activation Assays

For activation assays of PPARg in COS-1 cells transiently cotransfected with pSG5-haPPARg1

and pPPRE-CAT, normalized CAT activity is plotted after incubation in the presence of the indicated concentration of each compound for 48 h.

Lipogenesis Assays

The lipogenic activity of PPAR agonists can be assessed by determining radioactive glucose uptake into the total lipids of cells. The amount of glucose incorporation into lipids in these cells provides a measure of cell differentiation. Moreover, the expression of adipocyte marker genes (aP2, lipoprotein lipase, leptin) and the accumulation of triglycerides in the cytoplasm confirmed by oil red O staining are other methods to evaluate the lipogenic activity of PPARγ agonists.

Protease Digestion Assay

For evaluation of the protease digestion assay, the partially protease-resistant conformation product of PPARγ is visualized by autoradiography on SDS-PAGE after incubation with the thiazolidinedione and increasing concentrations of trypsin.

Modifications of the Method

Berger et al. (1999) reported distinct biological effects of PPARγ and PPARδ ligands.

Desvergne and Wahli (1999) reviewed nuclear control of metabolism by peroxisome proliferator-activated receptors.

Lin et al. (1999) reported a fluorescence-based method for investigating the interactions of PPAR with ligands.

Brown et al. (2001) described identification of a subtype selective human PPARα agonist through parallel-array synthesis.

Xu et al. (2001) investigated the structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors.

Lee et al. (2003) reviewed metabolic disorders and peroxisome proliferator-activated receptors.

Ram (2003) underlined the therapeutic significance of peroxisome proliferator-activated receptor modulators in diabetes.

Norris et al. (2003) found that muscle-specific PPARγ-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidines.

Vikramadithyan et al. (2003) studied a peroxisome proliferator-activated receptor alpha (PPARα) and PPARγ agonist as a body weight lowering, hypolipidemic, and euglycemic agent. HEK 293 cells were used to conduct the reporter-based transactivation of PPARα and PPARγ.

Matsusue et al. (2003) found that liver-specific disruption of PPARγ in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes.

Stanley et al. (2003) studied subtype-specific effects of peroxisome proliferator-activated receptor ligands on corepressor activity.

Wurch et al. (2002) described the pharmacological analysis of wild-type α, γ, and δ subtypes of the human peroxisome proliferator-activated receptor.

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Antidiabetic Effects of Liver X Receptor Agonists

Purpose and Rationale

The nuclear receptors liver X receptor (LXR)α and LXRβ which are sensors of cholesterol metabolism and lipid biosynthesis are also regulators of inflammatory cytokines, suppressors of hepatic glucose production, and involved in different cell-signaling pathways. LXRα is a target gene of the peroxisome proliferator-activated receptor-γ, a target of drugs used in treating elevated levels of glucose seen in diabetes (Steffensen and Gustafsson 2004). Furthermore, insulin induces LXRα in hepatocytes, resulting in increased expression of lipogenic enzymes and suppression of key enzymes in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK). Mukherjee et al. (1997) observed sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Treatment of diabetic rodents with an LXRα agonist resulted in dramatic reduction of plasma glucose (Cao et al. 2003).

Procedure

Oral Glucose Tolerance Study in fa/fa Rats

Obese insulin-resistant female Zucker (fa/fa) rats, 10 weeks of age, are orally gavaged for 9 days with either vehicle of the LXRα agonist. Eight h after the last dose, animals are fasted overnight and on the following morning subjected to an oral glucose tolerance test. Blood was obtained from the animals via the tail vein at time 0 and times 15, 30, 60, and 120 min after an oral glucose challenge (2.5 g of glucose/kg body weight). Plasma glucose and insulin levels are analyzed on all samples, and the results are expressed as the product of glucose AUC and insulin AUC.

Glucose Output with ZDF Rat Liver Slices

Precision-cut liver slices are generated from control, treated for 7 days with the LXRα agonist after

an overnight fast. After preincubation and wash phases, the slices are incubated for 2 h at 29 °C in Krebs-Henseleit bicarbonate buffer containing 40 mM mannitol in either the presence or absence of 10 mM lactate. Incubation media glucose levels are assessed at 2-h time by subtracting the basal rate of glucose output per gram liver tissue from the substrate-stimulated rate of glucose output per gram of liver tissue.

Nuclear Run-On Experiment

A nuclear run-on experiment (Cao et al. 2002) is performed in the livers of diabetic db/db mice treated for 7 days with the LXRα agonist. Mouse liver nuclei are isolated (Schibler et al. 1983) and in vivo elongation reaction is performed (Goldman et al. 1985). The radiolabeled RNA is then subjected to slot blot (Mauvieux et al. 1998) in probes.

mRNA Measurement

Total RNAs are prepared from frozen tissue samples or cells with TRIzol reagent (Invitrogen) or Qiagen RNA prep kit. Mouse phosphoenolpyruvate carboxykinase and G6P mRNA are measured by RNase protection assay and quantified with a Molecular Dynamics Phosphorimager Model 51. Rat mRNA is subjected to reverse transcription reactions using the Omniscript reverse transcriptase kit (Qiagen). The resulting cDNA is amplified using TaqMan 2 × PCR master mix (Applied Biosystems). The PCR products are detected in real time using an ABI-7900HT sequence detection system (Applied Biosystems). The rat PEPCK branched cDNA is analyzed according to Burris et al. (1999).

Critical Assessment of the Method

Chisholm et al. (2003) reported that an LXR ligand induces severe lipogenesis in db/db diabetic mice.

Modifications of the Method

Stulnig et al. (2002a, 2002b) concluded that LXR ligands mediate beneficial metabolic effects in insulin resistance syndromes including type 2 diabetes by interfering with peripheral glucocorticoid activation.

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Measurement of Energy Metabolism

General Considerations

The epidemic of obesity has generated a large interest in understanding the physiological mechanisms regulating energy metabolism (Woods et al. 2000). This understanding has been facilitated by the knockout (KO), knockdown, or overexpression of specific genes, many of which result in altered body weight (BW) and/or body composition (BC) owing to a perturbation of energy balance. These advances, however, have led to some controversy over how best to assess these phenotypes and how to interpret the data (Arch et al. 2006, Butler and Kozak 2010, Kaiyala et al. 2010). In this perspective, we provide information on the best available and standardized measures for both energy intake and energy expenditure. Such an approach will allow progress in understanding complex biological questions about the consequences of genetic or pharmacological manipulations for energy balance and BW. This standardization is particularly important considering ambitious large-scale operations ongoing in the United States and Europe with the goal of assessing the consequences of all mouse and rat gene deletions. As a consequence, Tschöp and coworkers presented the following recommendations (Tschöp et al. 2011).

Purpose and Rationale

Genetically modified, pharmacologically treated, and nutritionally challenged laboratory mice, as well as spontaneous mutants, often exhibit alterations of BW and/or BC. It is often unclear whether food intake or energy expenditure (or both) is the major determinant of the phenotype because a small imbalance between intake and expenditure, if maintained over the course of weeks or months, can result in dramatic changes in body weight and fat mass. Furthermore, once obesity or leanness has developed, behavioral and metabolic alterations triggered by the BW change may obscure or confound the processes that caused the changes in BW and/or BC in the first place. Thus, assessing BW and BC phenotypes requires a careful analysis of the various factors that might affect these phenotypes.

Recommending a single approach to metabolic phenotyping for all different types of studies, models, and scientific questions is almost impossible. Nevertheless, several general considerations can help minimize artifacts and achieve comparability of data across studies and laboratories and therefore help understand the overall biology underlying the perturbation in BW and BC.

Studies defining the causal factors leading to altered BW and BC should ideally be initiated early in the life of the mouse, when BW and BC are still identical or minimally different. If changes in caloric intake or energy expenditure are observed before the change in BW or fat mass, it is more likely that one can attribute the difference to these changes. To dissect these mechanisms, it is sometimes helpful to conduct pair-feeding studies, in which mice from a "pair-fed" group are given an amount of food that

matches that eaten by the comparison group, which is given ad libitum access to the same food. If the pair-fed group consumes all the food and maintains a normal physiological status (e.g., does not enter torpor) and if the body-fat differences persist under conditions of equalized caloric intake, one has prima facie evidence that the alteration in BW is due to altered energy expenditure.

A second approach, which does not require normalization, is careful quantification and subtraction of the cumulative calories burned over a given period of time from the cumulative number of ingested and assimilated calories in the experimental and control groups. Also, mathematical modeling of cumulative intake and BW over several weeks can provide considerable insight into differences in energy balance (Guo and Hall 2009). If a mutant mouse differs in weight from wild-type littermates at birth or weaning, the above methodological approach is more difficult. In either case, measurements of energy intake and energy expenditure should be performed over several days and nights and, if possible, at more than one time point during growth. Additional data on BC, body temperature, physical activity, body length, organ, and tissue weights and ex vivo tissue-specific oxygen consumption data are all of value. In such cases, one should then analyze these parameters together with energy intake and energy expenditure data considering relevant covariates in an ANCOVA or generalized linear model as described below.

Genetic Background

As genetic backgrounds can lead to dramatic differences in response to experimental procedures or genetic manipulations, studying mice with a homogeneous genetic background simplifies the interpretation of the energy-balance data. In contrast, differences between mouse strains can be of great interest. The C57BL/6 (B6) mouse is the most commonly used strain in obesity and metabolism research because it is prone to diet-induced obesity with high-fat diets and develops severe insulin resistance. By contrast, A/J, FVB, C3HeB/FJ, and 129v strains are relatively resistant to diet-induced obesity and exhibit lower weight gain on a high-fat diet (Almind and Kahn 2004, Champy et al. 2008). As 129/Sv and FVB mice are often used to create knockout and transgenic lines, many investigators perform extensive backcrossing onto the B6 background to eliminate these background gene effects. In such cases, one needs to backcross for at least 8–10 generations to establish a sufficiently homogeneous background. For some studies, it is important to determine the effect of these background genes, and in these cases, comparative studies on different genetic backgrounds, mixed genetic background, or outbred backgrounds can provide additional information.

Environmental Conditions

Institutional and governmental guidelines define housing conditions for laboratory mice, and these conditions can affect energy balance. The general recommendations of the EUMORPHIA Consortium for animal housing are useful as a starting point (Champy et al. 2008). Mice are most frequently housed with a constant light and dark phase of 12 h at 20–23 °C. Because the lower critical temperature of the mouse is around 30 °C, this means in most circumstances mice are housed under mild thermoregulatory stress, which may not be the ideal ambient temperature for energy-metabolism studies aiming to mimic the situation in humans, who normally exist much closer to thermoneutral conditions. The temperature in a cage may be substantially higher than the ambient temperature outside of a cage, and this temperature gradient increases from singly housed to multiply housed mice.

It is important to realize that mouse facilities, including commercial breeders, may use different "normal" chow diets. One example source for diet inconsistencies would be the soy-isoflavone content, which is often only partially removed when proteins are extracted. Seasonal differences in diet soy-isoflavone content can affect study outcomes based on their effects on estrogen receptor activation and AMPK. Such influences can make a

considerable difference in baseline measurements and affect the interpretation of the effect of changing to high-fat or other experimental diets. It is therefore critical for the investigator to know what constitutes normal chow, especially if mice are housed in more than one facility or are being compared to those in the previous literature. Unfortunately, there also is no clear "gold standard" for high-fat diets. Protein and carbohydrate content should ideally be kept constant within and between studies. High-energy diets should be modeled to principally match diets relevant for human metabolic disease, but it can be advantageous to also match diets used in published studies to allow for relevant comparisons of mouse phenotypes.

Most experimental procedures can induce stress responses in animals, and proper acclimation and recovery intervals should be given before food intake or energy expenditure are recorded. Mice with impaired health often decrease their food consumption, and mice with fever have elevated energy expenditure. Although mice in energy-metabolism studies are normally housed singly, measurements can also be derived from group-housed mice if housing space is a limiting factor, but this is far from ideal. There is little evidence that single housing induces stress because fecal corticosterone levels of singly housed mice do not differ from those of group-housed individuals (Arndt et al. 2009, Champy et al. 2004, Hunt and Hambly 2006). However, singly housed mice may have altered activity patterns relative to group-housed individuals (Martin and Brown 2010). In contrast, if mice are group housed, factors such as spilling of food, aggression behavior, or social dominance can introduce considerable bias and variability in a range of traits, including those linked to energy balance (Bartolomucci 2007, Bartolomucci et al. 2004 and 2009, Cohn and Sa-Rocha 2009, Moles et al. 2006, Schmidt et al. 2007). Moreover, group-housed individuals can huddle, reducing their thermoregulatory requirements, and this may have a major effect on energy expenditure and intake. Cage position (top of rack versus lower) and group size have been shown to affect development of diabetes in mice (Ader et al. 1991). Ideally, when group housed, mice should be littermates and together from early age (Bartolomucci et al. 2004), and social groups should not be disrupted. Removing individual mice from a group to measure energy intake and/or energy expenditure can cause problems when that mouse is reintroduced to the group, especially in groups of males. Moving boxes of mice around different rooms or rack positions seems not to be related to significant effects on stress or BW (Dahlin et al. 2009) ($P > 0.05$).

Body Composition

Gold-standard methods for determining the BC of animals were established over 50 years ago and involve killing the mouse (Reynolds and Kunz 2001). For longitudinal measures, there are various options such as isotope dilution, dual-energy X-ray absorptiometry, total body electrical conductivity, magnetic resonance spectroscopy, magnetic resonance imaging, and computed tomography. Generally, it is good practice to use both destructive and nondestructive methods as they provide complementary information, including individual organ and fat depot weights as well as measures of fat cell size and number. When high accuracy is required, destructive methods of BC analysis are the methods of choice. Magnetic resonance spectroscopy and dual-energy X-ray absorptiometry systems provide the next best alternatives, in that order. Total body electrical conductivity and isotope-dilution methods are relatively poor. All indirect methods should be calibrated against destructive methods to yield the best results.

Energy Intake

Exact measurement of food intake is a key component in analysis of energy metabolism. Techniques are based on weighing of food and vary greatly in accuracy, reproducibility, practicality, and cost. Not all ingested calories are introduced into metabolism, making assimilation efficiency studies (e.g., using bomb calorimetry) a

recommended addition. Investigators need to be aware of other factors that can complicate estimates of energy intake including the exact form in which diets are delivered to mice.

Energy Expenditure

With the development of several different commercial systems, measures of energy expenditure in mice are now much more common than 10–15 years ago, but results are sometimes misinterpreted (Arch et al. 2006, Butler and Kozak 2010, Kaiyala et al. 2010). Using direct calorimetry, energy expenditure is assessed by the direct measurement of the body's heat production in a calorimeter (Faber et al. 1998, Levine 2005, Spinnler et al. 1973). Despite high reproducibility and measurement errors of only 1–3 %, these calorimeters are expensive, have slow response time (Levine 2005), and do not provide information about the nature of the oxidized substrates. In indirect calorimetry, energy expenditure is calculated based on the amount of oxygen consumed and carbon dioxide produced. The most common indirect calorimeter types are ventilated, open-circuit systems, in which the animals are placed in gas-tight metabolic cages through which a flow of fresh air is passed. The system collects and mixes the expired air, measures the flow rate, and analyzes the gas concentration of the incoming and outgoing air for both O_2 and CO_2 (Levine 2005). Another indirect method of calorimetry is the doubly labeled water method, an isotope-elimination technique developed in the 1950s (Lifson et al. 1955, Lifson and McClintock 1966, Speakman 1997). This method has been traditionally used to measure the metabolic rate of small free-living animals, which are released in the field between two time points: it is often referred to as field metabolic rate (Speakman and Krol 2010). In the laboratory, the main advantage of the method is that it allows the measurement of energy demands of an animal embedded in a social environment (Krol et al. 2007). However, the time intervals between blood sampling are often too long to permit measurements of short-term or diurnal changes of the metabolic rate.

Evaluation

In most circumstances, the analysis of energy balance should be made on the raw data expressed per mouse. In many circumstances, however, researchers are interested in whether a particular difference between mice occurs simply because the mice differ in BW or because treatment or genetic differences affect energy intake or energy expenditure. Often corrections for BW differences are made only on energy expenditure measurements, and this can lead to confusion, especially when combined with analyses of energy intake that have been made without normalization. There is no reason to limit normalization to energy expenditure because energy intake also varies with BW. However, it is important that methods of normalization not be mixed on the two sides of the energy-balance equation (Arch et al. 2006).

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Measurement of Insulin and Other Glucose-Regulating Peptide Hormones

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Radioimmunoassays for Insulin, Glucagon, and Somatostatin

Purpose and Rationale

Insulin activity is an important laboratory parameter in the clinical evaluation of several diseases such as diabetes mellitus types I and II, states of impaired glucose tolerance, and insulin-producing tumors (insulinomas), where the insulin secretion released from pancreas β-cells is altered.

The first description of an immunoassay of endogenous plasma insulin in man has been given by Yalow and Berson (1959, 1960). Yalow and coworkers (1960) provided evidence that the bioassays hitherto being used (isolated rat diaphragm, epididymal fat pad tissue) measure insulin-like activity but not true insulin levels in blood. Since that time, the method has been used and modified by many investigators, e.g., Grodsky and Forsham (1960), Morgan and Lazarow (1963), Hales and Randle (1963), Melani and coworkers (1965, 1967), and Wright and coworkers (1968). Survey on the radioimmunoassay of insulin has been given by Ditschuneit and Faulhaber (1975) and Freedlender and coworkers (1984). The introduction of radioimmunoassay (RIA) by Berson and Yalow (1959) provided a novel method for measuring insulin in plasma and serum. In order to improve reliability and practicability, various technologies have appeared in the field of immunoassays. Variety is seen in the choice of labels (radioactive, enzymatic, fluorescent, etc.), separation methods (precipitation of antigen-antibody complexes, coated solid-phase antibody technology, etc.), detection methods (spectrophotometry, fluorometry, amperometry, potentiometry, and calorimetry), and principles such as the competitive or noncompetitive immunoassay. These immunoassay techniques have become important analytical methods in clinical chemistry laboratories for the selective detection of drugs or proteins as well as hormones such as insulin at trace levels.

Procedures

Immunization

Semisynthetic or biosynthetic human insulin is used as immunogen and as standard. Formerly, porcine insulin has been used since Yalow and Berson (1960) and subsequently many other authors have shown that antisera raised against porcine insulin react identically with human and porcine insulin. Guinea pigs weighing 350–450 g are injected subcutaneously with 0.4 ml of an emulsion of 5 mg human insulin dissolved in 1.0 ml 0.01 N HCl and 3.0 ml complete Freund's adjuvant (Difco Laboratories). For boostering, 0.2 ml of an identically prepared emulsion is injected in monthly intervals. Fourteen days after the third booster injection, the animals are slightly anesthetized, and 8–10 ml blood is withdrawn by cardiac puncture. Boosting is continued at monthly intervals, and the animals are bled 2 weeks following each booster injection.

The optimal antiserum titer for use in the radioimmunoassay is determined using conditions identical to those employed in routine immunoassays. The percentage binding of 1 μU 125I-insulin is determined for dilutions of antisera ranging from 103- to 106-fold. The steepness of the antisera dilution curve is a measure of the affinity of the antiserum and therefore the potential sensitivity of the radioimmunoassay. Antisera with the steepest slopes, but not necessarily the highest titer, are selected for further study. The selected antisera dilutions are then run in an immunoassay using a full range of standards. A reduction in the percent 125I-insulin bound to antibody from 50 % (in the absence of unlabeled insulin) to 45 % (in the presence of unlabeled insulin) (B/Bo = 0.9) is a reasonable measure of assay sensitivity.

Assay

The antibody-bound 125I-insulin prepared as described above can be separated from free 125I-insulin in various ways, such as by paper electrophoresis, as originally described by Yalow

and Berson (1960), or by a two-antibody system, as described by Morgan and Lazarow (1963) and Starr and coworkers (1979). In this method, the soluble insulin-anti-insulin complex is precipitated by an anti-guinea pig serum antibody. The following procedure is recommended (Freedlender et al. 1984):

- A buffer is prepared from a solution of 8.25 g boric acid and 2.70 g NaOH dissolved in 11 water. After dissolving 5.0 g of purified bovine serum albumin (BSA), pH is adjusted with concentrated HCl to 8.0.
- In disposable plastic tubes, 10 × 75 mm, the following volumes are added:
  - 100 µl serum or standard
  - 900 µl buffer
  - 100 µl 1 mU 125I-insulin in assay buffer
  - 100 µl guinea pig anti-insulin antiserum diluted in assay buffer (at a concentration to bind 50 % of the 125I-insulin in the absence of unlabeled hormone)
- The mixture is incubated at 4 °C for 72 h. Then, the following solutions are added:
  - 100 µl normal guinea pig serum diluted 1:400 in the assay buffer
  - 100 µl rabbit anti-guinea pig globulin serum diluted in assay buffer
- The mixture is again incubated at 4 °C for 72 h and then centrifuged (2,000 × g, 20 min, 4 °C). The supernatant is decanted and radioactivity counted in the precipitate for 5 min.

Evaluation

Counts in the nonspecific binding tubes are subtracted from counts in all other tubes. Data are linearized using an unweighted logit-log transformation (Rodbard and Frazier 1975). Microunits of insulin in a logarithmic scale are plotted against the ratio B/Bo 125I-insulin on a logit scale. The range of B/Bo between 0.4 and 0.9 is the most suitable for determination of insulin concentration in plasma.

Critical Assessment of the Method

The immunoassay of insulin as described by Yalow and Berson (1960) has been a breakthrough for many immunological assays of peptide hormones and other drugs. At present, not only guinea pig anti-insulin antisera but also complete RIA kits are available from a number of commercial firms.

Modifications of the Method

Cam and McNeill (1996) published a sensitive radioimmunoassay optimized for reproducible measurement of rat plasma insulin. Relatively small volumes (25 µl) of plasma from control, diabetic, and fasted rats can be assayed reproducibly with charcoal in the final separation step.

The hormones, insulin, glucagon, and somatostatin are determined radioimmunologically: insulin with the RIA-gnost kit and rat insulin as standard; glucagon with a rabbit antibody, 125I-glucagon as tracer, and polyethylene glycol as precipitant; and somatostatin with a rabbit antibody, 125I-tyrosyl-somatostatin as tracer, and charcoal dextran for separation of free and bound hormone. At least three experiments per concentration are performed. The values of each collection period of 1 min are averaged and plotted vs. time. The effects of the test compound (increase or decrease of the secreted hormones) are compared with the control periods and the effect of elevated glucose.

Bioassay for Glucagon

Purpose and Rationale

Glucagon is a 29-amino-acid, single-chain polypeptide which is secreted by the α-cells in the islets of Langerhans. It is synthesized from preproglucagon, a 180-amino-acid precursor (Bell et al. 1983). Proglucagon is processed in islet and intestinal cell lines (Tucker et al. 1996).

Glucagon interacts with a 60-kDa glycoprotein receptor on the plasma membrane of target cells (Sheetz and Tager 1988).

A biological assay of glucagon is described in the British Pharmacopoeia 1988. The potency of glucagon is estimated by comparing its hyperglycemic activity with that of the standard preparation of glucagon using the rabbit blood sugar assay as performed for insulin determinations.

Procedure

Rabbits of either sex, weighing 1.8–2.8 kg, are maintained under uniform conditions and an adequate uniform diet for at least 1 week. Forty-eight hours before the beginning of the test, each rabbit is injected with 1 ml of cortisone acetate injection. The animals are deprived of food, but not water, from 16 h before each test day until the withdrawal of the last blood sample on that day. The rabbits are randomly distributed into four groups of at least six animals.

The standard preparation to be used is the first International Standard for Glucagon, porcine, established in 1973, consisting of freeze-dried porcine glucagon with lactose and sodium chloride (supplied in ampoules containing 1.49 units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

The entire contents of one ampoule of the standard preparation are reconstituted with 2 ml of saline solution, acidified to pH 3.0 with hydrochloric acid and diluted with the same solvent to a convenient concentration, for example, 100 milliunits per ml. Two dilutions are made containing 24 and 6 milliunits per ml, respectively, and at the same time, two dilutions are made of the preparation being examined. The rabbits are injected subcutaneously with doses of 1 ml of each of the four solutions, giving the doses in random order following a twin crossover design on 2 consecutive days at the same time each day. At 20 and 60 min after injection, a blood sample is taken from a marginal ear vein of each rabbit. Blood glucose concentrations are determined using a suitable method, such as the glucose-oxidase procedure.

Evaluation

The result of the assay is calculated by standard statistical methods using the means of the two blood glucose levels found for each rabbit.

Modifications of the Method

Glucagon can be determined by radioimmunoassay (Unger et al. 1959; Harris et al. 1978; von Schenk 1984). Commercial kits are available.

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Receptor Binding and In Vitro Activity of Glucagon

Purpose and Rationale

The binding of glucagon to its receptor is assayed with rat liver plasma membranes (Neville 1968; Pohl et al. 1971; Goldstein and Blecher 1976). Displacement of 125I-labeled glucagon is measured for synthetic glucagon analogs in comparison to natural glucagon. Cyclic AMP formation as the first step in glucagon action on liver is measured as a bioassay in liver plasma membranes.

Procedure

Preparation of Membranes

Male Sprague–Dawley rats weighing 160–200 g are decapitated and the livers rapidly removed and trimmed of fat and connective tissue. The pooled livers are placed on a prechilled glass plate and chopped finely with a stainless steel blade. Ten-gram portions of the minced liver pieces are suspended in 35 ml of ice-cold 1 mM NaHCO3 and homogenized in a loose fitting Dounce homogenizer. Two homogenates are combined, brought to 500 ml with ice-cold medium, stirred magnetically for 5 min, and finally filtered once through two layers of cheesecloth. The filtrate is centrifuged (1,500 × g, 10 min). Supernatant fluid is aspirated to waste using a serum pipette attached to a water pump. Homogenizations and centrifugations are continued until all of the tissue has been processed. The pooled pellets are again homogenized. The suspension is adjusted to 44 ± 0.1 % (wt/wt) sucrose solution by the addition of 70 % sucrose solution. Sucrose gradients are prepared in 1 × 3.5-in. tubes by pipetting 26 ml of the tissue suspension followed by an

overlay of 13 ml of 42.3 ± 0.1 % sucrose. Centrifugation is carried out (95,100 × g, 2 h). The float containing the plasma membranes can be removed by pinching the tube slightly above the float and then lifting off the float with a spoon-shaped Teflon-coated spatula. These floats are transferred to a pre-weighed plastic centrifuge tube (50 ml) and the well-mixed suspension centrifuged at 40,000 × g, 30 min. Following aspiration of the supernatant to waste, the tubes are reweighed in order to estimate the yield of plasma membranes. After addition of an equal volume of medium, the pellet is aspirated repeatedly through a 20-gauge needle fitted to a syringe. The plasma membrane suspension is distributed in 0.2 or 1.0 ml aliquots to screw cap plastic vessels for storage in liquid nitrogen.

Radioiodination of Glucagon

Three nmol glucagon is allowed to react with a 1.0 nmol sample of carrier-free Na125I (2.0 mC) in the presence of 1.5 nmol chloramine-T, added at a regular interval of 30 s (0.5 nmol each time). Reaction is terminated by addition of 0.5 % sodium metabisulfite solution (Hagopian and Tager 1983). By chromatographic purification (Jørgensen and Larsen 1972), fractions of 2 ml are collected, and the monoiodinated glucagon as determined by reverse phase HPLC is stored at −20 °C for receptor binding assays.

Receptor Binding

Membrane suspensions adjusted to 50 μg protein in 400 μl of Tris–HCl buffer (25 mM, pH 7.5, with 1 % BSA) are incubated for 10 min with 50 μl peptide solution (Tris–HCl buffer) and 50 μl of [125I]glucagon (106 cpm, 25 fmol). The samples are then filtered through Oxoid filters and washed three times with 1 ml of Tris–HCl buffer. The radioactivity retained in the filters is counted by a γ-counter.

Adenylate Cyclase Assay

The assay is carried out with a membrane suspension containing 25–30 μg protein in a volume of 0.1 ml of 25 mM Tris/HCl (pH 7.5) containing 1 % BSA, 1 mM ATP with 4 × 106 cpm [α32P] ATP, 5 mM MgCl2, 1 nM cAMP containing 10,000 cpm of [3H]cAMP, 10 mM GTP, 20 mM phosphocreatine, and 0.72 mg/ml creatinine phosphokinase(100U/ml). Assays are run in triplicate.

Evaluation

Results are expressed as the percentage inhibition of [125I]glucagon-specific binding for receptor binding assays. For adenylate cyclase assays, the results are expressed as percent potency relative to the maximal stimulation by glucagon which is defined as 100 %.

Modifications of the Method

Azizeh and coworkers (1995, 1997) synthesized and tested a glucagon antagonist and multiple replacement analogs of glucagon by adenylate cyclase assay according to Lin and coworkers (1975) and receptor binding assay according to Wright and Rodbell (1979).

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Glucagon-Like Peptide I

Purpose and Rationale

Several intestinal peptides have been described to have insulinotropic or incretin activity, e.g., GIP (gastric inhibitory peptide, glucose-dependent insulin-releasing peptide) (Creutzfeldt and Ebert 1985; Baer and Dupré 1989; Volz et al. 1995). More recently, glucagon-like peptide-1 (7–37) or the (7–36)amide is described as a new incretin (Kreymann et al. 1987; Fehmann et al. 1989, 1990, 1991a, 1992, 1995; Holz et al. 1993). The insulinotropic activity has been confirmed in diabetic and nondiabetic subjects (Gutniak et al. 1992; Nathan et al. 1992; Nauck et al. 1993; Ørskov et al. 1993). The insulin stimulatory effect of glucagon-like peptide-1(7–36) amide is glucose dependent (Göke et al. 1993a). The peptide does not only stimulate insulin release but also inhibit glucagon secretion (Komatsu et al. 1989). Cloning and functional expression of the rat (Thorens 1992) and human (Dillon et al. 1993) glucagon-like peptide1 (GLP-1) receptor has been achieved. The sequence of glucagon-like peptide-1 (7–36) amide is completely conserved in all mammalian species studied, implying that it plays a critical physiological role. Intracerebroventricular administration of GLP-1 powerfully inhibits feeding in fasted rats, which is blocked by the GLP-1-receptor antagonist exendin(9–39) (Turton et al. 1996; Meurer et al. 1999).

Peptides isolated from reptile venoms, such as exendin-4, were found to have similar activity as glucagon-like peptide-1(7–36)amide (Göke et al. 1993b; Adelhorst et al. 1994; Schepp et al. 1994). Gedulin et al. (2005) found that exendin-4 improves insulin sensitivity and β-cell mass in insulin-resistant obese fa/fa Zucker rats independent of glycemia and body weight. Shechter et al. (2003) described [2-sulfo-9-fluorenylmethoxycarbony]3-exendin-4 as a long-acting glucose-lowering prodrug. Analogs and antagonists of glucagon-like peptide-1(7–36)-amide have been synthesized and evaluated in pharmacological experiments (Watanabe et al. 1994; Hjorth and Schwartz 1996; Montrose-Rafizadeh et al. 1997).

Besides insulin release from perfused pancreas (see chapter "► Assays for the Expression and Release of Insulin and Glucose-Regulating Peptide Hormones from Pancreatic β-Cell"), the receptor binding according to Göke and Conlon (1988) on rat insulinoma-derived cells (RINm5F cells), the cAMP formation, and the insulin release was studied (Göke et al. 1989a, b).

Procedure

Binding Studies with RINm5F Cells

The RINm5F cell line is derived from a radiation-induced insulin-producing rat tumor (Gazdar et al. 1980). The RINm5F cells are grown in plastic culture bottles (Praz et al. 1983). They are detached from the surface of the bottles before the experiment using phosphate-buffered saline (PBS, 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.3) containing 0.7 mM EDTA and centrifuged (100 × g, 5 min). The pelleted cells are resuspended in an incubation buffer (2.5 mM Tris–HCl, 120 mM NaCl, 1.2 mM MgSO4, 1.5 mM KCl, and 15 mM

CH3COONa, pH 7.4) containing 1 % human serum albumin, 0.1 % bacitracin, and 1 mM EDTA. Approximately 3 × 106 cells/tube are incubated for 5 min at 37 °C, followed by the addition of unlabeled peptide (final concentration range from 10 pmol to 1 mmol) and radiolabeled tracer (approximately 40,000 cpm). Iodination of the glucagon-like peptide-1(7–36)amide is carried out using the lactoperoxidase method. The total volume of incubation is 0.3 ml. After incubation for 60 min, aliquots (200 μl) of the cell suspensions are centrifuged (11,500 × g, 2 min) through an oil layer (dibutyl phthalate/dinonyl phthalate, 10/4, v/v). Cell surface-associated radioactivity in the pellet is counted using a γ-counter (e.g., Gamma 5500, Beckman).

Determination of cAMP Production in RINm5F Cells

Approximately 1 × 106 cells in 0.45 ml buffer (113 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 10 mM HEPES, 2.5 mM CaCl2, and 1.2 mM MgSO4, pH 7.4) containing 1 % human serum albumin are preincubated for 10 min at 37 °C and then incubated for 10 min after the addition of 2 μl 3-isobutyl-1-methylxanthine (IBMX, 50 mM) in order to prevent the breakdown of cAMP. The reaction is then started by the addition of 50 μl of a peptide solution dissolved in the above buffer (final concentration range from 10 μM to 1 μM). After incubation for 10 min at 37 °C, the reaction is stopped by the addition of 200 μl 12 % trichloroacetic acid (TCA). The reaction mixture is sonicated for 30 s at 25 W (e.g., heat system, ultrasonics) and centrifuged (11,500 × g, 2 min). 25 μl of 1 M HCl is added to 0.5 ml supernatant. TCA dissolved in the supernatant is removed by diethyl ether (3 × 1 ml), and the resulting supernatant is stored at −40 °C until cAMP assays being performed by the use of a RIA kit.

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Insulin-Like Growth Factors

Purpose and Rationale

Three different lines of research have led to the discovery of insulin-like growth factors (Froesch et al. 1985): Growth hormone does not stimulate growth processes by itself but induce factors, named sulfation factors or somatomedins, that mediate the message of growth hormone (Salmon and Daughaday 1957; Nevo 1982; Laron 1999). Pierson and Temin (1972) extracted factors from serum with multiplication-stimulating activity (MSA) for chicken fibroblasts in the cell culture and with non-suppressible insulin-like activity. Serum exerts insulin-like effects on insulin target

tissues such as muscle and adipose tissues to a much greater extent than it can be expected on the basis of the insulin content in serum. The activity of factors other than insulin in the epididymal fat pad assay was proven by the persistence of serum insulin-like activity after pancreatectomy (Steinke et al. 1962). Since this activity could not be suppressed by insulin antibodies, the factors were called "non-suppressible insulin-like activity" or NSILA.

They were finally identified as insulin-like growth factors I and II (Rinderknecht and Humbel 1978a, b) structurally related to insulin. These polypeptide hormones are present in serum in high concentrations but are bound to specific carrier proteins (Zapf et al. 1975). Most of IGF and also the IGF-binding protein are synthesized and secreted, but apparently not stored by the liver (Schwander et al. 1983; Sara and Hall 1990).

Insulin-like growth factor-1 (IGF-1) is a polypeptide of 70 amino acids with more or less clearly defined functions (Cascieri et al. 1988; Moxley et al. 1990; Salamon et al. 1989; Schmitz et al. 1991; Vikman et al. 1991; Laron 1999). The metabolic activity of IGF-1 is regulated by at least 6 IGF-binding proteins, the most important being IGFBP-3. The effects of growth hormone on protein metabolism are mediated by IGF-1, whereas these 2 hormones are antagonistic in their effects on insulin and some aspects of lipid metabolism. IGF-1 has been shown to improve glycemic control and to reduce insulin requirements in both IDDM and NIDDM (Simpson et al. 1998). One important clinical use is replacement therapy in primary IGF-1 deficiency, such as Laron syndrome, which is characterized by dwarfism due to primary GH resistance or insensitivity (Laron 1999). IGF-1 improves bone healing in vivo (Kobayashi et al. 1996). A truncated variant of human IGF-1 with the tripeptide Gly-Pro-Glu absent from the N-terminus has been isolated from bovine colostrum, human brain, and porcine uterus which is about tenfold more potent than IGF-1 at stimulating hypertrophy and proliferation of cultured cells (Ballard et al. 1996).

The function of insulin-like growth factor II is less defined (Roth 1988). IGF-II has an insulin-like effect in vitro and in vivo (Shizume et al. 1996; Burvin et al. 1998). IGF-II, administered acutely, affects glucose homeostasis in a manner very similar to insulin, probably via the insulin receptors, although with significantly lower potency.

Two subtypes of IGF receptors exist which are different from the insulin receptor and different in their affinity for IGF I and IGF II (Rechler 1985; Verspohl et al. 1988). They belong to the insulin receptor family of receptor tyrosine kinases (Ullrich and Schlessinger 1990; Schlessinger and Ullrich 1992; Fantl et al. 1993). Although insulin and IGF bind with high affinity to their own specific receptors, each can also bind to the heterologous receptor with reduced binding affinity (DeMeyts 1994).

With recombinant DNA technology, insulin analogs with modified amino acid sequence can be produced which may bind differently to the insulin and IGF receptor resulting in different biological activities (Drejer 1992; Schäffer et al. 1993). It has been speculated whether the unexpected carcinogenic effect of an insulin analog (Dideriksen et al. 1992) is related to the mitogenic effect in mouse NIH 3 T3 fibroblasts which express IGF receptors but not insulin receptors (Gammeltoft and Drejer 1991). Biosynthesis of 10 kDa and 7.5 kDa insulin-like growth factor II in a human rhabdomyosarcoma cell line has been described by Nielsen et al. (1993).

For new insulin analogs, the kinetics of association and dissociation to the receptors should be studied, and in vivo metabolic activity should be compared with insulin and IGF I.

The in vivo metabolic action of insulin-like growth factor I can be compared with insulin in adult rats using the following methods (Schmitz et al. 1991): dose dependence and time dependence of blood sugar decrease after intravenous injection, antilipolytic effect (decrease of nonesterified fatty acids) after i.v. injection, and stimulation of glucose disposal during euglycemic clamping after intravenous infusion. Furthermore, all other assays described for insulin can be applied. Gazzano-Santoro and coworkers (1998) described a cell-based potency assay for insulin-like growth factor-1.

Procedure

Cells from the human cell line HU-3, established from the bone marrow of a patient with acute megakaryoblastic leukemia, are adapted to grow in the presence of human thrombopoietin. The removal of thrombopoietin results in decreased proliferation and rapid loss of viability. Cells are cultured in RPMI-1640 medium with 2 % heat-inactivated human serum, 2 mM glutamine, 10 mM HEPES (pH 7.2), and 5 ng/ml thrombopoietin in culture flasks. They are grown in suspension at 37 °C in a humidified 5 % CO2 incubator and are routinely subcultured every 2 or 3 days when densities reach 0.8–1.5 × 106 cells/ml.

The cell growth assay is performed under serum-free conditions in assay medium consisting of RPMI-1640 supplemented with 0.1 % BSA, 10 mM HEPES (pH 7.2), and 50 μg/ml gentamycin. Cells are washed twice in the assay medium and resuspended at a density of 0.25 × 106 cells/ml. In a typical assay, 100 μl of a cell suspension (25,000 cells/well) and 100 μl of IGF-1 at varying concentrations are added to flat-bottomed 96-well tissue culture plates at 37 °C and 5 % CO2 and cultured for 2 days. 40 μl of alamarBlue™ (undiluted) is then added and the incubation continued for 7–24 h. The plates are allowed to cool to room temperature for 10 min on a shaker, and the fluorescence is read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm.

Evaluation

Results, expressed as relative fluorescence units, are plotted against IGF-1 concentrations using a 4-parameter curve-fitting program. Test compounds are compared with the standard.

Modifications of the Method

Boge and coworkers (1994) described an enzyme immunoreceptor assay for the quantitation of insulin-like growth factor-1 and insulin receptors in bovine muscle tissue.

Hodgson and coworkers (1995) tested mutations at positions 11 and 60 of insulin-like growth factor-1 using (a) quantification of affinities for the human insulin receptor overexpressed on NIH 3 T3 cells, (b) quantification of affinities for the type 1 IGF receptor via competition for binding to a monolayer of MDA-MB-231 cells, (c) promotion of the in vitro mitogenesis of growth-arrested MCF-7 cells in the presence of 17-β-oestradiol, and (d) a competition assay for binding to IGF-binding proteins secreted by MCF-7 cells.

In order to investigate the influence of insulin-like growth factor-1 and IGF-binding protein-1 on wound healing, Lee and coworkers (1996) measured the contraction of collagen gels with embedded fibroblasts.

Ernst and White (1996) studied the hormonal regulation of IGF-binding protein-2 expression in C2C12 myoblasts.

Frystyk and Baxter (1998) described a competitive assay for rat insulin-like growth factor (IGF)-binding protein-3 (rIGFBP-3) based on the ability of IGFBP-3 to form a ternary complex with the acid labile subunit (ALS) in the presence of IGF. Human ALS was bound to test tubes pre-coated with antihuman ALS antibody. The assay depends on a competition between a covalent complex of 125I-hIGF-I and hIGFBP-3, added as tracer, and hIGFBP-3 or rIGFBP-3 in standards or test samples, for binding to the immobilized hALS. Purified natural hIGFBP-3 served as standard.

Damon and coworkers (1997) used the C2 skeletal myogenic cell line to characterize an insulin-like growth factor-binding protein, named Mac25/IGFBP-7.

To explore the possible relationship between insulin-like growth factor I and diabetic retinopathy, Naruse and coworkers (1996) examined the effects of glucose on IGF-I-stimulated thymidine incorporation into DNA and IGF-I binding in cultured bovine retinal pericytes.

Jonsson and coworkers (1997) used a fluorometric proliferation assay with alamarBlue to study the proliferative capacity of isolated human osteoblasts which were dose-dependently stimulated by IGF-I.

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Amylin

General Considerations

Amylin, also named islet amyloid polypeptide, is a pancreatic islet peptide consisting of 37 amino acids with a role in the maintenance of glucose

homeostasis. The peptide is predominantly present in the β-cells of the pancreas and to a lesser extent in the gastrointestinal tract and in the nervous system, where amylin mRNA is also present along with specific binding sites.

Amylin has structural and functional relationships to two other messenger proteins, calcitonin and calcitonin gene-related peptide (Rink et al. 1993; Pittner et al. 1994; van Rossum et al. 1997; Wimalawansa 1996).

Amylin inhibits food intake in rodents when given centrally as well as peripherally (Morley et al. 1997; Lutz et al. 1998). Bhavsar and coworkers (1998) found a synergy between amylin and cholecystokinin for inhibition of food intake in mice.

Amylin reduces insulin-stimulated incorporation of glucose into glycogen in skeletal muscle. Young and coworkers (1992) found that increasing concentrations of amylin progressively depressed the maximal insulin-stimulated radioglucose incorporation into soleus muscle glycogen.

Castle and coworkers (1998) reported an inhibition of 3-O-methyl-D-glucose transport in perfused rat hind limb muscle under hyperinsulinemic conditions.

Bryer-Ash and coworkers (1995) found that amylin-mediated reduction in insulin sensitivity corresponds to reduced insulin receptor kinase activity in the rat in vivo.

Transgenic mice expressing human islet amyloid polypeptide develop diabetes mellitus by 8 weeks of age, which is associated with selective β-cell death and impaired insulin secretion (Janson et al. 1996).

Mulder and coworkers (2000) showed that amylin-deficient mice develop a more severe form of alloxan-induced diabetes.

In single β-cells exhibiting normal glucose sensing, amylin causes membrane hyperpolarization and increases net outward current and reductions in insulin secretion. In contrast, in cells with abnormal glucose sensing (e.g., from db/db diabetic mice), amylin has no effect on electrical activity or secretion (Wagoner et al. 1993).

Amylin suppressed the cAMP generation induced by glucagon-like peptide 1 (GLP-1) in RINm5F cells dose dependently (Göke et al. 1993).

Amylin, also named human islet amyloid polypeptide (hIAPP) according to van Hulst and coworkers (1997), may be implicated in the pathogenesis of pancreatic islet amyloid formation and type 2 diabetes mellitus (Wimalawansa 1997). Van Hulst and coworkers (1997) used transgenic mice for the study of (patho)physiological roles of hIAPP in vivo. Leckstrom and coworkers (1997) studied plasma levels and immunoreactivity of insulin and islet amyloid polypeptide in *Psammomys obesus* (sand rat) under low-energy and high-energy diet.

Amylin given peripherally or centrally inhibits acid gastric secretion in a dose-dependent manner and has a protective effect against indomethacin- or ethanol-induced ulcers only when injected centrally. Subcutaneous or central injection of amylin produces a dose-dependent inhibition of gastric emptying, which may contribute to the activity of amylin in the regulation of carbohydrate absorption. In addition, amylin inhibits food intake both when injected peripherally or centrally. Amylin is considered to take part in the rapid endocrine response during digestion to maintain euglycemia (Guidobono 1998).

Amylin slows the rate of gastric emptying in spontaneously diabetic BB/Wistar rats (Macdonald 1997) and inhibits gastric acid secretion in rats (Rossowski et al. 1997).

Guidobono and coworkers (1997) reported a cytoprotective effect of amylin against indomethacin- and ethanol-induced ulcers when given intracerebroventricularly, but not when given subcutaneously.

Clementi and coworkers (1997) found a protective, dose-dependent effect of amylin against reserpine- and serotonin-induced gastric damage in rats, however, no antiulcer effect after pylorus ligation.

The search for a superior compound with the biological actions of human amylin resulted in the identification of [Pro25,28,29]human amylin, assigned the USAN name pramlintide. Young and coworkers (1996) compared this compound with human and rat amylin in various pharmacological tests, such as receptor assays (binding to

rat nucleus membranes, amylin receptors; binding to membranes from SK-N-MC cells, CGRP receptors; binding to membranes from T46D cells, calcitonin receptors), gastric emptying in rats, and plasma glucose after oral glucose gavage. When studying glycogen metabolism in the isolated soleus muscle of rats, pramlintide was slightly more potent than human amylin, but not different from rat amylin in inhibiting insulin-stimulated incorporation of [U-14C]glucose into muscle glycogen. After intravenous infusion of amylin or pramlintide, a dose-dependent increase of plasma glucose, plasma lactate, and ionized calcium was found. Mean arterial blood pressure decreased with higher doses.

Clementi and coworkers (1995) found an anti-inflammatory activity of amylin in mouse ear edema induced by croton oil and acetic acid-induced peritonitis in the rat, but not in serotonin-induced rat paw edema and plasma protein extravasation induced by dextran in rat skin.

Bell and coworkers (1995) and Bell and McDermont (1995) reported that amylin has an hypertrophic effect in rat ventricular cardiomyocytes and exerts the contractile response via CGRP1-preferring receptors.

In anesthetized rats, amylin increased after intravenous infusion urine flow, sodium excretion, glomerular filtration rate, and renal plasma flow (Vine et al. 1998).

Clementi and coworkers (1996) reported that intracerebroventricularly injected amylin induced in rats a dose-dependent decrease of locomotor activity without affecting grooming and sniffing.

Amylin dose-dependently stimulated cell proliferation of human osteoblast (hOB)-like cells and increased osteocalcin production (Villa et al. 1997). Cornish and coworkers (1998) found in adult male mice after daily subcutaneous injection of amylin for 4 weeks an increase of histomorphometric indices of bone formation and a reduction of bone resorption. Muff and coworkers (1995) and Poyner (1997) identified, characterized, and cloned the receptors for calcitonin, calcitonin gene-related peptide, and amylin.

Purpose and Rationale

Binding sites with high affinity for amylin are present in several brain regions, with the nucleus accumbens and surrounding tissue containing more than twice as many banding sites as any other regions (Beaumont et al. 1993).

Procedure

Membrane Preparation

Membranes are prepared from male Sprague–Dawley rats (150–200 g). Following decapitation, the basal forebrain regions (nucleus accumbens) are removed to PBS (pH 7.4) at 4 °C. The tissues are weighted, then placed in 10 ml/g tissue of ice-cold 20 mM HEPES/KOH (pH 7.4), and homogenized with a Polytron (10 s at setting 4). An additional 30 ml of cold HEPES/KOH is added, and the homogenate centrifuged (48,000 × g, 15 min). After discarding the supernatant fluid, membrane pellets are resuspended by homogenization in 40 ml of fresh HEPES/KOH and centrifuged as before. Membranes are washed again by homogenization in buffer and centrifugation. The final membrane pellet is resuspended in a volume of 20 mM HEPES/KOH containing 0.2 mM PMSF added immediately before use from a stock 0.2 M solution in ethanol. A volume of buffer is used to yield a concentration of about 80 mg original tissue/ml. Membranes are kept frozen at −80 °C until use.

Binding Assay

Membranes from 4 mg of original wet weight of tissue are incubated with 125I-BH-amylin (rat amylin, BH-labeled at the amino-terminal lysine and obtained from Amersham Corp.) in 20 mM HEPES/KOH (pH 7.4), containing 0.5 mg/ml bacitracin, 0.5 mg/ml BSA, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), for 60 min at 23 °C. Incubations are carried out in duplicate tubes and are started by addition of membranes. Incubations are terminated by filtration through glass fiber filters that have been presoaked in 0.3 % polyethyleneimine, followed by washing with 15 ml of cold PBS.

Evaluation

Competition curves are generated by measuring binding of 13 pM 125I-BH-amylin in the presence of 10–11 to 10–6 unlabeled peptide. Data are fitted to a four-parameter logistic equation to derive half-maximal inhibitory concentrations (IC50 values) and slope factors (Hill coefficients).

Modifications of the Method

Sheriff and coworkers (1992) characterized amylin binding sites in the human hepatoblastoma cell line, HepG2. Perry and coworkers (1997) studied amylin and calcitonin receptor binding in the mouse α-thyroid-stimulating hormone thyrotroph cell line.

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Insulin Target Tissues and Cells

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Adipose Tissue and Adipocytes

General Considerations

Rodent adipose tissue and cells represent the targets exhibiting the most prominent insulin sensitivity (i.e., lowest EC/IC50) and responsiveness (i.e., highest fold stimulation/inhibition above basal) of the relevant insulin signaling cascades (e.g., insulin receptor activation) and metabolic end effector systems (e.g., lipolysis) in comparison to liver (e.g., gluconeogenesis) and muscle cells (e.g., glucose transport). This might be based in part on technical advantages of the adipose tissue/adipocyte preparation in comparison to that of muscle/myocytes. But more likely, it reflects the exquisite physiological role of the adipose tissue in the regulation and coordination of glucose and lipid metabolism, i.e., insulin stimulation of lipid synthesis (lipogenesis) and insulin inhibition of lipolysis. On the basis of their relatively easy technical preparation, functional adipose tissue fragments (epididymal fat pads) and primary adipocytes (isolated epididymal adipocytes) from rats as well as adipocyte cell lines derived from mice (3T3-L1, F442A) are the first choice for the development of robust and reliable cell-/tissue-based assay systems for insulin-like activity.

Epididymal Fat Pads of Rats

General Considerations

Insulin-like activity can be measured by the uptake of glucose into fat cells, which at low extracellular glucose concentrations (see section "Cellular Esterification" in chapter "► Assays for Insulin and Insulin-Like Activity Based on Adipocytes") represents the rate-limiting step for the conversion of glucose in and its storage as lipids or for the oxidative (glycolysis plus citric acid cycle) and non-oxidative (glycogen synthesis) metabolism of glucose. Adipose tissue from the epididymal fat pads from rodents has been found to be very suitable for the analysis of non-oxidative and oxidative glucose metabolism. Early studies (Beigelman 1959; Steelman et al. 1961) determined the difference of glucose concentration in the medium after incubation of pieces of epididymal rat adipose tissue for the direct determination of glucose uptake (encompassing non-oxidative and oxidative glucose metabolism) or measured oxygen consumption in Warburg vessels (Ball and Merill 1961; Froesch et al. 1963) or the release of radiolabeled $^{14}CO_2$ from [^{14}C]glucose after its trapping and counting (Martin et al. 1958; Slater et al. 1961; Gliemann 1965, 1967b, 1972) monitoring glucose oxidation, exclusively. The latter principle was used by Martin and coworkers (1958) using epididymal fat pads from the rat. This method has been used by various authors (Humbel 1959; Ditschuneit et al. 1959) and described in detail by Renold and coworkers (1960) and Siess and coworkers (1965). Sönksen and coworkers (1965) found a close correlation between "suppressible" insulin-like activity measured by the fad pad method and insulin concentration determined by immunoassay. Ball and Merrill (1961) used the manometric measurement of the net gas exchange of rat adipose tissue to quantitate small amounts of insulin. A survey of the biological assays of insulin-like serum activity was given by Faulhaber and Ditschuneit (1975).

The use of serum from pancreatectomized animals revealed the presence of factors apparently exerting insulin-like activity other than insulin (Steinke et al. 1962, 1965). Subsequently, the nature of these insulin-like activities has been characterized (see section "Glycosyl-Phosphatidylinositol-Specific Phospholipase (GPI-PL) and Insulin-Like Signaling" in chapter "► Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes"), but so far, they escaped their unequivocal characterization and molecular identification, presumably due to their structural heterogeneity and low abundance.

Purpose and Rationale

The epididymal fat pad assay, originally developed as a bioassay of insulin-like activity in serum samples, can also be used for measuring activity of synthetic insulin derivatives and analogs as well as for the evaluation of peripheral insulin-like effects of antidiabetic compounds, such as

sulfonylureas and biguanides, which have long been assumed to exert their blood glucose-decreasing activity exclusively via stimulation of insulin release and improvement of peripheral insulin sensitivity, respectively. However, in the subsequent decades, this tissue-based system has been replaced by cell-based assays (see below) which for most applications offer the advantages of higher sensitivity, accuracy, reproducibility, and test capacity (throughput) despite lower expenditure in the number of animals (per data point) required.

Primary Rat Adipocytes

Purpose and Rationale

The worldwide dramatic increase in obesity with its associated health problems and the identification of adipocyte-secreted proteins that apparently have major impact on the regulation of whole-body glucose, lipid, and energy metabolism have generated major interest in adipocyte biology. Adipocytes are the primary storage site for energy in vertebrate animals. During fasting, adipocytes release energy-rich molecules that provide metabolic fuels to other tissues. Adipocytes also secrete hormones that orchestrate the storage, release, and oxidation of energy-rich molecules throughout the body and that control behavior, including feeding and appetite. Primary adipocytes maintain a large dynamic pool of triacylglycerol (TAG) and express a specific set of proteins to maintain circulating metabolic fuel levels. Primary adipocytes and adipose tissue have been used to study basic adipocyte biology.

Rodbell (1964) and Gliemann (1965, 1967a, b) used primary adipocytes isolated by digestion of epididymal fat pads from rats with collagenase. The conversion of (3-^{3}H) glucose into total adipocyte TAG has been used as a very sensitive parameter in an improved assay for insulin-like activity by Moody and coworkers (1974) which during the subsequent decades turned out to represent the golden standard for assays for insulin-like activity. Alternatively to primary rat adipocytes, Etherton and Chung (1981) and Etherton and Walker (1982) characterized the insulin sensitivity of isolated swine adipocytes.

Preparation of Rat Adipocytes

Male Wistar or Sprague–Dawley rats weighing 140–200 g are sacrificed, and both epididymal fat pads are removed under sterile conditions. The fat pads are cut into pieces and incubated for 20 min at 37 °C with 1 mg/ml collagenase (e.g., type CLS II, Worthington, NJ) in Krebs-Ringer HEPES bicarbonate buffer, KRHB (25 mM HEPES/KOH, pH 7.4, 0.1 mM glucose, 1 % wt/vol BSA). The cell suspension is filtered through a 100-μm nylon screen and washed three times by flotation (accumulation of a thin cell layer on top of the medium after centrifugation at 1,000× g, 1 min, swing-out rotor) with KRHB lacking glucose and finally suspended in the same solution. The suspension is adjusted to a final titer of 3.5 × 105 cells/ml.

Stimulation of Rat Adipocytes with Insulin

Adipocytes are suspended in buffer S (Dulbecco's Minimal Essential Medium ([DMEM] for primary culture to induce insulin resistance, see section "Insulin-Resistant Primary Rat Adipocytes") or KRHB (for short-term measurement of insulin-like activity) both containing 5 mM glucose, 0.5 M mM sodium pyruvate, 4 mM L-glutamine, 200 nM (1-methyl-2-phenylethyl)adenosine, 100 μg/ml gentamicin, 1 % BSA, and 25 mM HEPES/KOH, pH 7.4) at 5 % cytocrit (corresponding to about 7 × 105 cells/ml). For the determination of the packed cell volume, small aliquots of the cell suspension are aspirated into capillary hematocrit tubes and centrifuged for 90 s in a microhematocrit centrifuge in order to measure the fractional occupation of the suspension by the adipocytes (lipocrit). A 20-ml portion of the adipocyte suspension (5 % lipocrit) is added to 20 ml of buffer S containing human insulin as indicated. Incubations (20 min, 37 °C) are performed under 5 % CO_2 in 200-ml polyethylene vials during shaking at 110 cycles/min with a stroke length of 3.5 cm.

Permeabilization of Rat Adipocytes

Studies of intermediary metabolism and its regulation by insulin often require the use of agents that do not readily penetrate the plasma membrane

of intact cells due to the size and charge of substrates (e.g., sugar phosphates, nucleotides, hydrophilic inhibitors, antibodies). Such studies would greatly benefit if the permeability of the plasma membrane to these compounds or the release of freely diffusible cytoplasmic molecules (e.g., metabolites, proteins) across the plasma membrane could be increased. Thus, permeabilized cells can be useful for the analysis of complicated metabolic processes as well as for the study of the insulin-like activity of agents which do not readily pass the plasma membrane of adipocytes including the elucidation of the molecular mechanism(s) of the insulin(-like) action induced or inhibited by a certain compound/drug. For this, it may be useful to block known signal transduction or metabolic pathways in adipocytes by introducing specific inhibitory antibodies (against key signaling proteins/metabolic enzymes) or peptide substrates (competing for the endogenous protein substrate, e.g., synthetic peptide as kinase substrate) prior to stimulation of the adipocytes with the compound/drug. In conclusion, permeabilized cells should be looked upon as a method that rapidly makes the interior of the cells accessible.

In general, the widely used methods of short incubation under hypoxic conditions (Gordon et al. 1985) and electroporation (i.e., subjecting cells to a short series of high-voltage electrical discharges) permeabilize cells transiently. These cells are characterized by the formation of small protuberances (blebs) indicative of localized cell surface damage. It is conceivable that the stretched plasma membrane of such blebs acts as a high-permeability region. Disappearance of blebs and restoration of normal plasma membrane impermeability are achieved again by short (15-min) incubation at 37 °C. During the period of enhanced permeability, small compounds can be introduced. Upon alternative physical treatment, such as osmotic shock and brief freezing/thawing, the cells are also temporarily permeabilized. Such cells reseal under appropriate conditions (Lepers et al. 1990). These physical methods are probably not selective for the plasma membrane necessitating other permeabilization techniques if intact intracellular membranes are required.

In addition, intactness of intracellular organelles will also benefit from the use of permeabilizing media that are isotonic with the cell content (Bijleveld and Geelen 1987). The cholesterol-sequestering approach produces very fast holes of about 8 nm (Schulz 1990) and is most suited to rapidly measure cellular enzyme activities as the lesions are sufficiently large to allow entry of large molecular substrates or proteins and to allow cytosolic proteins of at least 285 kDa to leave the cell (Bijleveld and Geelen 1987). Apart from transient permeabilization, all the other techniques for permeabilizing cells have severe limitations due to the fact that cellular integrity is destroyed. The degree of loss of integrity determines the application of a particular permeabilizing procedure. The use of digitonin-permeabilized mammalian cells for measuring enzyme activities in the course of studies on lipid metabolism has been reviewed by Geelen (2005).

Electroporation of Rat Adipocytes

For the introduction of antibodies and peptides as well as compounds/drugs into isolated rat adipocytes without significant loss of cell viability and insulin sensitivity, the method of electroporation has been used successfully. For this, 0.4 ml of buffer E (4.74 mM NaCl, 118 mM KCl, 0.38 mM $CaCl_2$, 1 mM EGTA, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mg/ml BSA, 3 mM sodium pyruvate, and 25 mM HEPES/KOH, pH 7.4) is placed in a 0.4-cm-gap-width electroporation cuvette (Bio-Rad, Munich, Germany) together with the antibodies or peptides or compounds/drugs. 0.4 ml of adipocyte suspension (50 % cytocrit in buffer E) is added to each cuvette and gently mixed. Electroporation is performed using a Gene Pulser Transfection Apparatus (Bio-Rad, Munich, Germany) which is set at a capacitance of 25 μF and voltage of 800 V (2 kV/cm), at 25 °C for six shocks (Shibata et al. 1991; Quon et al. 1993). After the third treatment, the adipocyte suspension is gently stirred with a plastic stick, and the electric polarity is reversed. The time constant of electroporation is typically 0.6 ms during the final shock. Routinely, 4 ml of adipocyte suspension (25 % cytocrit) is

electroporated in five cuvettes. The time required for treatment of the five cuvettes is about 3 min. After electroporation, the cells from five electroporations are pooled and transferred to 50-ml polystyrene tubes. After incubation (30 min, 37 °C) in 5 % CO_2/95 % O_2, the cells are centrifuged (200× g, 1 min, swing-out rotor), and the infranatant is aspirated. Thereafter, the cells are washed once with 40 ml of buffer E containing 5 mM glucose and 4 % BSA, suspended in 20 ml of the same buffer, and then incubated (1 h, 37 °C) under 5 % CO_2 prior to challenge with the appropriate stimulus. Using this electroporation technology, Müller and coworkers (2000) introduced into rat adipocytes inhibitory antibodies against tyrosine kinases mediating insulin-like signaling in response to stimuli different from insulin (see K.6.3.6.4).

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Separation According to Size

Adipocytes are isolated by collagenase digestion of epididymal fat pads from male Sprague–Dawley rats and washed according to published procedures (see above). Adipocytes prepared from 1-month (120–140 g)- to 6-month (320–380 g)-old male rats are collected by flotation (200× g, 2 min, 30 °C) and then filtered through serial nylon mesh screens with pore sizes of 75, 150, and 400 μm to obtain small (diameter <75 μm from 1-month-old rats) and large (diameter >400 μm from 6-month-old rats) adipocytes, respectively. After fixation of aliquots of the adipocyte suspension with osmic acid, cell number is determined using a Coulter counter. The adipocyte suspension is adjusted to the desired titer with KRH buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES/KOH, pH 7.4) supplemented with 0.5 % bovine serum albumin (BSA), 100 μg/ml gentamicin, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate. Total lipid content of the adipocyte suspension ("lipocrit") is measured as described above.

Differentiation from Stromal Vascular Cells

Stromal vascular cells (SVCs) are isolated upon collagenase digestion of rat adipose tissue as described above with the following modifications. Freshly harvested epididymal adipose tissue (~5-g samples) from untreated Wistar rats (200–240 g, 9–11 weeks old) is digested (1 h, 37 °C) with collagenase (250 U/ml, type II, Worthington, St. Katharinen, Germany) in phosphate-buffered saline (PBS) containing 2 % BSA under constant agitation. After filtration through a strainer, the digested adipose tissue was centrifuged (500× g, 10 min, 20 °C). The pellet was suspended in 10 ml of adipocyte buffer and then passed through a 150-μm mesh for the removal of undigested clumps, debris, and lysed adipocytes. The pass-through is centrifuged (500× g, 10 min, 20 °C). The pellet is suspended in 50 ml of erythrocyte

lysis buffer (155 mM NH_4Cl, 5.7 mM KH_2PO_4, 0.1 mM EDTA in water) and then consecutively filtered through nylon mesh screens with pore sizes of 75 and 30 μm. The latter pass-through was centrifuged (500× g, 10 min, 20 °C). The pelleted SVCs were washed once in 5 ml of PBS/2 % BSA/2 mM EDTA, recentrifuged, and then suspended in 1 ml of adipocyte buffer supplemented with 100 μg/ml gentamicin, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate at 3.5 × 106 cells/ml and plated in collagen-coated 6-well (for differentiation/incubation), 96-well (for RNA analysis), or 12-well (for protein analysis) microplates at 50,000 cells/cm^2.

For differentiation into adipocytes, the SVCs are plated in duplicate onto circular cover glasses inserted in 6-well plates and cultured in DMEM/Ham's F-12 (1:1) containing 10 % fetal calf serum, 100 μM ascorbic acid, 33 μM biotin, 17 μM pantothenate, 50 nM triiodothyronine, 2.5 μM niacinamide, 1 μM octanoic acid, and 10 μg/ml insulin for 5 days. Subsequently, the incubation is continued in adipogenic medium (DMEM/Ham's F-10, 3 % fetal bovine serum, 33 μM biotin, 17 μM pantothenate, 25 nM insulin, 1 μM dexamethasone) supplemented with 0.2 mM isobutylmethylxanthine, 10 nM L-thyroxine, and 0.1 μM rosiglitazone for the first 3 days and then in adipogenic medium for the following 9 days. Cells are used upon 80–90 % differentiation according to Oil Red staining and light microscopy.

Mean cell diameters of the primary and differentiated rat adipocytes are determined by measurement of 100 cells, each, under the light microscope. The cell volume is calculated from the diameter using the formula 4/3 × d3 × π.

Flow Cytometry of Stromal Vascular Cells

Purpose and Rationale

The structure and morphology of adipose tissue with its large lipid droplets and honeycomb structure create challenges in identifying and studying smaller, often widely dispersed non-adipose cells. These cells, such as infiltrating macrophages, may play key roles in regulating local inflammatory responses when exposed to a high-fat diet (Weisberg et al. 2003). There are strong links between the expression of proinflammatory cytokines, such as tumor necrosis factor-α and the progression of insulin resistance associated with obesity (Hotamisligil et al. 1993; Xu et al. 2003). Identifying which cell types are responsible for the regulation and secretion of these inflammatory molecules is an area of active investigation. Flow cytometry allows investigation of size, granularity, and extracellular and intracellular expression of proteins on single cells, isolated from adipose tissue. Antibodies against cell markers, such as CD3, CD11b, F4/80, CD11c, and CD34, can be used to identify T cells, myeloid cells, macrophages, dendritic cells, and stem cell-like cells, respectively. Recent publications have used flow cytometry to identify CD3+ lymphocytes, CD34+ cells, and subsets of F4/80+ macrophages in the stromal vascular fraction of white adipose tissue (Robker et al. 2004; Brake et al. 2006; Caspar-Bauguil et al. 2005; Wu et al. 2007). Flow cytometry on fresh adipose cells has been used to investigate the potential of transplanted bone marrow-derived progenitor cells to migrate to adipose tissue and differentiate into adipose cells (Crossno et al. 2006). Studies have also been performed to characterize freshly isolated cells from human tissue (Yoshimura et al. 2006).

Analysis of fresh adipose tissue presents difficulties because of fewer SVCs per gram of adipose tissue as well as the relative fragility of these cells. For this, the adipose tissue cells must be in a single-cell suspension, which requires collagenase digestion of the adipose tissue. Optionally, the cells can be purified to study a specific population through magnetic bead-assisted cell sorting (MACS). The following protocol presents a method of isolating and preparing freshly isolated stromal vascular tissue for flow cytometric analysis.

Procedure

Adipose Tissue Isolation and Stromal Vascular Fractionation

Adipose depots (perigonadal, periepididymal, retroperitoneal, or subcutaneous) are isolated with sterile techniques. Any lymph nodes are carefully removed if present and the tissue placed on ice in

KRB solution supplemented with glucose and BSA (100 ml consist of 13.16 ml of 0.77 M NaCl, 0.52 ml of 0.77 M KCl, 0.132 ml of 0.77 KH_2PO_4, 0.132 ml of 0.77 $MgSO_4$, 2.76 ml of 0.77 M $NaHCO_3$, 4.16 ml of 0.3 M glucose, and 0.38 ml of 0.275 M $CaCl_2$ supplemented with sterile H2Obidest and stored at 22 °C) ($CaCl_2$ can cause the solution to precipitate and therefore should be added slowly and lastly, while swirling the flask before gassing the solution). The solution may appear slightly clouded. Glucose is made daily fresh and added to the KRB solution; the solution is then gassed with 95 % O_2/5 % CO_2 for 20–30 min; BSA should be added to a final concentration of 4 % after gassing, since it will cause the solution to bubble over if added before, and mixed well; the complete KRB solution is kept on ice for the isolation and the washing procedures. The minimal amount of adipose tissue recommended for this procedure is 1 g. Depending on the fat pad isolated, it is recommended to pool tissue from three or more mice. Mice of 8–12 weeks of age on a normal chow diet have between 0.1 and 0.4 g of perigonadal fat. The tissue is then washed with KRB solution, weighed, and minced with scissors, razor blades, or disposable scalpels. The cell yield is highly dependent on how finely minced the tissue is when added to the collagenase solution. The greater is the surface area, the more efficiently the collagenase will work resulting in higher yield. The minced adipose tissue is added to KRB solution in a ratio of 1 g/3 ml of KRB. Collagenase (type I, Worthington Chemicals) solution (lot-to-lot variance in collagenase can widely affect results, and it is recommended to try several lots or different manufacturers and use only one lot of collagenase for all experiments) is added to minced adipose tissue at a concentration of 280 U/ml of KRB, and the mixture is incubated in a shaking water bath at 200–300 rpm at 37 °C for 40 min. The incubation time of collagenase may vary. Incubation times longer than 1 h are not recommended because of increased cell death and apoptosis. Alternatively, the collagenase solution may be fractionated after 30 min, with undigested tissue being mixed with fresh collagenase and KRB and incubated for another 30 min. The single-cell supernatant can be stored on ice during that time. Thereafter, the cells are filtered through chiffon or a 25-μm nylon mesh cut into 2- to 3-in. squares. (Chiffon is a low-cost alternative to nylon mesh. It can be bought at any fabric store in large quantities. If culturing of cells is desired, it may be sterilized by washing with 70 % ethanol and exposing to UV light inside a laminar flow hood overnight. For immediate antibody staining and flow cytometric analysis, sterilization is not required. Chiffon is not recommended if the corresponding experiment is endotoxin sensitive.) Subsequently, the cells are washed with 2× initial volume of KRB solution. The adipose cells are separated from stromal vascular (S-V) cells by centrifugation at 500× g for 5 min. The adipose cells will float, while the S-V fraction will pellet. SVCs are removed from the bottom of a conical tube and resuspended and washed twice with 15 ml of KRB solution and then centrifuged again at 500× g for 5 min. SVCs are resuspended in 1 ml of PBS and counted with a Coulter counter or a cell-counting chamber.

Positive Selection of Cells from the S-V Fraction Using Magnetic Cell Sorting

The cell suspension is centrifuged at 300× g for 10 min. The supernatant is gently aspirated as the pellet will be easy to disturb. After the S-V fraction has been isolated, all subsequent centrifugation steps are done at 4 °C. One hundred seven or fewer cells (more cells require a proportional upscaling) are resuspended in 90 μl of MEC buffer consisting of PBS without Ca^{2+} or Mg^{2+}, 0.5 % BSA, and 2 mM EDTA, filtered through a 0.22-μm-pore filter, and stored at 4 °C (MEC buffer contains an ion chelator and EDTA and no Ca^{2+} or Mg^{2+}). Although this greatly increases the efficacy of antibody binding resulting in highly pure populations, this buffer makes the cells fragile and prone to apoptosis. Move without delay through these steps and keep the solutions and cells on ice at all times. High centrifugation speeds at this stage will greatly reduce the yield. 10 μl of CD11b MicroBeads, i.e., MicroBeads conjugated to monoclonal rat anti-mouse CD11b

(Mac-1) antibody, Clone M1/70, isotype IgG2b, stored at 4 °C, and protected from light (Miltenyi Biotec 9), are added. After mixing by flicking the tube or very gentle vortexing, the cells are incubated on ice for 15 min, then washed by adding 1 ml of MEC buffer, and finally centrifuged at 300× g for 10 min. The supernatant is aspirated off. The pelleted cells are resuspended in 500 µl of MEC buffer for use in MACS MS separation columns (for positive selection, Miltenyi Biotec has a wide variety of MACS columns and separators that will work for this procedure. The MS columns described are designed for positive selection for cell quantities less than 107. That is, the primary cells of interest will remain in the column attached to the antibody of interest, while all unlabeled cells will fall through. Once removed from the magnetic field, the magnetically conjugated antibodies and the cells will easily and with high efficacy be released from the column). The column is placed in the magnetic field of the MiniMACS separator (Miltenyi Biotec) and equilibrated with 500 µl of MEC buffer. Thereafter, the cell suspension is applied onto the column. The unlabeled cells that pass through are collected and washed with 500 µl of 3× MEC buffer allowing the column to empty each wash, but without leading to dryness. This fraction is the CD11b-negative fraction that can be assayed by flow cytometry if desired. The column is removed from the magnetic field of the separator and placed in a 1-ml microfuge tube. After addition of 1 ml of MEC buffer to the column, the fraction is expelled into the microfuge tube with the provided plunger. This represents the CD11b-positive fraction that can be assayed by flow cytometry. The cell fractions are centrifuged at 300× g for 5 min. After aspirating off the supernatant, the pelleted cells are resuspended in 300 µl of PBS containing Ca^{2+}, Mg^{2+}, and glucose (this is important since MEC buffer will greatly reduce yield if used for incubation and washing steps. If forward versus side scatter plots show a high percentage of debris in the lower left corner, this could be due to being in the MEC buffer for too long or due to too high centrifugation speeds).

Analysis of Cell Surface Markers by Flow Cytometry

5 × 105 cells are added per test to 400 µl of PBS containing 10 mM glucose. 1–2 µg of appropriate antibodies are added to separate cell samples, e.g., CD11b clone M1/70 with fluorescein isothiocyanate (FITC) conjugated as fluorophore (BD Biosciences), ICAM-1 clone YN1/1.7.4 with phycoerythrin (PE) conjugated as fluorophore, FITC-conjugated CD14 clone Sa2-8 (eBioscience), FITC-conjugated isotype control IgG2a and IgG2b, and PE-conjugated isotype control IgG2b (BD Biosciences). If two-color staining is desired, the corresponding primary antibodies are incubated at the same time (When staining with multiple antibodies in the same sample, the fluorophores have to emit different wavelengths. Fluorophores PE and FITC are commonly paired together because of their emission spectra. However, light emissions from PE will be detected in the FITC channel; therefore, compensation will be required. PE- and FITC-conjugated isotypes can be used to set the appropriate compensation settings. For this reason, it is recommended that the brighter or more highly expressed protein be FITC labeled). After mixing by vortexing and subsequent placing on ice for 30 min under protection from light, 1 ml of PBS containing 10 mM glucose is added. (The quantity of the antibody and duration of incubation should always be optimized for each antibody and the amount of cells being stained. Usually, less than the manufacturer's recommended amount may be used with satisfactory results. The appropriate isotype control antibody has always to run in parallel for each set of experiments as this is essential in establishing the background fluorescence for nonspecific binding. Isotypes should be run for each condition, as a treatment may alter nonspecific binding, resulting in potential false-positives.) Following centrifugation at 300× g for 2 min, the supernatant is gently removed, and the washing procedure is repeated twice. The last cell pellet is resuspended in 700 µl of 1× FACS Lysing Solution (BD Biosciences) to remove red blood cells and then incubated for 10 min at 22 °C in the dark. After addition of 1 ml of PBS

containing 10 mM glucose, the cells are centrifuged at 300× g for 2 min. The supernatant is gently removed and the washing procedure is repeated twice. The last pellet is resuspended in 400 µl of 4 % paraformaldehyde as fixative. (The final resuspension volume can vary depending on the limits of the flow cytometer. If the flow rate or counts per second are low, the cells have to be resuspended in a smaller volume.) During the final analysis of sample portions on the flow cytometer (e.g., BD-FACScan or similar equipment with a laser that emits at 488 nm), the samples are kept on ice and occasionally gently vortexed to keep cells from settling. It is possible to gate or select certain regions and look at cell surface expression of only those populations.

Evaluation

Adipose tissue contains cell types other than adipocytes that may contribute to complications linked to obesity and T2D. For instance, macrophages have been shown to infiltrate adipose tissue in response to a high-fat diet. Isolation of the stromal vascular fraction of adipose tissue allows one to use flow cytometry to analyze cell surface markers on leukocytes. Flow cytometry and magnetic cell sorting are sensitive, reliable, and efficient technologies to identify subsets of vascular stromal cells, such as leukocytes, that differentially express cell surface markers.

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Insulin-Resistant Primary Rat Adipocytes

Purpose and Rationale

Primary rat adipocytes can be maintained in culture for long-term treatment with compounds/drug candidates to study their insulin-like activity upon chronic challenge or for the induction of insulin resistance to study the insulin-sensitizing activity of compounds/drugs for up to 24 h without significant loss of viability by incubation in buffer S (see K.6.3.5) containing 5 mM glucose and 0.5 mM sodium pyruvate under 95 % O_2/5 % CO_2 and continuous gentle shaking with moderate

loss of insulin sensitivity and responsiveness, only (Müller and Wied 1993). The conditions can be modified for the (subsequent) induction of insulin resistance in vitro, which might reflect the hyperglycemic and hyperinsulinemic state promoting insulin resistance in vivo.

Procedure

For induction of insulin resistance, primary rat adipocytes are incubated in buffer S in the presence of 25 mM glucose, 0.5 mM sodium pyruvate, and 10 nM insulin for 4–20 h at 37 °C under slow shaking. Cells are assayed for insulin-stimulated glucose transport following a 15- to 30-min incubation period in buffer S containing 0.5 mM glucose and sodium pyruvate but lacking insulin (which enables downregulation of the glucose transport system from the chronically insulin stimulated to basal levels).

Evaluation

The adipocytes made insulin resistant in vitro show a rightward shift of the concentration-response curve (decreased insulin sensitivity) and a reduced maximal glucose transport velocity at constant or only slightly elevated basal glucose transport (decreased insulin responsiveness) (see Müller and Wied 1993). During prolonged incubation in suspension culture, the adipocytes tend to increase their basal glucose uptake which might reflect elevated energy demands due to stress conditions or loss of plasma membrane integrity. It is therefore most critical to choose culture conditions which preserve the energy status of the rat adipocytes and are compatible with five- to six-fold stimulations of glucose transport, at least, at low glucose concentrations corresponding to the normal insulin-sensitive state. This allows monitoring of the induction of insulin resistance by culturing of the adipocytes at high glucose and insulin and its blockade by the concomitant presence of compounds/drugs which may act as insulin sensitizers. The use of 25 mM glucosamine instead of high glucose/insulin as initially proposed by Marshall and coworkers (1984; see K.6.3.5) on the basis of the apparent activation of the hexosamine pathway during glucose-/insulin-induced desensitization of rat adipocytes in culture is not recommended. It has meanwhile been demonstrated that intracellular accumulation of glucosamine results in depletion of cytosolic ATP and accompanying induction of the stress response (Hresko et al. 1998) causing apparent loss of insulin responsiveness which is based on massive increment of the basal glucose transport rate rather than on impairment of insulin signaling to the glucose transport system.

Cultured Mouse Adipocytes

General Considerations

Primary adipocytes have several limitations. They do not propagate in culture, they are difficult to transfect with DNA, they usually have a single huge TAG lipid droplet (LD) that interferes with biochemistry and microscopy, their properties vary as a result of the genetics and conditions of the animals from which they have been isolated, and the isolation procedure is tedious and introduces variation. In addition, harvesting primary adipocytes or adipose tissue from animals generally requires the euthanasia of a vertebrate animal and the expense of specialized facilities and protocols. For these reasons and likely others, cell lines have been developed that can be induced to store TAG, to express proteins that are hallmarks of primary adipocytes, to exert many of the functional and physiological features of primary adipocytes, and presumably to recapitulate key events in adipocyte ontogeny.

3T3-L1 Adipocytes

Purpose and Rationale

Three decades ago, Green and Meuth (1974) reported that a clonal subline of mouse 3T3 cells had a propensity to differentiate into adipocytes when in a "resting state." This 3T3-L1 cell culture model of adipogenesis has been exploited extensively to investigate the molecular mechanisms of adipocyte differentiation, lipid metabolism, insulin signaling, and glucose transport as well as to identify physiologically important adipocyte-secreted proteins (adipokines or adipocytokines), such as adiponectin, visfatin, and resistin

(for reviews, see Banerjee and Lazar 2003; Stefan and Stumvoll 2002; McGillis 2005).

Green and Kehinde (1974) isolated from the established mouse fibroblast line 3T3 two subclonal lines that accumulate large amounts of TAG after they had undergone stimulus-induced differentiation from confluent fibroblasts via the state of preadipocytes to mature adipocytes. The cell line 3T3-L1 has been used extensively by many authors (Spooner et al. 1979; Frost and Lane 1985; Zuber et al. 1985; Chan et al. 1988; Clancy and Czech 1990; Wieland et al. 1990; Müller et al. 1994). For some purposes (e.g., analysis of the endogenous leptin production), the F442A mouse fibroblastic cell line has been used successfully (Zhang et al. 2002; Teta et al. 2005). Kletzien and coworkers (1992) studied the effect of pioglitazone, an insulin-sensitizing agent, on the expression of the adipocyte fatty acid-binding protein in ob/ob mice and 3T3-L1 cells.

Procedures

Culture and Differentiation

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) are seeded in 12-well (60,000 cells/well) or 96-well (15,000 cells/well) plates and maintained in DMEM (high glucose), 10 % fetal bovine serum (FBS), 5 mM L-glutamine, and 2 % BSA. Following 3 days at 90–100 % confluence, differentiation is initiated by the addition of DMEM containing 10 % FBS, 400 nM human insulin, 1 μM dexamethasone, and 1 mM IBMX. Three days later, the medium is replaced with DMEM, 10 % FBS, and 100 nM insulin. After additional 2 days, the medium is changed to DMEM (low glucose) and 10 % FBS. The presence of putative insulin-sensitizing compounds, such as glitazones (e.g., 1–10 μM pioglitazone), in the various differentiation media considerably may improve the degree of differentiation as has been shown in numerous studies since the introduction of these drugs into the antidiabetic therapy. The differentiated adipocytes are used 5–12 days after completion of the differentiation protocol, when more than 85 % of the cells expressed the adipocyte phenotype (LD accumulation as observed with "Oil Red" staining). Prior to experimentation, the cells are rinsed two times with low-serum medium (DMEM containing 5 mM glucose, 0.5 % BSA, 0.1 % FBS, 25 mM HEPES/KOH, pH 7.4, 10 mM glutamine, and 100 U/ml streptomycin/penicillin), then incubated (12–14 h) in this medium lacking FBS (for downregulation of the insulin signal transduction cascade), and finally washed twice with PBS containing 2 mM sodium pyruvate prior to incubation (30 min, 37 °C) with insulin or compounds/drug candidates.

OP9 Adipocytes

Purpose and Rationale

The 3T3-L1 adipocyte model has significant limitations. First, from the time of initial plating, the generation of 3T3-L1 adipocytes from preadipocytes requires at least 2 weeks (Student et al. 1980). Second, if 3T3-L1 cells become confluent and are further propagated or if they are passaged extensively, they no longer differentiate robustly into adipocytes. These issues make culturing of 3T3-L1 cells demanding and limit their utility in the generation of stable cell lines. Third, it is difficult to efficiently detect RNAs and proteins encoded by transiently transfected DNA in 3T3-L1 adipocytes. This limitation derives from the facts that most plasmid transfection protocols require subconfluent cells and that levels of RNAs and proteins expressed from such transfections dramatically decline before 3T3-L1 cells become adipocytes. Finally, because the 3T3-L1 cell line originated from a single clone and thus has clone-specific traits, it fails to recapitulate the primary cells it models. Thus, an alternative, tractable adipocyte model system would be desirable. A new adipocyte cell culture model, OP9 mouse stromal cells, has been characterized recently that provides a tractable alternative system for studies of adipocyte biology (Wolins et al. 2006). The OP9 cell line was established from the calvaria of newborn mice genetically deficient in functional macrophage colony-stimulating factor (M-CSF) (Nakano et al. 1994).

Procedure

Propagation and Differentiation

OP9 cells are available from the American Type Culture Collection (ATCC, catalog no. CRL-2749) but, however, have to be tested and selected for those capable of consistent differentiation into adipocytes. OP9 cells are grown in OP9 propagation medium (α-MEM, 20 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). The optimal differentiation potential of OP9 cells is achieved when cells are replated every 3 days at a density of at least 7,000 cells/cm^2. OP9 cells grown in this manner are flat with broad cell processes. In contrast, OP9 cells maintained at low cell density adopt a spindly morphology and differentiate into adipocytes poorly. Three methods can be used to differentiate OP9 preadipocytes into adipocytes.

Serum Replacement (SR) Method

OP9 cells are grown to confluence and then cultured for two additional days in either OP9 or 3T3-L1 propagation medium as described above. The cells are then cultured up to four more days in serum replacement medium (α-MEM, 15 % KnockOut™ SR [Invitrogen], 100 U/ml penicillin, and 100 μg/ml streptomycin). For the purpose of studying effects of insulin/compounds/drugs, it is important to change the SR medium to OP9 propagation medium after day 2 of differentiation, because KnockOut™ SR contains very high concentrations of insulin (final concentration of 1.7 μM) and 5 ng/ml bone morphogenic protein 4 (BMP-4; R&D Systems). However, BMP-4 makes no significant difference in adipocyte differentiation, so all experiments can be performed without BMP-4 with the exception of the assay for insulin-stimulated glucose uptake.

Insulin/Oleate (IO) Method

OP9 cells are plated at 5,000 cells/cm^2. When cells adhered to the plate, the OP9 propagation medium is replaced with insulin/oleate medium (α-MEM, 0.2 % FBS, 175 nM insulin, 900 μM oleate bound to BSA [5.5/1 M ratio] prepared according to Wolins and coworkers (2005), 100 U/ml penicillin, and 100 μg/ml streptomycin).

Adipogenic Cocktail (AC) Method

This method is very similar to that used for 3T3-L1 cells (see section "3T3-L1 Adipocytes" and Student et al. 1980). Cells are grown to confluence and then cultured for two additional days in 3T3-L1 adipocyte medium (DMEM, 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells are then cultured for 2 days in DM1 (DMEM, 10 % FBS, 175 nM insulin, 0.25 μM dexamethasone, 0.5 mM IBMX, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cells are cultured for two additional days in DM2 (DMEM, 10 % FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Differentiated OP9 cells are maintained in OP9 propagation medium.

Evaluation

OP9 mouse stromal cells represent a new model of rodent adipocytes that will be useful for further studies of the mechanisms of adipocyte differentiation and of mature adipocyte function (Wolins et al. 2006). When OP9 cells are given any one of the three adipogenic stimuli, they rapidly accumulate TAG to form numerous, large LD; exert adipocyte morphology; express adipocyte late marker proteins, including glucose transporter 4 (GLUT4), adiponectin, perilipin, and S3-12; and exhibit robust insulin-stimulated glucose transport. OP9 cells can differentiate into adipocytes within 2 days. This rapid rate of differentiation allows for the detection of transiently expressed proteins in mature OP9 adipocytes. Adipogenesis in OP9 cells involves the master transcriptional regulator of adipocyte differentiation, peroxisome proliferator-activated receptor gamma (PPAR-γ). OP9 cells are late preadipocytes in that, before the addition of adipogenic stimuli, they express the adipocyte proteins CCAAT/enhancer-binding proteins α and β, PPAR-γ, sterol-regulatory element-binding protein-1, S3-12, and perilipin. OP9

differentiation is not diminished by maintenance in culture at high cell density or by long periods in continuous culture, thereby facilitating the generation of stable cell lines that retain adipogenic potential.

Several practical features of OP9 cells make this cell line a particularly convenient model for adipocyte studies. First, OP9 adipocytes can be rapidly produced from either preconfluent cells (IO method) or confluent cells (SR and AC methods). Second, in contrast to 3T3-L1 cells, OP9 cells can be maintained at high density without loss of potential to differentiate into adipocytes. Third, OP9 cells continue to differentiate well even at high passage number. These features permit large numbers of OP9 cells to be conveniently maintained in culture and OP9 adipocytes to be produced for experiments within a few days.

Cultured Human Adipocytes

Murine cell models, most notably the 3T3-L1 cell line, have been the basis for the majority of studies of lipogenesis and lipolysis including their regulation as well as of adipogenesis at the transcriptional and posttranscriptional levels. However, there is a growing concern that the regulation of lipid metabolism may differ between human and murine adipocytes. For example, the resistin gene and its secreted protein product as well as its putative role in the regulation of lipid synthesis and degradation were first identified in the 3T3-L1 cells. Subsequent in vivo analysis in mice demonstrated an association between resistin levels, obesity, and type II diabetes. In contrast, clinical studies do not demonstrate a comparable association between serum resistin levels, obesity, and insulin resistance in nonobese and obese human subjects. Likewise, the expression of the agouti gene in adipose tissue and its involvement in lipolysis regulation differ between man and mouse. These discrepancies argue for the increased use of human preadipocyte cell models in exploratory research and drug discovery relating to obesity and type II diabetes. Recently, the culturing of human adipocytes has been introduced successfully. The dramatically increasing incidence of diabetes and obesity in the industrialized countries immediately argues for the need of in vitro studies with human adipocytes, which justifies a more detailed evaluation of their generation and characterization. Two different sources for differentiated human adipocytes are available so far which differ in their accessibility for daily use and reflection of the primary adipocyte phenotype.

Adipocytes Derived from Human Adipose-Derived Adult Stem (ADAS) Cells

Purpose and Rationale

ADAS cells can be reproducibly isolated from liposuction aspirates through a procedure involving collagenase digestion, differential centrifugation, and expansion in culture. A single milliliter of tissue yields over 400,000 cells (Aust et al. 2004). The undifferentiated human ADAS cells express a distinct immunophenotype based on flow cytometric analyses and, following induction, produce additional adipocyte-specific proteins (Aust et al. 2004; Gronthos et al. 2001; Halvorsen et al. 2001; Sen et al. 2001; Zuk et al. 2002). The human ADAS cells display multipotentiality with the capability of differentiating along the adipocyte, chondrocyte, myogenic, neuronal, and osteoblast lineages (Aust et al. 2004; Zuk et al. 2002). In the presence of dexamethasone, insulin, IBMX, and a thiazolidinedione, the undifferentiated human ADAS cells undergo adipogenesis. Between 30 % and 80 % of the cells, based on flow cytometric methods, accumulate LD, which can be stained for neutral lipids with the “Oil Red” dye.

Procedure

LD proteomics procedures for the analysis of human (ADAS) and rodent fat cells have been used by DeLany and coworkers (2005), Lee and coworkers (2006), Chen and coworkers (2005), and Brasaemle and coworkers (2004). For the evaluation of ADAS cells (DeLany et al. 2005), liposuction aspirates from subcutaneous adipose tissue sites can be obtained from male and female subjects (30–40 years, 25–30 BMI) undergoing elective procedures in local plastic surgical

offices. Tissues are washed three to four times with PBS and suspended in an equal volume of PBS supplemented with 1 % bovine serum and 0.1 % collagenase type I prewarmed to 37 °C. The tissue is placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged (300 × g, 5 min, room temperature). The supernatant is removed, and the pelleted stromal vascular fraction (SVF) is resuspended in stromal medium (DMEM/Ham's F-12, 10 % FBS, antibiotics/antimycotics) and plated at a density of 0.156 ml of tissue digest/cm^2 of surface area in T225 flasks using stromal medium for expansion and culture according to the procedure of Hauner and coworkers (1989). This initial passage of the primary cell culture is referred to as "passage 0." Following the first 48 h of incubation at 37 °C at 5 % CO_2, the cultures are washed with PBS and maintained in stromal medium until they achieve 100 % confluence (mean cell density of ~30,000 cells/cm^2 after 4 days in culture). The cells are passaged by trypsin/EDTA digestion and seeded at a density of 30,000 cells/cm^2 ("passage 1") on 10-cm plates.

One day after seeding, plates are either harvested for protein (day 0) or the medium is replaced with an adipogenic differentiation (AD) medium composed of DMEM/Ham's F-12 with 3 % FBS, 33 μM biotin, 17 μM pantothenate, 1 μM bovine insulin, 1 μM dexamethasone, 0.25 mM IBMX, 5 μM rosiglitazone, and 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of Fungizone. After 3 days, AD medium is changed to adipocyte maintenance medium, which is identical to the AD medium except for the removal of both IBMX and rosiglitazone. Cells are fed every third day and usually maintained in culture for 9 days prior to harvest.

Evaluation

The studies published so far provide a composite profile of the proteome of undifferentiated human ADAS cells obtained from multiple donors. Overall, the results confirm the adipocyte-specific protein expression. However, not all of the proteins identified derive from ADAS cells and may be qualified as contaminants. The most striking examples are hemoglobins A and B, reported in the undifferentiated cell lysates. The presence of hemoglobin apparently reflects the cell isolation procedure. The SVF population prepared by collagenase digestion of the adipose tissue and subsequent centrifugation routinely contains erythrocytes that are washed away after a 48-h period during which the ADAS cells adhere to the plastic surface. Typically, nucleated hematopoietic cells, expressing the marker CD45, account for less than 1 % of the ADAS cell population by passage 1 according to previous flow cytometric studies (Aust et al. 2004; Gronthos et al. 2001). Nevertheless, it is feasible that some hemoglobin remains bound to the adherent ADAS cell population either as intact erythrocytes or as membrane-bound protein.

Proteomic analyses of total cell lysates from murine 3T3-L1 adipocytes have identified between 8 and 100 protein features by one- and two-dimensional gel electrophoresis/mass spectroscopy (Welsh et al. 2004; Wilson-Fritch et al. 2003; Brasaemle et al. 2004; Choi et al. 2004). The ADAS cell proteome includes a high percentage of identical or similar proteins (DeLany et al. 2005).

Adipocytes Derived from Commercially Available Human Preadipocytes

Procedure

Frozen human preadipocytes (PromoCell) are thawed and cultured in preadipocyte expansion medium (DMEM/Ham's F-12 supplemented with 10 % FBS, human epidermal growth factor (2.5 ng/ml), hTGF-β (0.25 ng/ml), bovine fibroblast growth factor (bFGF, 0.5 ng/ml), and antibiotics) until the cells are confluent. For adipocyte terminal differentiation, preadipocytes are induced with differentiation medium containing DMEM/Ham's F-12, 3 % rabbit serum, biotin (33 μM), pantothenate (17 μM), human recombinant insulin (0.1 μM), dexamethasone (1 μM), IBMX (0.5 mM), and a peroxisome proliferator-activated receptor (PPAR)-γ ligand, such as troglitazone (3 μM) or F-moc-L-leucine (30 μM). After a 6-day induction period, with fresh medium added every third day, the cells are

fed with the same medium without IBMX or a PPAR-γ ligand (adipocyte medium) every 3–4 days for 6 additional days. Cells at passages 3–4 are routinely used for adipocyte differentiation. TAG accumulation is confirmed by staining formaldehyde-fixed cells with the neutral lipid-specific dye, BODIPY, at 1 μg/ml. Microscopic images are obtained using an inverted microscope with an illuminator and image acquisition system (Carl Zeiss, Inc., Germany).

Evaluation

Differentiated adipocytes are fixed in formaldehyde (5 %), and TAG is stained with BODIPY. After the fluorescence of each well has been read at 530 nm, the BODIPY-stained lipid is removed, and cells are permeabilized using isopropanol. Isopropanol is subsequently removed, and cellular DNA is stained with EthD-1 (2 μM) for 30 min before measuring EthD-1 fluorescence at 620 nm. The EthD-1 readout is directly correlated with cell density between 2,000 and 40,000 cells/cm^2. Fluorescence units of BODIPY and EthD-1 per well are used to determine the lipid/DNA ratio.

Adipocytes Derived from Mesenchymal Stem Cells

Human MSCs (mesenchymal stem cells) between passages 4 and 10 (Cambrex Bioscience, Verviers, Belgium) derived from resection of abdominal subcutaneous (external to the fascia superficialis, mesenteric, and omental) fat tissue (2–10 g per subject) during gastric bypass surgery for the management of obesity (29–61 years, body mass index of 54 + 2 kg/m^2, fasting blood glucose lower than 120 mg%, no malignancies, no administration of thiazolidinediones and steroids) are seeded in 12-well plates at a density of 4 × 104 cells/cm^2 and incubated in DMEM/Ham's F-12 (1:1, Invitrogen, Basel, Switzerland) containing 0.5 % BSA and antibiotics for 3 days and then grown in MSC basal medium (Cambrex) until confluency (medium change every 2 days). Macrophages are rare (<10 per 106 cells) as assessed by phase-contrast microscopy.

For differentiation from confluence, first- to fourth-passage cells were differentiated using a previously described method (Fasshauer et al. 2000, 2001) with the following modifications: Cultures were incubated (15–20 days) in 6-well plates in DMEM/Ham's F-12 (1:1) containing 0.5 % BSA, 3 % FCS, antibiotics, and the following supplements until completion of differentiation – 250 μM isobutylmethylxanthine (removed after 2 days), 1 μM dexamethasone, 0.2 nM 3,3,5-triiodo-L-thyronine, 5 μM transferrin (Sigma, Deisenhofen, Germany), 500 nM human insulin, and 1 μM rosiglitazone (GlaxoSmithKline, Worthing, UK). Medium is changed every 2 days. Two to 4 days after completion of differentiation, the cells are washed three times with warm PBS and used for the experiments. Differentiation of MSCs into adipocytes is considered to be successful in case of detection of (i) doubly refractile inclusions by low-power phase-contrast microscopy that displayed LD nature according to staining with Oil Red O; (ii) expression of the adipocyte-specific mRNAs, PPARγ2, leptin, adiponectin, and GLUT4 by qRT-PCR; and (iii) isoproterenol (1 μM)-induced glycerol release and insulin (100 nM)-stimulated glucose transport.

Adipocyte–Myocyte Coculture

Purpose and Rationale

There is growing evidence that paracrine/endocrine communication between adipose and muscle cells may be involved in the induction of insulin resistance during the pathogenesis of type II diabetes. This negative cross talk, which may be mediated by soluble factors secreted by the adipocytes (e.g., adipokines), can be mimicked in vitro by using a coculture based on human primary skeletal muscle and human primary adipose cells as has been recently introduced by Eckel and coworkers (Dietze et al. 2002).

Procedure

Culture of Human Skeletal Muscle Cells

Satellite cells are isolated from M. rectus abdominis by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination

with fibroblasts. After two passages, the myoblasts are characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100 % confluence (8 days). Primary human skeletal muscle cells of four healthy donors are usually supplied as proliferating myoblasts (5 × 105 cells). These cells are grown up to confluence in 25-cm^2 flasks, trypsinized, and subsequently seeded in 175-cm^2 flasks at a density of 1 × 106 cells. After two passages, 5–7.5 × 107 cells are harvested and stored until further use as frozen aliquots containing 2 × 106 myoblasts. For an individual experiment, myoblasts are seeded in 6-well culture dishes (9.6 cm^2/well) at a density of 105 cells per well and are cultured in α-MEM/Ham's F-12 medium containing "Skeletal Muscle Cell Growth Medium Supplement Pack" to up to near confluence. The cells are then differentiated and fused by culture in α-MEM for 4 days.

Isolation and Culture of Human Adipocytes

Adipose tissue samples are obtained from the mammary fat of normal or moderate overweight women (BMI 20–30 kg/m^2, 20 and 50 years) undergoing surgical mammary reduction. All subjects should be healthy and have no evidence of diabetes according to routine laboratory tests. Adipose tissue samples are dissected from other tissues and minced into pieces of about 10 mg in weight. Preadipocytes are isolated by collagenase digestion as described (see section "Primary Rat Adipocytes"). Isolated cell pellets are resuspended in DMEM/Ham's F-12 medium supplemented with 10 % FCS, seeded on membrane inserts (3.5 × 105/4.3 cm^2) or in a 6-well culture dish, and kept in culture for 16 h. After washing, culture is continued in an adipocyte differentiation medium. After a period of 15 days, 60–80 % of seeded preadipocytes develop into differentiated adipose cells as defined by cytoplasm completely filled with small or large LD.

Coculture

Coculture of human fat and muscle cells is conducted according to a recently published protocol (Dietze et al. 2002; Dietze-Schroeder et al. 2005). Briefly, after in vitro differentiation of preadipocytes on membrane inserts, the adipocytes are washed once with PBS and then incubated for 24 h in skeletal muscle cell differentiation medium containing 1 pM insulin. Thereafter, adipocytes are washed twice with PBS, and individual membrane inserts are subsequently transferred to the culture plates containing differentiated (4 days) myocytes in α-MEM containing 1 pM insulin. This results in an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert. The typical distance from the bottom of the culture dish to the membrane is 0.9 mm. Coculture is conducted for 48 h.

Evaluation

Integrity of both cell types is routinely checked by light microscopy at the end of the coculture period. Following coculture, the effect of insulin or compounds/drugs is studied as described below for normal myocytes and adipocytes.

Adipocytes Derived from Mouse Embryonic Fibroblasts (MEF)

It is often useful to study the effects of insulin/compounds/drugs in adipocytes derived from knockout (KO) mice deficient for one (or several) gene(s) relevant for adipocyte differentiation or function. This may lead to the confirmation/exclusion of the affected gene (product) in the molecular mechanism of insulin/compound/drug action on the regulation of lipid metabolism or differentiation in adipocytes. For this, MEF from the KO mice are isolated and differentiated to adipocytes in vitro. Stable lines of MEF adipocytes are generated from 12.5- to 14.5-day embryos of wild-type or homozygous KO mice as described. Selected MEF cells are cultured in DMEM with 10 % FBS. At confluence, cells are exposed to a pro-differentiation regimen 1 μM dexamethasone, 5 μg/ml insulin, 0.5 mM IBMX, and 5 μM rosiglitazone. After 2 days, cells are maintained in a medium containing insulin for 4 days and then in a medium without insulin for an additional 3–4 days before use.

Brown Adipocytes, Primary Culture, and Immortalization

Brown fat precursor cells are isolated from the interscapular brown adipose tissue of 5–6-week-old C57BL/6 wild-type, KO, or transgenic mice. Cells are cultured in a maintenance medium (DMEM, 10 % FBS, 10 mM HEPES, 100 nM sodium selenite, 3 nM insulin, 15 mM ascorbic acid, 50 μg/ml tetracycline, 50 μg/ml streptomycin, 50 μg/ml ampicillin, 1 μg/ml Fungizone [Invitrogen]). Precursors are seeded at 15–20,000 cells/cm^2 in 12-well plates or in 35-mm coverslip bottom dishes for biochemical and cell biological assays. After they attained confluence (d 0), cells are cultured for 2 days in a differentiation medium (DMEM, 0.5 mM IBMX, 0.5 mM dexamethasone, 125 μM indomethacin) after which they are maintained in a maintenance medium for an additional 3 days before use.

Purpose and Rationale

To prepare primary brown preadipocytes for immortalization which may be very helpful for metabolic studies relying on knockout mice, the following five protocols are used (Fasshauer et al. 2000, 2001).

Procedure

The following reagents are required: primary cell culture medium (500 ml) consisting of 385 ml of glucose (DMEM-high) (450 mg/dl glucose), 100 ml of fetal bovine serum (final conc. 20 %), 10 ml of 1 M HEPES/KOH (pH 7.4, final conc. 20 mM), and 5 ml of 100× penicillin/streptomycin (Life Technologies) and isolation buffer (100 ml) consisting of 4.1 ml of 3 M NaCl (final conc. 123 mM), 3.25 ml of 0.154 M KCl (final conc. 5 mM), 1.3 ml of 100 mM $CaCl_2$ (final conc. 1.3 mM), 10 ml of 50 mM glucose (final conc. 5 mM), 10 ml of 1 M HEPES/KOH (pH 7.4, final conc. 100 mM), 70.35 ml of H_2Obidest, 1.0 ml of 100× penicillin/streptomycin (filter sterile, 0.2 μm, Life Technologies), and bovine serum albumin (final conc. 4 %, Fisher, fraction V, catalog # BP 1605-100), stored at −20 °C in 100-ml aliquots.

Primary Culture

100 ml of isolation buffer is thawed using a 37 °C water bath, supplemented with collagenase (final conc. 1.5 mg/ml, type A, Roche Biochemicals, catalog #103 578), and vortexed. Primary cell culture medium (which includes 20 mM HEPES and 20 % FBS) is warmed up. Interscapular brown fat pad is removed from mice (age: late fetal to postnatal day 2). The tissue is dissected and minced on Parafilm with 500 μl PBS (sterile) using sterile pipette tips and a Parafilm indented in the center (so PBS will stay in the center) and then transferred into a 500 μl collagenase solution in Eppendorf vials (total volume now about 1,000 μl). After vortexing for 10 s, Eppendorf vials are put into a shaking water bath at 90 cycles/min for 30 s and vortexed for 10 s every 5 min. The digestion procedure should not exceed 40 min. Subsequently, the digested tissue is filtered through 100-um filters (Labcor Products, Inc., P.O. Box 7277, Gaithersburg, Maryland, 20898-7277, catalog # 30-1) or sterile Nytex membrane filter (38 × 38 mm, 100 μm, 50/pkg, individually wrapped, order from: PGC Scientifics, 7311 Governors Way, Frederick, Maryland, 21704, 800-424-3300, catalog # 358–201) into autoclaved Eppendorf vials. The vials are centrifuged at 1,500 rpm (Eppendorf centrifuge) for 5 min at 25 °C. During the centrifugation, 2 ml prewarmed culture medium is added to 12-well plates. The supernatant (use 1-ml automatic pipette) is removed. The pellet is resuspended in 1 ml of culture medium per sample (use 1-ml automatic pipette) and transferred into a 12-well plate. The cells are left overnight to get plated (day 1). On the next day, the cells are washed with 3 ml of culture medium and supplemented with 3 ml of fresh culture medium (day 2) (the cells are sensitive to cold and aspirating).

Immortalization and Establishing Cell Lines

After cells are plated overnight (day 1), the cells are washed on the next day (day 2) and then on day 3 supplemented with virus (pBabe SV40 large T antigen puromycin; for preparation of viral stock, see below). For this, a virus solution consisting of 50 % primary culture medium, 50 % viral stock, and 1× polybrene/

hexadimethrine bromide (Sigma, catalog # H-9268, 8 mg/ml stock solution dissolved in H_2O (bidest), final conc. 4 μg/ml, 1:2,000 dilution) is added up to approximately 0.9 ml of total volume containing primary culture media, viral stock, and polybrene per well. Thus, for three wells, 2.7 ml of primary culture media, 2.7 ml of viral stock, and 2.7 μl of polybrene stock are mixed by pipetting and then sterile filtered using a 0.45-μm filter. Then, for each well, the media are pipetted off without using a vacuum (vacuum will disrupt cells too much). Each well is rinsed well with 2 ml of prewarmed primary culture medium, which is then pipetted off. Subsequently, virus solution is added very slowly. On the next day, the cells are splitted into 15-cm dishes. Thereafter, the virus solution is pipetted. Each well is rinsed well with 2 ml of DMEM-high (without FBS), which is then pipetted off. 0.2 ml of trypsin is added to each well for 3 min (until the cells just come off the plate). After supplementation of 2 ml of primary culture medium, the cells are transferred to 15-cm dishes containing 25 ml of primary culture medium. Two days later, puromycin (Sigma, catalog # P 8833) selection is started (2 μg/ml puromycin into DMEM-high containing 20 % FBS). The puromycin medium is left for 1 week (at this point, some cells can be split off to differentiate or do some experiments with). After puromycin selection, cells need to be put into a medium directed against mycoplasma infection (Cyclin, Roche Biochemicals, catalog # 799 050). This will take approximately 2–3 weeks. Once cells have been in puromycin and mycoplasma selection, they can be frozen down.

For the production of SV40 large T antigen, Pharmacia's Cell Phect protocol with slight modifications (Cell Phect Transfection Kit, Pharmacia Biotech, Cat. # 27-9268-01) is used. For this, ϕNX cells (see below) are grown in 15-cm plates to 50–70 % confluency. 15 μg of SV40T-pBabe-puro is dissolved in 480 μl of distilled water. 480 μl of buffer A is added. After brief vortexing and incubation for 10 min at room temperature, 960 μl of buffer B is added with blue pipette tip while at the same time the solution is "bubbled" with an automatic pipette. After incubation for 15 min at room temperature, the precipitate is added to the ϕNX cell culture (in 15-cm plates, 50–70 % confluency) as evenly as possible. Distribution is supported by rotating the dish in a figure eight configuration a few times. On the next morning (i.e., 10 a.m.), the medium is aspirated, and the cells are rinsed carefully twice with 25 ml of DMEM-high (no FBS, at room temperature) and, subsequently, 25 ml of DMEM-high containing 10 % FBS to the cells. On the same day (i.e., 4 p.m.), the medium is changed to 25 ml of fresh DMEM-high containing 10 % FBS at room temperature. On the next evening (24 h later, i.e., 4 p.m.), the viruses are harvested for the first time. For this, the supernatant is aspirated with a 50-ml sterile syringe equipped with an 18G sterile needle. The needle is removed and a 0.45-μm filter is placed in front of the syringe. The supernatant is pressed through the filter into a 50-ml blue top tube. Subsequently, polybrene (4 μg per ml) is added to the supernatant. After vortexing, 1-ml aliquots are transferred into sterile freezing vials and stored in a −80 °C freezer. Finally, 25 ml of DMEM-high containing 10 % FBS is added to the ϕNX cells. On the next evening (24 h later, i.e., 4 p.m.), the second harvest is performed exactly like the first one.

For handling of ϕNX cells, the following notes are given: (i) The cells are grown in DMEM-high containing 10 % FBS at 5 % CO_2. (ii) The cells grow rapidly. (iii) The cells come off of the dish very easily so washing/feeding has to be performed carefully. (iv) The cells are frozen in 90 % FBS containing 10 % dimethly sulfoxide. (v) It is recommended that the cells are not allowed to reach confluence and that they are periodically grown in selection media to maintain their retroviral packaging "goodies." As ϕNX cell selection medium DMEM-high containing 10 % FBS, 300 μg/ml hygromycin B, and 1 μg/ml diphtheria toxin is recommended. (vi) The cells need to be in the selection for only about 4–7 days, then they have to be placed back into a regular medium and allowed to "recover" to their normal morphology before using and/or freezing them. Additional information and more details regarding the ϕNX cells can be found on the Nolan Lab homepage at www.Stanford.edu/group/Nolan/.

Culturing Brown Preadipose Cells

The following media are required: DMEM-high containing 10 % FBS, DMEM-high lacking FBS (for rinsing cells), DMEM-high lacking FBS (for starving cells), DMEM-high containing 10 % FBS and T3 (for differentiation), and induction medium (see below). For the preparation of the differentiation medium, 450 ml of DMEM-high containing 10 % FBS in a 500-ml filter bottle is supplemented with 50 ml of FBS. Then 10 µl of 1 mM human insulin and 50 µl of 10 µM T3 (Sigma) are added. For sterile filtration, 0.2-µm filters are used. For the preparation of the induction medium (prepared freshly only on the day of use), 200 µl of 0.125 M indomethacin (1,000×, Sigma) and 200 µl of 2 mg/ml dexamethasone (1,000×) are mixed and vortexed and then supplemented with 400 µl of 0.25 M IBMX (500×, Sigma). All three are dissolved in 100 % ethanol and stored frozen at −20 °C. Indomethacin and IBMX must be heated to 75 °C (using a heating block).

For a typical differentiation time course, on day 1 cells are split into differentiation medium; on day 4 the confluent cells are induced; on day 6 the cells are put back into differentiation medium; on day 8 the old medium is aspirated off, and fresh differentiation medium is added; on day 10 the fully differentiated cells are ready to be used for Oil Red O staining, RNA/DNA extraction, Western analysis, etc.

As a master plate, one large dish of 15 cm is kept always in DMEM-high containing 10 % FBS. For splitting, confluent cells are subjected to a 1:20 split (i.e., 1 ml cells plus 25 ml medium will reach confluence in 3 days). Subsequently, the medium is aspirated off. Then the cells are rinsed using DMEM-high lacking FBS and a 25-ml pipette filled to top. Each master plate is rinsed with 18 ml of FBS. 2.5 ml trypsin is added to each plate (always using new pipette). The plates are put back into CO_2 for at least 20 min. Next, the kind and number of plates needed have to be fixed: 6-well plate gets 4 ml of differentiation medium plus 12 drops of cells, 12-well plate gets 2 ml of differentiation medium, 10-cm dish gets 8 ml of differentiation medium plus 3 ml cells, and 15-cm dish master plate gets 25 ml of DMEM-high containing 10 % FBS.

After labeling of the plates, the trypsinized master plate is supplemented with 25 ml of DMEM-high containing 10 % FBS. After pipetting up and down three times to disperse cells and break up clumps, 1 ml is added into the master plate, 12 drops into 4 ml differentiation media and 6-well plate, and 3 ml cells into 8 ml of differentiation media and 10-cm dish. While plating out one cell type, others can remain in trypsin in the hood. When "splitting" of the cells has been finished, the master plate and 10-cm dish are taken and the cells "distributed" evenly by moving dishes in a "figure eight" configuration ten times (for 6-well plates a "faster" figure eight is used). Cells are frozen in DMEM-high containing 20 % FBS and 10 % DMSO.

For induction of the adipocyte phenotype, the differentiation medium is aspirated off, and 11 ml of induction medium per plate is added without any rinsing. The adipocyte phenotype is monitored on the basis of Oil Red O staining of some "control" plates.

Oil Red O Staining

This protocol is used to test for lipid accumulation in fully differentiated cells. A stock solution of Oil Red O is required: 0.5 g Oil Red O (Sigma) in 100 ml isopropanol. Prepare Oil Red O working solution (make fresh each time, as working solution is unstable) by adding 6 ml stock to 4 ml sterile H_2Obidest and then mix and filter through Whatman #1 filter paper. (The filtration step takes a while; it is not advisable to hasten the process by applying a vacuum because there is too much stuff to filter out.) About 5 ml per 10-cm plate to be stained is required.

The plates are rinsed with PBS. Cells are fixed by covering with PBS containing 10 % formaldehyde or with commercially available buffered formalin (Sigma). The plates were incubated for at least 15 min to up to overnight at room temperature. Alternatively, the plates can be left at 4 °C until multiple plates are collected. Subsequently, the fixation agent is removed and Oil Red O stain is added. After incubation for 1 h (or longer) at

room temperature, the plates are carefully rinsed several times with H2Obidest to remove excess stain and any precipitate that forms. The dishes are maintained in H_2O, if intact cells are desired. H_2O is aspirated and the dishes are allowed to air-dry.

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Conditionally Immortalized Cell Strains

Purpose and Rationale

Cell lines provide the cell count and homogenous populations for throughput applications, but do not behave as a primary cell can. It is well accepted that primary cells behave more normally than cell lines. Yet, key limitations exist that preclude one from using primary cells during initial phases of drug discovery. The limitations include cost, homogeneity of cell population and total cell count achievable, donor-to-donor variability, and loss of key cellular behaviors when the cell is outside its home organ.

Conditionally immortalized cell strains are primary cells that behave normally in cell culture and can be expanded beyond 40 population doublings, yet maintain normal behaviors throughout the culture time. This is achieved by combining the telomerase rejuvenating effect with a specifically modified temperature-sensitive large T (ts LT) antigen in the same cell. The ts LT mutant codes for a gene product that is functional at 33 °C but nonfunctional at 37 °C. Though the construct overcomes the cells' natural senescence behavior at low passage and provides a strong proliferative signal, the cells are not able to exhibit a full range of differentiated functions. By switching the culture conditions to 37 °C, one stops cell proliferation, and the cells are responsive to media growth factors that drive differentiation. If the line is carried at 37 °C for sufficient time, the strain permanently loses its ability to proliferate. A second mutation in the construct controls SV40's ability to auto-excise. Consequently, this mutant conveys a higher level of karyotype stability to the host genomic background over multiple population doublings. Telomerase, also called telomere terminal transferase, elongates chromosomes by adding TTAGGG sequences to the end of existing chromosomes, thereby preventing chromosomes from losing base pair sequences at their ends and from fusing to each other. However, each time a cell divides, some of the telomere is lost (25–200 bp per division). When the telomere becomes too short, the chromosome reaches a critical length and can no longer replicate. At this point cells senescence and may be killed by apoptosis. In somatic and aging cells telomerase activity is usually very low. If telomerase is activated in the cell, the cell will continue to grow and divide. Consequently, the conditionally immortalized cell strain with active telomerase acts like a primary cell but provides homogenous cell populations at the high cell counts of cell lines.

Experimental Procedures

Sequential retroviral (MMLV-based vector possessing the neomycin selection marker) transfection at low passage using ts LT (u197sA58), followed by the telomerase construct hTERT, leads to highly proliferative strains which are highly responsive to differentiation. The conditional immortalization allows at a permissive temperature production of large uniform cell populations while active telomerase is reversing telomere shortening. Concurrently, at the permissive temperature, ts LT is actively driving cell proliferation while maintaining karyotype stability over >40 population doublings. By increasing the culture temperature, the cells stop dividing.

One then converts to a differentiation medium so that the cells can express normal differentiated function and phenotype once again.

Conditionally Immortalized Human Skeletal Muscle Cells

The XM13A1 cell line introduced from Cambrex, Clonetics Bio Science Walkersville Inc., contains a mixed cell population derived from human skeletal muscle of normal 30–40-year-old female subjects. These cells can finally be differentiated from myoblasts into well-developed myotubes (e.g., in 96-well plates) that have morphological and phenotypic characteristics of differentiated muscle. These include creatine kinase activity and stimulation (two- to 2.5-fold above basal) of glycogen synthesis by 0.1–1 μM insulin up to passage 21. Other markers of differentiation found include myogenin, GLUT4, uncoupling protein-3, and GS. Functional activities include stimulation of glycogen synthesis by LiCl and IGF-1, stimulation of glucose transport by AICAR, and induction of insulin resistance by glucosamine (see section "Insulin-Resistant Primary Rat Adipocytes") or fatty acids.

Conditionally Immortalized Human Preadipocytes

The XA15A1 and XM18B1 cell lines introduced from Cambrex, Clonetics Bio Science Walkersville Inc., represent mixed cell populations derived from subcutaneous adipose tissue of normal 31–40-/60-year-old male subjects. Both strains are highly responsive, given the correct media conditions (even in the absence of PPAR-γ ligands glitazones), to differentiate from the primary normal human preadipocytes into functional mature adipocytes after more than 40 population doublings in culture. Markers of differentiation found include glycerol-3-phosphate dehydrogenase and aP2. The differentiated cells secrete leptin and adiponectin into the culture medium and exhibit insulin stimulation of glycogen synthesis (up to twofold with EC50 of 10–50 nM) and of glucose transport (up to 2.5-fold at 1 μM).

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Liver and Hepatocytes

General Considerations

In type II diabetic patients with moderate fasting hyperglycemia, the liver production of glucose is increased by about 50 g/day above normal (DeFronzo et al. 1992). This modest increase is the consequence of reduced suppression of hepatic glucose production by insulin (Firth et al. 1987) and could be nullified by reducing dietary carbohydrate by 50 g/day. The increased hepatic glucose production in type II diabetes is probably caused by a combination of lack of an insulin-mediated reduction in glucagon secretion (Müller et al. 1970) and hepatic resistance to insulin action at the level of both the insulin signaling transduction cascade and its coupling to the metabolic end effector enzymes of gluconeogenesis. The importance of increased hepatic glucose production is underlined by the fact that when phosphoenolpyruvate carboxykinase (PEPCK), the key rate-limiting regulatory enzyme of gluconeogenesis, is overexpressed in mice carrying a transgene expressing the protein, hyperglycemia results (Valera et al. 1994). Independent of whether increased hepatic glucose production plays a primary or only secondary role in the pathogenesis of human type II diabetes, analysis of insulin signaling and metabolic action in the liver is important for gaining a complete picture of the pathophysiology of type II diabetes. The model of the isolated perfused rat liver offers advantages in determining insulin action, which, compared to primary and cultured liver cells, are the intact tissue, the near physiological function of the liver, and, compared to the in vivo situation, the separation from other effects which may also affect hepatic metabolism, like basal glucagon secretion from α-cells, increased sympathetic activity, and/or hypothalamic effects (Nonogaki 2000). Taken together, liver cells harbor a number of important targets for compounds/drugs with insulin-like and antidiabetic activity, which can be assayed using the intact liver and primary or cultured hepatocytes of human and rodent origin.

Perfused Rat Liver

Male Wistar rats weighing 200–250 g are anesthetized with 150 mg/kg hexobarbital intraperitoneally. After opening the abdomen, two ligatures are tied around the stomach, one around the esophageal end to include the adjacent blood vessels and the other around the pylorus. The stomach is removed between these ligatures, a procedure that facilitates the remaining dissection and the subsequent removal of the liver from the animal. A ligature is placed around the bile duct. The portal vein is tied and cannulated with PP10 tubing. The thorax is opened and the vena cava is cannulated via the right atrium. The lower inferior vena cava is tied and the liver is removed from the animal. From the portal vein, the liver is washed with 100 ml heparinized (5 IU/ml) physiological saline solution at 37 °C for 3 min while the outflow occurs via the vena cava. The preparation is then transferred to a perfusion apparatus, where the portal vein cannula is attached to tubing containing the oxygenated medium.

KRBH with 25 % bovine erythrocytes, 1.6 % BSA, and 22.5 mM Na-L-lactate is used as perfusion medium. The perfusion rate is 30 ml/min. 70 ml of this solution is used for recirculation over 2 h. The test compounds/drug candidates are added in a concentration of 40–100 μM to the perfusate medium. The central element of the perfusion apparatus is a gastight, thermostatized, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas tube of 18-cm length and 10-mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer safety joint. The suction filter for perfusion of the liver has an internal diameter of 95 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 μm). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 μl/ml 0.1 % Genapol PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

Primary Rat Hepatocytes

Sprague–Dawley rats (100–200 g) are fed ad libitum prior to the hepatocyte isolation. Hepatocytes are isolated from rat livers thoroughly perfused with buffer containing collagenase, hyaluronidase, and trypsin inhibitor as described by Van den Berghe and coworkers (1980). The rat livers are perfused for 4 min at 25 ml/min flow rate with perfusion buffer and then with collagenase buffer until the digestion is complete (~6 min). At the end of the perfusion, connective tissue and large blood vessels are removed. The hepatocytes are then passed through a 100-μm nylon mesh sieve. The cells are washed twice with 125 ml of wash buffer and collected by centrifugation (50 × g, 2 min). The hepatocytes are suspended in a plating medium (DMEM, 10 % FBS, 100 nM insulin, 25 nM dexamethasone, 6.3 μg/ml transferrin, 22 μg/l gentamicin) and passed through a 100-μm nylon mesh sieve. Cells (1.6 × 105) are plated in 48-well cell culture plates for the various metabolic assays.

Cultured Human Hepatocytes

HepG2/C3A (ATCC) human hepatoma cells are cultured in minimal essential medium (α-MEM, GIBCO) supplemented with 10 % FBS, 100 μM nonessential amino acid mix, 1 mM sodium pyruvate, and 1 mM L-glutamine and are seeded in 48-well plates at 1.4 × 105 cells per well 3 days prior to the experiment.

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Muscle Tissue and Myocytes

General Considerations

Muscle cell lines have been used to study skeletal muscle glucose metabolism in response to acute insulin stimulation. Subcellular fractionation followed by immunoblotting as well as photoaffinity labeling followed by immunoprecipitation has demonstrated that in cultured muscle cells, insulin causes rapid translocation of the glucose transporter isoform, GLUT4, to the plasma membrane as has been well established for primary and cultured adipocytes. Over decades, numerous studies have demonstrated that insulin affects many signaling events (e.g., IR and insulin receptor substrate tyrosine phosphorylation) as well as metabolic end effector systems (e.g., glucose transport and glycogen synthesis) in similar fashion. Despite the considerably higher complexity in gaining access to insulin-responsive cells/tissues from muscle compared to adipose tissue, the eminent physiological role of the human skeletal muscle as the predominant acute and short-term storage site for the daily carbohydrate intake necessitates the use of adequate cell- and tissue-based muscle model systems for assaying the insulin-like activity of compounds/candidate drugs. Moreover, these systems may be helpful for the analysis of the molecular mechanisms of muscle insulin resistance and of its abrogation by putative insulin-sensitizing compounds/drugs. According to recent findings culminating in the "lipotoxicity hypothesis" (see K.6.3.4), peripheral insulin resistance seems to be correlated to the deposition or loss, respectively, of TAG or its precursors/degradation products in the cytoplasm of skeletal muscle cells, the so-called intramyocellular lipids. Thus, cell- and tissue-based muscle assay systems are also prerequisite for the analysis of myocellular lipid metabolism.

Perfused Rat Hind Limb

Female Wistar rats weighing 170–230 g are starved 48 h before the experiment. They are anesthetized by IP injection of 50 mg/kg pentobarbital. After a midline incision, the skin is reflected and the superficial epigastric vessels are ligated. The abdominal wall is then incised from the pubic symphysis to the xyphoid process using electrocautery. After ligation of the uterine, ovarian, and inferior mesenteric arteries, the upper half of the uterus, the ovaries, and part of the descending colon are excised, together with adhering adipose tissue. Next, branches of the hypogastric and pudoepigastric trunks supplying pelvic viscera are ligated. Ligatures are also placed around the neck of the bladder and the residual portions of the uterus and descending colon. Adipose tissue in the perineal and retroperitoneal regions is removed.

Two pairs of ligatures are placed around the aorta and the vena cava, one just above the origin of the iliolumbar vessels and the other above the origin of the renal vessels. The inferior epigastric, iliolumbar, and renal vessels are then ligated as are the coeliac axis and the portal vein. A ligature is also placed around the tail. The ligatures previously placed around the vena cava and aorta above the origin of the renal vessels are then tied. The aorta is incised between the left renal

and iliolumbar vessels, and a no. 18 polyethylene catheter filled with 0.85 % NaCl containing 200 units of heparin/ml is introduced, passed to a point midway between the iliolumbar vessels and the aortic bifurcation, and after flushing with heparin–NaCl solution finally tied in place. The vena cava is cannulated with a no. 16 needle which is secured in a position so that its tip is at the same level as the aortic catheter. The needle is connected with a transparent vinyl tubing. The preparation is then transferred to a perfusion apparatus, where the aorta cannula is attached to a tubing containing the oxygenated medium.

KRBH with 25 % bovine erythrocytes, 4 % BSA, and 10 mM D-glucose is used as a perfusion medium. The perfusion rate is 8 ml/min. 70 ml is used for recirculation over 2 h. The test compounds/drug candidate is added in a concentration of 40–100 μM to the perfusate medium. The central element of the perfusion apparatus is a gas-tight, thermostatized, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas tube of 18-cm length and 10-mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer safety joint. The suction filter for perfusion of the hind limb has an internal diameter of 145 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 μm). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 μl/ml 0.1 % Genapol PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

Rat Diaphragms

Sprague–Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia, and the diaphragms still attached to the rib cages are carefully removed, released from the rib cages and adhering connective and fat tissues, washed in PBS, spread out, and divided into two equal pieces as described by Müller and coworkers (1994). For assaying the effects of insulin/compounds/drugs, the hemidiaphragms are incubated in KRH buffer gassed with carbogen (95 % O_2/5 % CO_2) in the presence of 5 mM glucose or other ingredients as indicated for the specific assays. In some cases, diaphragms attached to the rib cage instead of hemidiaphragms are used to prevent spontaneous contraction to less than physiological length.

Rat Soleus and Extensor Digitorum Longus

In studies of soleus muscle and extensor digitorum longus (EDL), the hindquarters are perfused for 3 min (25 ml/min) to wash out the blood. The two soleus or EDL muscles from each rat are removed with tendons intact, placed in perforated baskets, and incubated in separate test tubes. Muscles from three rats are incubated together with insulin/compounds/drug candidates. The perfusing and incubation medium is KRB (pH 7.4) containing 8 mM glucose, 1 mM pyruvate, and 0.2 % (w/v) BSA. The incubation medium was gassed continuously with 95 % O_2/5 % CO_2. At the end of the incubation, muscles are freeze-clamped with aluminum tongs cooled in liquid nitrogen and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to further ensure that findings reflected the biology of muscle cells, these are before analysis in most experiments isolated from other tissue components by microdissection using a stereomicroscope after freeze-drying.

Human Muscle Strips

A human muscle preparation has been introduced successfully by Dohm and coworkers (1988). For this, immediately after the surgical incision, muscle strips (abdominal rectus muscle) are mounted at in vivo length by using one set of clamps, which

is 2.5-cm wide and constructed from two pairs of hemostats. The clamp is placed by the surgeon on the muscle, and a 0.5–1 g muscle piece is clamped, excised, and immediately transferred to oxygenated KRBH for immediate transfer to the laboratory. Muscle fiber strips, weighing between 20 and 50 mg, are dissected free from the mounted specimen, secured with a small Plexiglas support, and cut free. Particular care has to be taken to remove all visible fat. From one muscle piece, it is possible to obtain five to ten muscle strips. After preparation, the muscle strips are washed for 30 min in KRBH supplemented with 38 mM mannitol, 2 mM pyruvate, and 10 mg/L BSA. The viability of the muscle is usually investigated by analysis of 3–0-methylglucose transport (see below). Typically, insulin-stimulated glucose transport follows a concentration-dependent fashion with the maximum effect of about twofold above the basal glucose transport rate (~1 μmol/ml • h).

Cultured Human Skeletal Muscle Cells

Human skeletal muscle cell cultures are handled as described by Aas and coworkers (2004). A cell bank of satellite cells is established from muscle biopsy samples of the vastus lateralis muscle from four healthy volunteers (age 25–30 years, BMI 22–25, fasting glucose and insulin within the normal range, and no family history of diabetes). The biopsies have to obtained with informed consent and approval by the National Committee for Research Ethics. Muscle cell cultures free of fibroblasts were established as previously described (Henry et al. 1995) with minor modifications. Briefly, muscle tissue is dissected in Ham's F-10 media at 4 °C, dissociated by three successive treatments with 0.05 % trypsin/EDTA, and then satellite cells were resuspended in skeletal cell growth medium with 2 % FCS and no added insulin. The cells are grown on culture wells coated with extracellular matrix gel (Gaster et al. 2001). After 2–3 weeks at about 80 % confluence, fusion of myoblasts into multinucleated myotubes is achieved by growth for 8 days in α-MEM with 2 % FCS. Hyperglycaemic medium can be made by the addition of glucose (to a concentration of 10 or 20 mM) to α-MEM with 2 % FCS. All cells used in the experiments should be at passage 3–6.

L6 Myotubes

Stock cultures of L6 rat skeletal muscle cells from the ATCC are grown in α-MEM containing 10 % (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and antimycotics (growth medium) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. For the experiments, stocks are trypsinized and the myocytes reseeded on 6-well cell culture plates or 60 × 15 mm Petri dishes at a density of 4,000 cells/cm^2. After 24 h (~80 % confluence), the medium is changed to α-MEM containing 2 % (v/v) FBS and antibiotics/antimycotics as described above (differentiation medium) that is replaced after 2, 4, and 6 days of culture. After 7 days, myotube differentiation is complete, and experimental procedures are initiated. In all experiments, L6 myotubes are serum-starved for 4 h before exposure to insulin/compounds/drugs. All controls are incubated with equal concentrations of the vehicle (e.g., DMSO) and the respective concentrations of fat-free albumin (e.g., BSA) as present in the treated cells.

L6 Myotubes Transfected with GLUT4

L6 myotubes express a detectable but rather moderate amount of the insulin-regulated GLUT4 which may account, in part, for the limited insulin stimulation of their glucose transport system. To increase the insulin responsiveness of glucose transport, L6 myocytes are transfected with myc-tagged GLUT4 (L6 GLUT4–myc cells) (Dr. Amira Klip, Division of Cell Biology, The Hospital for Sick Children, Toronto, Canada). After growth in 96-well tissue culture plates using 200 μl of α-MEM containing 2 % FBS, 100 U/ml penicillin G, and 100 mg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 8.5 % CO_2, myoblasts are seeded in a medium

containing 2 % (v/v) FBS at a density of ~2 × 104 cells/ml and used 6–8 days postseeding. Cells are fed fresh medium every 48 h and used at the stage of myocytes or after the differentiation to myotubes.

BC3H1 Myocytes

General Considerations

The BC3H1 cell line is a non-fusing spontaneously and reversibly differentiating mouse muscle cell line derived from a mouse neoplasm (Schubert et al. 1974). As for other cultured cell lines, the BC3H1 myocytes demonstrate intermediate characteristics, possessing electron-microscopic features of both smooth and skeletal muscle but with a nicotinic acetylcholine receptor and an action potential more characteristic of skeletal muscle (Standaert et al. 1984). Unfortunately, BC3H1 myocytes do not express GLUT4, and therefore, the limited insulin stimulation of glucose transport relies on the glucose transporter isoform 1, GLUT1, exclusively.

Procedure

BC3H1 myocytes are cultured to confluence in 100-mm dishes over 10–14 days in DMEM supplemented with 15 % "Process Serum Replacement-I" (Sigma), and 25 mM glucose is added 18 h before the experiment. Cells are rinsed and preincubated at 37 °C for 20 min in Dulbecco's PBS with 0.1 mM $CaCl_2$ and 1 mg/ml BSA, then treated with vehicle or compounds/drug candidates in vehicle for 30 min or overnight.

C2C12 Myotubes

Purpose and Rationale

C2C12 cell, a mouse skeletal muscle cell line, has been isolated from dystrophic mouse muscle by Yaffe and Saxel (1977), McMahon and coworkers (1994), and Ernst and White (1996). Subsequently, the myocytes have been shown to express a limited amount of GLUT4 and to be suitable for stable transfection experiments of exogenous cDNA, making this cell line a candidate for stable transfections of cDNAs that encode mutant skeletal muscle and cardiac protein isoforms.

Procedure

Monolayers of mouse C2Cl2 myoblasts (ATCC CRL-1772) are grown in Dulbecco's Modified High Glucose Eagle's Medium (DMEMH; 90 %/10 % (v/v) FBS/4.0 mM glutamine/50 μg/ml gentamicin, in a humidified incubator at 37 °C, 5 % CO_2 as has been described by Muoio and coworkers (1999). Cells are grown in 100-mm dishes, subcultured at 60–80 % confluence, and split at a ratio of 1:10 using trypsin (0.25 % (w/v) in MEM with 1.0 mM EDTA). Cells grown to 60 % confluence are subcultured at a ratio of 1:15 into 6-well dishes that had been coated with 0.01 % (w/v) collagen. When cells are 80 % confluent, myoblasts are induced to differentiate into myotubes by changing to low-serum differentiation medium (98 % DMEMH/2 % (v/v) horse serum/4.0 mM glutamine/25 mM HEPES/50 μg/ml gentamicin). Differentiation medium is changed daily. By day 5, cells are typically fully confluent and had differentiated into multinucleated, contracting myotubes.

Cardiomyocytes

Cardiomyocytes are isolated by perfusion of hearts from adult rats with collagenase according to established protocols (Eckel et al. 1983, 1991; Bähr et al. 1995).

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Pancreas and Pancreatic β-Cells

General Considerations

Concurrent with the obesity epidemic, the incidence of type II diabetes is increasing at an alarming rate. Type II diabetes arises when the endocrine pancreas fails to secrete sufficient insulin to cope with the metabolic demand, because of acquired β-cell secretory dysfunction and/or decreased β-cell mass. Insulin secretory dysfunction is well documented. Whether insulin secretory dysfunction is a cause or consequence of the disease (in response to chronic challenge for compensation of the peripheral insulin resistance) is still debated, but there is mounting evidence that it may be symptomatic of changes in β-cell mass. Although proposed 50 years ago, the hypothesis that β-cell loss plays an important role in the pathogenesis of type II diabetes has only recently come to the fore. β-cell mass in the adult is plastic, and adjustments in β-cell growth and survival maintain a balance between insulin supply and metabolic demand. For example, obese individuals who do not develop diabetes exhibit an increase in β-cell mass that appears to compensate for the increased metabolic load and obesity-associated insulin resistance. However, this β-cell adaptation eventually fails in the subset of obese individuals who develop type II diabetes. Indeed, most individuals with type II diabetes whether obese or lean show a net decrease in β-cell mass. Thus, type II diabetes is a disease of relative insulin deficiency. Given the pivotal role of β-cell mass in determining whether an individual will progress to type II diabetes, there is growing interest in understanding the mechanism that controls the life and death of β-cells. The molecular mechanisms involved in the decision between

β-cell life and death have been reviewed by Rhodes (2005). Furthermore, assay systems based on the isolated perfused pancreas, perifused islets, insulinoma cells, and cultured β-cells are required to study the effects of compounds/drugs on insulin secretion, β-cell growth, and β-cell survival.

Perfused Rat Pancreas

Procedure

Male Wistar rats (e.g., Hoe:WISKf, SPF 71 strain) weighing 200–250 g serve as donors. Prior to the experiment, the animals have free access to food and water. The pancreas is removed under pentobarbital (50 mg/kg IP) anesthesia. The mesenteric artery is doubly ligated and cut, and the entire intestine below the duodenum is separated and removed from the rat to simplify the exposure. The esophagus is ligated as high as possible and cut above the ligature. A loose ligature is placed, but not tied, around the entire gastrohepatic ligament. The aorta is cautiously exposed through the crura of the diaphragm well above the point of origin of the celiac axis. The ligature around the gastrohepatic ligament is then tied as tightly as possible and the ligament cut above the ligature. The aorta is cut between the double clamps and below the lower clamp, and the preparation is lifted out from the abdomen. The clamps are removed from the aorta which is slit open on the side opposite to the origin of the celiac axis, revealing the opening of the latter. The arterial cannula is inserted and tied in place. An opening is made close to the ligature, as near as the end of the portal vein as possible.

Evaluation

Circulation through the preparation is initiated, and fluid is observed to flow from the slit in the portal vein. After a minute or two, the flow is stopped and a cannula is inserted into the portal vein and tied in place. Flow is then resumed at a perfusion pressure of about 100 mmHg. Carbogenated Krebs-Ringer bicarbonate buffer with 2 % BSA and 5.5 mM glucose is used as perfusion medium at a temperature of 37.5 °C with a perfusion rate of 1.75 ml/min. The perfusate is collected every minute for 30 min. After 5 min perfusion with 5.5 mM glucose, the test compound is added until the 15th min (concentrations between 0.05 and 0.5 mM for highly active substances), followed by perfusion with 5.5 mM glucose and finally with 16.6 mM glucose. The perfusate medium samples are stored at −20 °C until further processing.

Perifused Islets

Male Wistar rats weighing 200–250 g serve as donors. Before the study, the animals have free access to food and water. Two donor rats are used for each test. The pancreas is removed under pentobarbital anesthesia. The islets are obtained by the collagenase (e.g., collagenase type IV, Worthington) method and collected under a stereomicroscope. In each test, up to ten chambers each with 15 islets are perifused. Cutoff Microfuge tubes, sealed with Tuohy-Borst adapters, serve as perfusion chambers. Two thick-walled, small-diameter Teflon catheters are passed through the adapter into the chamber. One of the catheters extends to the bottom of the chamber and acts as the perifusate inlet; the other extends to the lower edge of the adapter cone and acts as the outlet. The latter is connected to a multichannel peristaltic pump which delivers the perifusate to a fraction collector. The chamber volume is 0.15 ml. The perifusate flow rate is 0.1 ml/min. The perifusate consists of a carbogenated Krebs-Ringer bicarbonate buffer with 1.0 mM glucose, 0.25 % BSA, and 5 mM theophylline. The storage vessels for the perifusate, the chambers, and the inlet catheters are immersed in a water bath of 37 °C.

Evaluation

After a pre-perifusion phase of 1 h, the perifusate is collected every min for 46 min. From the 2nd until the 18th min, the test compound is added at concentrations between 0.1 and 2.5 μM, and from the 34th to the 46th min, the glucose concentration is raised to 20.0 mM. The hormones – insulin, glucagon, and somatostatin – are determined by

using a commercially available RIA kit or ELISA kit.

Insulinoma Cells

Purpose and Rationale

Chick and coworkers (1977) described a transplantable insulinoma in the rat which was originally observed as a primary tumor in the pancreas of an old male NEDH albino rat being previously irradiated during a parabiosis experiment. Fragments of this tumor were transplanted to young NEDH rats in many passages inducing severe hypoglycemia in host rats. Gazdar and coworkers (1980) reported the establishment of a continuous cell line of a rat islet cell tumor which secretes primarily insulin and some somatostatin. Bhatena and coworkers (1982) studied insulin, glucagon, and somatostatin receptors on cultured cells and clones from rat islet cell tumor.

Procedure

The insulinoma is transplanted when the tumor has reached a diameter of about 2 cm and the carrier animal exhibits distinct manifestations of insulin excess. The tumor is removed, cut open, and mixed briefly in a mortar, and 1/10th to 1/20th is injected between the shoulder blades of another animal. No metastases are observed. The histological examination also shows a nonmalignant adenoma. The tumors grow only in rats of the strain NEDH (New England Deaconess Hospital).

Cultured β-Cells

General Considerations

Reliable β-cell models are of paramount importance for diabetes research (see above). It is generally accepted that the use of primary cells is preferable. However, this requires large quantities of isolated pancreatic islets, which is work intensive and has the inherent inconvenience of representing a mixed population of β-, α-, δ-, and F cells. Consequently, rodent β-cell lines have proven their usefulness, and their continuous development is still essential until clonal human β-cells become available. The intrinsic challenge when establishing a β-cell line is the maintenance of tissue-specific differentiation combined with adequate cell proliferation. As a result, only a limited number of β-cell lines are available to date, all of rodent origin. Several insulin-secreting cell lines are reported in the literature (review by Poitout et al. 1996). The most widely used are RINm5F, βHC9, βTC6-F7, and, more recently, MIN6. Among the mouse-derived β-cell lines (Miyazaki et al. 1990; Knaack et al. 1994), MIN6 cells (Miyazaki et al. 1990; Ishihara et al. 1993) represent a valuable model, which was further improved by isolation of the clonal subline MIN6m9 (Minami et al. 2000). However, MIN6 cells exhibit secretory responses to pyruvate, which is not a secretagogue for normal islets (Skelly et al. 1998). Mouse βHC9 grows very slowly and is thus difficult to study. Of rat origin, RINm5F cells are poorly differentiated, have low insulin content, and do not respond to glucose in the physiological concentration range (Halban et al. 1983; Praz et al. 1983; McClenaghan et al. 1998), and BRIN-BD11 cells are poorly differentiated, exhibiting low insulin content and weak secretory responses to glucose (McClenaghan and Flatt 1999). Finally, loss of differentiated features as a function of time in tissue culture has been reported for several rodent cell lines, including RIN1046-38 and βTC6 (Clark et al. 1990; Ferber et al. 1994). Genetic engineering of RIN1046-38 cells results in clones with stable glucose responsiveness but with maximal insulin secretion occurring at subphysiological glucose concentrations because of a high level of low-Km hexokinase activity in these cells (Ferber et al. 1994; Hohmeier et al. 1997). Stable glucose responsiveness has also been reported for β-TC cells after clonal selection in soft agar (Knaack et al. 1994), but even these cloned cell lines (e.g., βTC6-F7) appear to lose glucose responsiveness after prolonged tissue culture (Zhou et al. 1998). Currently, clonal INS-1 cells (e.g., INS-1–832/13 or 1E; Hohmeier et al. 2000) appear to represent the most attractive vehicles for studying β-cell function.

Procedure

RINm5F

Insulin-producing cells from the RINm5F cell line are grown in RPMI medium containing 10 % (v/v) heat-inactivated FCS, 50 IU/ml penicillin, 0.25 μg/ml amphotericin B, and 50 μg/ml streptomycin at 37 °C in an atmosphere of humidified air/CO_2 (19:1) (Praz et al. 1983). The cells are seeded at a density of 3.5 × 104 cells/ml in 20 ml of medium (75-cm^2 culture flasks). The medium is replaced four times a week (one passage). Thereafter, the cells are treated with trypsin (0.02 % trypsin in 0.9 % NaCl/0.2 mM EDTA) for 2–5 min at 37 °C. The trypsin-treated cells are diluted, reseeded at a density of 2 × 106 cells per 75-cm^2 culture flask, and grown to 70 % confluency.

For single-cell patch clamp studies, RINm5F cells are maintained in RPMI 1640 tissue culture media, containing 11 mM glucose, supplemented with 10 % FCS, 2 mM glutamine, and 50 μg/ml gentamicin. Cells are seeded out every 2–3 days onto Petri dishes and kept in a humidified atmosphere of 95 % O_2 and 5 % CO_2 at a temperature of 37 °C. The cells are isolated by incubation in a Ca^{2+}-free medium containing 0.25 % trypsin for about 3 min. Single cells and clusters of two to three cells are obtained after centrifugation with 800 rpm and are stored on ice until use. The tight-seal whole-cell patch clamp technique can be applied to single cells.

MIN6

The MIN6 cell has been derived from transgenic mice expressing a comparable transgene (Miyazaki et al. 1990). MIN6 cells cultured on a gelatine layer have been shown to form spherical cell clusters (pseudoislets) similar to those in islets (Hauge-Evans et al. 1999). Two rodent insulin genes have been identified in MIN6 cell pseudoislets (Roderigo-Milne et al. 2002). The glucose-induced biphasic insulin secretion pattern observed in isolated mouse islets was also demonstrated in MIN6 pseudoislets. Differences between pancreatic islets representing a complete endocrine organ and the artificial MIN6 pseudoislets containing only β-cells were observed cautioning the use of the pseudoislets for evaluation of compounds/drugs (Brenner and Mest 2003).

For culture, MIN6 cells (starting passage 40) are maintained in DMEM containing 25 mM glucose and supplemented with 15 % heat-inactivated FBS in humidified 5 % CO_2/95 % air at 37 °C. Cells are exposed to glucose-free extracellular solution for 30 min prior to measurement of insulin secretion or intracellular calcium.

Palmitate-Treated MIN6

Busch and coworkers (2005) studied the effect of fatty acids on the viability and functionality of pancreatic β-cells. For this, palmitate-treated MIN6 cell pools are selected and routinely passaged in 75-cm^2 flasks with 20 ml DMEM containing 25 mM glucose, 24 mM $NaHO_3$, 10 mM HEPES, 10 % (by vol.) FCS, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 1 mM palmitate coupled to 2.3 % BSA (wt/vol). This selection procedure is carried out twice, giving rise to two independent pools of palmitate-treated cells. Both pools are used for transcript profiling experiments and functional studies. Control MIN6 cells are taken through the same selection process but using BSA medium without palmitate. Cells are seeded in DMEM as follows. For [^{14}C]palmitate labeling and protein measurement, 2 × 105 cells per well in 24-well dishes (0.5 ml) are used; for microarray experiments, 5 × 106 cells per 25-cm^2 flask (5 ml) are used; for analysis of lipid composition, 6 × 106 cells per 10-cm dish (15 ml) are used. At 48 h before the experiments (24 h after seeding), the medium is replaced with DMEM (as above but with 5 mM glucose) supplemented with either BSA alone or BSA coupled to palmitate. Couplings are prepared in DMEM (25 or 5 mM glucose) as described by Busch and coworkers (2002). The couplings are diluted 1:8 (for culture and for experiments), giving a final concentration of 1 mM palmitate to 2.3 % BSA (wt/vol), corresponding to a molar ratio of 3:1.

HIT-T15

The insulin-secreting HIT cell line has been developed by Santerre and coworkers (1981) by isolating pancreatic islets from the hamster, dispersing

the islets into single cells, transforming the cell isolates with the simian virus 40 (SV40), and cloning out the insulin-secreting cell lines. These clonal cells retain a differentiated function and respond to secretagogues and inhibitors of insulin secretion (Boyd et al. 1991).

INS-1/INS-2

Asfari and coworkers (1992) derived INS-1 cells and INS-2 cells from parental radiation-induced RINm5F cells. In the course of a coculture of lymphocytes and RINm5F cells in the presence of 2-mercaptoethanol, the authors observed the formation of free-floating cell aggregates which appeared to be morphologically different from the parental cells. These clusters were isolated and gave rise to the INS cell lines, whose viability is dependent on the presence of 2-mercaptoethanol in the media and whose secretory characteristics are similar to those of native islets with its modulation by free radicals, upon challenge of the cells by alloxan, has been reported by Asfari and coworkers (1992). Due to the nonclonal nature of these cells, limited stability over passages probably explains some of the discrepancies observed among the numerous laboratories using parental INS-1 cells worldwide. To circumvent this problem, Hohmeier and coworkers (2000) stably transfected INS-1 cells with the human proinsulin gene, followed by the selection of clones based on robust glucose-stimulated insulin secretion. The resulting INS-1–832/13 cells are highly glucose responsive. However, these cells overexpress human insulin driven by the ubiquitous cytomegalovirus promoter in addition to the endogenous rat insulin, rendering it impossible to judge the differentiated state based on insulin content. An alternative approach would be the cloning of well-differentiated INS-1 cells without genetic manipulation. Hence, the clonal INS-1E cells have been isolated from the parental cells based on both their insulin content and their secretory response to glucose as well as adequate proliferation (Chen et al. 2000).

INS-1E

Clonal INS-1E cells, derived from parental INS-1 cells (Asfari et al. 1992) on the basis of selection for insulin content and adequate proliferation (Janjic et al. 1999), are cultured in a humidified atmosphere containing 5 % CO_2 in complete medium composed of RPMI 1640 supplemented with 5 % heat-inactivated FCS, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. The maintenance culture is passaged once a week by gentle trypsinization, and cells are seeded at a density of 4 × 104 cells/cm^2, i.e., 3 × 106 cells, in 75-cm^2 Falcon bottles with 20 ml complete medium. The potential presence of mycoplasma is regularly checked using a photometric enzyme immunoassay for the detection of PCR-amplified mycoplasma DNA (Roche Diagnostics). INS-1E are seeded at 2 × 105 cells/ml in Falcon 24-well plates and used 4–5 days thereafter, with one medium change on day 3 or 4. For generation of INS-1E cell clusters, cells are seeded in nonadherent bacterial 10-cm Petri dishes and cultured in complete medium for 5–6 days before use. The stable differentiated INS-1E β-cell phenotype was reported for over more than 100 passages corresponding to a 2-year continuous follow-up. INS-1E cells can be safely cultured and used within passages 40–100 with high insulin contents. Glucose-induced insulin secretion is concentration dependent and similar to rat islet responses as are the secretion responses to amino acids and sulfonylureas. Moreover, INS-1E cells retained the amplifying pathway, as judged by glucose-induced augmentation of insulin release in a depolarized state. Finally, spheroid clusters, sometimes referred to as pseudoislets (Hauge-Evans et al. 1999), composed of reaggregated INS-1E cells, can be prepared and tested for their ability to respond to secretagogues. Thus, INS-1E cells represent a stable and valuable β-cell model for the analysis of insulin secretion induced by compounds/drug/candidates.

β-TC

Hanahan (1985) applied gene transfer technology to the establishment of β-cell lines. In order to target the expression of viral DNA to the β-cell, recombinant oncogenes have been designed by fusion of the 5′-regulatory region of the rat insulin

II gene with the early coding region of SV40. The transgenes have been microinjected into fertilized mouse embryos, which were then implanted in the oviduct of pseudopregnant females. The offspring specifically expressed SV40 in their β-cells and spontaneously developed β-cell tumors at 10–20 weeks of age. Tumors were subsequently excised, isolated, and propagated in culture, giving rise to β-tumor cell (β-TC) lines (Erfrat et al. 1988). Because these cells do not grow in culture at low density, they have not been cloned, and several subpopulations have been derived. Hamaguchi and coworkers (1991) applied this technique to the development of the NIT1 cell line from transgenic NOD mice in order to establish an immortalized source of NOD β-cells.

Betacyte

The betacyte, also called the HepG2Ins/β-cell, is a genetically engineered insulin-secreting human liver cell line that is glucose responsive (Simpson et al. 1995, 1996; Tuch et al. 1997). The clone was constructed by doubly transfecting the HepG2 cell line with insulin cDNA under the control of the constitutive CMV promoter and the cDNA for the glucose transporter isoform 2 (GLUT2), which is specific to β-cells and hepatocytes. Interestingly, this cell is capable not only of synthesizing, storing, and secreting insulin in a regulated fashion when challenged with physiological concentrations of glucose but also of acting as a liver cell and secreting albumin.

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Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms

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Contents

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General Considerations

Despite the eminent importance of studies with primary and cultured adipocytes or adipose tissues on the basis of their physiological role in the regulation of lipid and carbohydrate metabolism in humans in combination with the relative low expenditure in preparing adipocytes of high quality and number, compounds and drug candidates for future antidiabetic and antiobesity therapy have to be analyzed for their effects in primary and cultured hepatocytes and myocytes or liver and muscle tissues, too. In principle, the majority of the assays described above for adipocytes can be adapted for the use with hepatocytes and myocytes. However, the following selection takes into account the relative contribution of each process monitored to its role in the whole-body regulation of intermediary metabolism in the normal and disease state. Moreover, technical aspects, such as requirement for a special equipment and applicability in throughput screening assays for drug discovery, were additional criteria.

Glucose Oxidation

Procedure

Cultured Myocytes and Hepatocytes

For the measurement of glucose oxidation on basis of $^{14}CO_2$ release, varying amounts of [^{14}C] glucose (0.1 mCi/ml) are used. Alternatively, glucose oxidation is determined as the formation of [^{3}H]water from [5-^{3}H]glucose. After culture in DMEM medium in 24-well culture dishes, cells are incubated at 37 °C for 2 h in 10 µl of KRB solution containing [5-^{3}H]glucose at various concentrations of glucose as described by Wang and coworkers (2004). Control wells containing no cells are included to allow for correction of the conversion of [^{3}H]water into [^{3}H]glucose.

Diaphragm

Glucose utilization by isolated diaphragms can be monitored according to the procedure described by Jeoung and coworkers (2006). For this,

diaphragms are removed from 48-h starved mice, rinsed in KHB buffer, blotted, weighed, and placed in 10-ml Erlenmeyer flasks containing 1.5 ml of KHB buffer (pH 7.4), 5 mM glucose, and 0.2 % BSA. The flasks are flushed with 95 % O_2/5 % CO_2 sealed with rubber stoppers, placed in a shaking (60 cycles/min) water bath at 37 °C, and preincubated for 30 min. Diaphragms are removed from the flasks, blotted, and transferred to new flasks containing 1.5 ml of KHB buffer supplemented with 5 mM glucose containing 20–500 μCi/mmol [U-^{14}C]glucose and 80 μCi/mmol [5-^{3}H]glucose and 1 mU/ml insulin as described by Clark and coworkers (1987). Flasks are flushed with 95 % O_2/5 % CO_2, sealed with rubber serum caps fitted with hanging center wells, and incubated for 1 h with shaking at 37 °C. Reactions are terminated by the injection of 0.25 ml of phenylethylamine/methanol (1/1, by vol.) into the center wells and 0.1 ml of 60 % (w/v) perchloric acid into the incubation medium.

Evaluation

The rate of glucose oxidation is determined from the production of $^{14}CO_2$, the rate of glycolysis from the difference between the rate of $^{3}H_2O$ formed (Ashcroft et al. 1975) and the rate of substrate cycling, and the rate of substrate cycling from the difference between the rates of glycogen synthesis from [U-^{14}C]glucose and [5-^{3}H]glucose. Glucose utilization rates are calculated as pmol of glucose utilized/hour/cell or mg tissue as glucose utilized (pmol) = [^{3}H]water formed (cpm)/specific radioactivity of [5-^{3}H]glucose (cpm/pmol).

Pyruvate Oxidation

Purpose and Rationale

Pyruvate oxidation can be monitored in isolated rat adipocytes as the incorporation of the acetyl-CoA generated into lipophilic products along the fatty acid synthesis and esterification (into TAG) pathways. Under conditions of glucose deprivation, pyruvate dehydrogenase (PDH) is rate limiting for the overall conversion of 3-[^{14}C]pyruvate into radiolabeled products (e.g., fatty acids, TAG) which are separated from the unincorporated ^{14}C-labeled pyruvate, acetyl-CoA, and hydrophilic intermediates by partitioning into the toluene phase.

Procedure

Isolated rat adipocytes are prepared by collagenase digestion as described above (chapter "▶ Insulin Target Tissues and Cells"), however, in KRHB lacking glucose. 3.5 × 104 cells are incubated (2 h, 37 °C) with 0.1 mM [3-^{14}C] sodium pyruvate (0.5 μCi) in 1 ml of KRHB lacking glucose. After addition of 5 ml toluene, the samples are vortexed and incubated overnight at 4 °C. One milliliter of the upper toluene phase is removed, supplemented with 10 ml toluene-based scintillation cocktail (e.g., Beckman Quickszint 501), and counted for radioactivity.

Evaluation

Dichloroacetate (DCA), which acts as unspecific inhibitor of PDH kinase leading to activation of PDH and thereby stimulation of pyruvate oxidation, should be used as control (0.2–5 mM final conc.). Data each corrected for a blank value lacking cells are calculated as fold stimulation of ^{14}C incorporation into toluene-soluble products in response to DCA versus basal. In isolated rat adipocytes, 5 mM DCA stimulates pyruvate oxidation by three- to fourfold. The assay may be useful for the identification of compounds/drug candidates which stimulate pyruvate oxidation by inhibition of PDK (PDK2 in adipocytes) or different modes of action.

Pyruvate Dehydrogenase Complex (PDC) Activity

PDC activity is determined according to the method of Jeoung and coworkers (2006). For this, deep-frozen and pulverized diaphragms are homogenized in five volumes of extraction buffer containing 30 mM HEPES/KOH (pH 7.5),

0.5 mM thiamine pyrophosphate, 3 % Triton X-100, 5 mM EDTA, 2 % BSA, 5 mM DTT, 10 μM tosyl-phenylalanyl chloromethyl ketone, 10 μg/ml trypsin inhibitor, 1 μM leupeptin, 2 mM dichloroacetate (DCA), and 50 mM KF. The supernatant obtained by centrifugation (10,000 × g, 10 min, 4 °C) is made 9 % (w/v) in PEG6000 to precipitate PDC. Pellet produced by centrifugation (12,000 × g, 10 min) is suspended in a suspension buffer containing 30 mM HEPES/KOH (pH 7.5), 1 % Triton X-100, 0.2 mM EDTA, 2 % BSA, 1 μM leupeptin, and 5 mM DTT. For determination of total PDC activity, diaphragms are homogenized in five volumes of the extraction buffer lacking DCA and KF. The supernatant (200 μl) obtained by centrifugation of the homogenate (10,000 × g, 10 min, 4 °C) is added to 100 μl of an activation buffer (suspension buffer containing 25 mM $MgCl_2$, 1.5 mM$CaCl_2$, and 1 μg of recombinant pyruvate dehydrogenase phosphatase one protein. After incubation (20 min, 30 °C), to dephosphorylate and activate PDC, samples are treated with PEG6000 (final 9 %, w/v) to precipitate PDC. An aliquot of an extract of the pellet is used to assay the total PDC activity. PDC activity is measured spectrophotometrically in a 96-well plate reader with a coupled assay based on the reaction catalyzed by arylamine acetyltransferase (Nakai et al. 1999; Coore et al. 1971). One unit of PDC activity corresponds to the acetylation of 1 μmol of p-(p-aminophenylazo)-benzenesulfonate per min at 30 °C.

Pyruvate Dehydrogenase Kinase (PDK) Activity

General Considerations

The PDC multienzyme complex (5–10 million Da) catalyzes a key regulatory step in oxidative glycolysis, the irreversible decarboxylation of pyruvate. Its activity is reduced under conditions of increased fatty acid oxidation, i.e., when tissue [acetyl-CoA]/[CoA] and [NADH]/[NAD+] ratios are elevated. Thus, PDC activity is downregulated during fasting and in pathological conditions associated with insulin resistance, such as diabetes and obesity (Fuller and Randle 1984; Orfali et al. 1993; Kelley et al. 1992).

The mammalian PDC is composed of multiple copies of three enzymes: pyruvate decarboxylase (E1 subunit), dihydrolipoyl acetyltransferase (E2 subunit, a modular protein which contains a transacetylase and so-called outer and inner lipoyl domains, E2c, E2L1, and E2L2, respectively), and dihydrolipoyl dehydrogenase (E3 subunit). It is regulated by reversible phosphorylation (Linn et al. 1969; Yeaman et al. 1978). Four pyruvate dehydrogenase kinase (PDK) and two pyruvate dehydrogenase phosphatase (PDP) isozymes have been identified to date (Gudi et al. 1995). PDKs inactivate PDC by catalyzing the ATP-dependent phosphorylation of three serine residues on the PDC E1 subunits.

Purpose and Rationale

The blood glucose-lowering effect of dichloroacetate (DCA) in diabetic rodents (Lorini and Ciman 1962) and type II diabetic patients (Stacpoole et al. 1978) has been ascribed to its ability to inhibit PDKs and thereby activate PDC in vivo. The primary structures of PDK isozymes 1–4 share little sequence homology with other eukaryotic protein kinases and are more homologous to prokaryotic histidine kinases (Popov et al. 1993). These findings increased the attractiveness for drug discovery projects to identify novel inhibitors of PDK activity as possible therapeutic agents for diabetes and other pathological conditions in which PDC activity is reduced (e.g., lactic acidosis, ischemia) (Bersin and Stacpoole 1997). Until recently, the only known small-molecule inhibitors of PDK activity were pyruvate, DCA, halogenated acetophenones, and analogs of ATP (e.g., adenosine 5′-[β, γ-imido] triphosphate (p[NH]ppA). The following assays may be useful for the identification of structurally novel PDK inhibitors.

Procedure

Functional PDK Assay

Compounds are assessed for their ability to inhibit the ATP-dependent inactivation of porcine heart PDC. The assay essentially consisted of three steps. First, a BSA-stabilized PDC preparation is acetylated to enhance its intrinsic PDK activity. Second, the PDK reaction is initiated by the addition of ATP. Third, after 7 min, the PDK reaction is terminated and the residual PDC activity is assessed by monitoring the formation of NADH at 340 nm. In some experiments, the PDC is either supplemented with rPDK or ATP is omitted during the second step.

E1 Subunit Phosphorylation Assay

For phosphorylation of the PDC E1 subunit by endogenous PDK (Jackson et al. 1998), the reaction is initiated by the addition of [γ-^{33}P]ATP (80 mCi/mmol) to intact porcine PDC. The reaction is terminated after 45 s by the addition of TCA. Precipitated protein is recovered by centrifugation and dissolved in 1 M NaOH, and the radioactivity of the entire sample is determined by liquid scintillation counting.

PDK Peptide Phosphorylation Assay

PDK activity is determined by measuring the PDK-catalyzed phosphorylation of an acetylated tetradecapeptide substrate, Ac-YHGHSMSDPGVSYR, as previously described (Jackson et al. 1998), except that the reaction volume is 25 µl. Effects of compounds/drug candidates on PDK activity are usually determined in the presence of 0.2 mM [γ-^{33}P]ATP (0.05 µCi/nmol ATP) and 0.5 mM peptide substrate. In experiments where the [γ-^{33}P]ATP (0.05–0.85 µCi/nmol ATP) concentration is varied, the final peptide concentration is 0.5 mM. When the peptide concentration is varied, the final [γ-^{33}P]ATP concentration is adjusted to 0.2 mM. Results are expressed as specific activity (nmol of phosphate transferred per mg of PDK protein per 30 min).

Kinase Autophosphorylation Assay

PDK1 or PDK2 (50 µg/ml) is incubated in a 25-µl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), $MgCl_2$ (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM), [γ-^{32}P]ATP (0.2 mM, 0.01 mCi/mmol), and compounds/drug candidates at 37 °C for up to 5 min. The reaction is terminated by the addition of 0.1 volume of a mixture of 425 mM H_3PO_4 and 5 mM p[NH]ppA. The sample is then adjusted to pH 7.4 by the addition of 125 µl of a mixture of 60 mM Tris (pH 8.7) and NaCl (500 mM). The entire sample is then applied to a nitrocellulose membrane and rinsed with TBS (20 mM Tris, 500 mM NaCl, pH 7.5) using a dot-slot blotting apparatus. Phosphorylated proteins are visualized using a phosphor screen and a phosphorimager (e.g., Storm 840).

Phosphorylation of E1 Protein by PDKs

PDK1 or PDK2 (7.5 µg) is incubated in the presence or absence of 95 µg of acetylated PDC without BSA (Jackson et al. 1998) in a 75-µl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), $MgCl_2$ (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM), and [γ-^{32}P]ATP (0.2 mM, 0.01 mCi/mmol). Following a 45-min incubation at 37 °C, the reaction is terminated by the addition of an equal amount of Laemmli sample buffer. Forty microliter portions are subjected to SDS-PAGE.

Assay for Direct Inhibition by Supplemental PDKs

PDK2 is added to the functional PDK assay described above, which is performed in the absence and presence of ATP. Alternatively, a PDC preparation which is neither stabilized with BSA nor pre-acetylated is incubated (14 µg/ml) in 180 µl of 40 mM MOPS (pH 7.4), 0.36 mM EDTA, 30 mM KCl, 1.5 mM $MgCl_2$, 120 mM ADP, 2 mM DTT, 10 mM NaF, and the additions indicated at 37 °C for up to 10 min. During this step, ADP is omitted in some experiments. The PDC reaction is initiated by the addition of 20 µl of 40 mM MOPS (pH 7.4), 0.36 mM EDTA,

1.5 mM $MgCl_2$, 1 mM DTT, 1.1 mM CoASH, 2.2 mM NAD+, 2.2 mM pyruvate, and 2.2 mM thiamine pyrophosphate. NADH formation over 5 min is monitored at 340 nm.

Evaluation

The functional PDK assay has a spectrophotometric readout and measures the effect of PDK activity on PDC function. However, the PDK isozyme composition and the stoichiometry of PDK relative to PDC are unknown. Obviously, this assay is more difficult to optimize and validate than a traditional kinase assay based upon a model fluorescent peptide substrate. However, the functional PDK assay should identify not only compounds acting at known or presumed sites on the kinase (ATP, lipoamide, or pyruvate binding sites) but also those interfering with interactions between PDK and other PDC protein components (e.g., E1 subunit or the inner lipoyl domain (E2L2) of the E2 subunit of PDC). Although inhibitors of protein–protein interactions are rare, it is desirable that an initial screening assay allows for this possibility.

In contrast to the functional PDK assay, the E1 phosphorylation assay enables PDK activity to be measured directly by testing the compounds/drug candidates for a more specific effect. The ability to inhibit phosphorylation of the E1 subunit by PDK activity is intrinsic to the PDC preparation. This assay was used to confirm PDK inhibition in the functional assay. At least one of the concentrations selected for each compound approximated its IC50 value determined in the functional assay. The recombinant inner lipoyl domain of the PDC dihydrolipoyl acetyltransferase (rE2L2) is a known inhibitor of PDK activity in this assay and is included as a control (Jackson et al. 1998).

The E1 phosphorylation assay is able confirm that apparent PDK inhibition in the functional assay is due to inhibition of E1 phosphorylation. However, this assay suffers from the drawback that the PDK isozyme composition and stoichiometry are undefined. Therefore, a much simpler assay with defined stoichiometry of a synthesized tetradecapeptide (a fragment of the E1 subunit which contains all three of the serines which are phosphorylated by PDK (Yeaman et al. 1978)) can be used. This assay has been modified by Mullinax and coworkers (1985) who demonstrated that this tetradecapeptide is a specific substrate for PDK and the PDH phosphatase. Therefore, this assay is in principle the most amenable to kinetic characterization of inhibitors acting at the active site of PDKs.

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Fatty Acid Oxidation

General Considerations

The development of obesity and the associated insulin resistance result from increases in the ratio of food intake to energy expenditure. Loss of body weight can be achieved through a reduction in food intake, a stimulation of energy expenditure, or a combination of both. At the same time, a reduction of fatty acid oxidation has been described in obese and diabetic patients, and the resulting accumulation of fatty acid oxidation has been described in obese and diabetic patients and the resulting accumulation of fatty acids or metabolites of fatty acids in tissues such as skeletal muscle and liver seems to play a direct role in the development of insulin resistance. Recent observations in animal models suggest that a stimulation of fatty acid oxidation reduces the accumulation of fatty acids in tissues and increases overall energy expenditure, leading to lean, insulin-sensitive phenotypes. The modulation of fatty acid oxidation is therefore an attractive new therapeutic strategy for the treatment of obesity and insulin resistance.

Fatty acid oxidation modulated by compounds/drug candidates can be measured with myocytes and hepatocytes by indirect (generation of $^{3}H_2O$) or direct (generation of $^{14}CO_2$) procedures with the advantages of lower expenditure in equipment and handling inherent to the former and higher accuracy and physiological relevance associated with the latter. In any case, current assays using cultured cells are cumbersome and of low throughput. Most published procedures utilizing ^{14}C-labeled long-chain fatty acid substrates (e.g., palmitate or oleate) are performed in tissue culture flasks, in which a center well captures the radioactive CO_2 product (Angelini et al. 1980; Alam and Saggerson 1998; Kaushik et al. 2001). Alternatively, ^{3}H-labeled fatty acids can be used, but a labor-intensive extraction of $^{3}H_2O$ is needed (Ibrahimi et al. 1999). These low-throughput assay methods are not suitable for rapid screening of potential drug candidates. More recently, a variant of the $^{14}CO_2$-capturing method has been

introduced by Collins and coworkers (1998) which facilitates the accurate, reproducible, and safe throughput measurement of the released of $^{14}CO_2$.

CO_2 Release

Purpose and Rationale

The measurement of fatty acid oxidation using a self-contained 48-well assay has been described by Wang and coworkers (2004). Palmitate is conjugated with essentially fatty acid-free BSA to generate a stock solution of 25 % (w/v) BSA and 6 mM fatty acid in serum-free medium. After conjugation with albumin, the concentration of fatty acids in the solution is measured using a NEFA kit (Wako Chemicals, Inc.). The stock solution is diluted into the final culture medium to obtain concentrations of 1–1,000 μM fatty acid. Palmitate oxidation is measured by the production of $^{14}CO_2$ from [1-^{14}C]palmitic acid.

Procedure

For throughput analysis, the cells are incubated for 1–3 h at 37 °C in 6- to 24-well culture dishes with medium containing 0.2–1 μCi/ml [1-^{14}C] palmitic acid and nonlabeled palmitate (1–1,000 μM), 2.5 mM glucose, and 0.8 mM carnitine in the presence or absence of compounds/drug candidates. Each culture dish is sealed using a special technique, for instance, by covering with Parafilm, which had a piece of Whatman paper taped facing the inside of the petri dish. Alternatively and for low-throughput application, the cells can be handled according to the protocol published by Fediuc and coworkers (2006). For this, the L6 myotubes are serum starved for 4 h before exposure to fatty acids and then incubated for 1 h in 60 × 15-mm culture dishes with medium containing 0.2 μCi/ml [1-^{14}C]palmitic acid (1–10 mM) in the absence or presence of compounds/drug candidates. Each culture dish is sealed with Parafilm, which has a piece of Whatman paper taped facing the inside of the petri dish. After the incubation, CO_2 generated during this period has to be trapped using a special technique, for instance, by wetting the Whatman paper with 100 μl of phenylethylamine/methanol (1/1) and subsequent addition of 200 μl of H_2SO_4 (4 M) to the cells. After additional incubation (1 h, 37 °C), the amount of $^{14}CO_2$ is determined by careful removal of the Whatman paper and transfer to scintillation vials for radioactivity counting as described by Ceddia and coworkers (2000, 2004).

Alternatively, cultured hepatocytes or myocytes are grown to confluence in the odd-numbered rows of 48-well plates. For standard assays, the cells are washed with PBS twice and then incubated with 125 μl of KRB buffer supplemented with 5 mM glucose at 37 °C for 1 h. After removal of this buffer, 125 μl of reaction mixture in KRB buffer containing 0.2 μCi [1-^{14}C] palmitate (~500,000 cpm) and 0.5 % BSA is added to the wells. Palmitate is conjugated with essentially fatty acid-free BSA to generate a stock solution of 25 % (w/v) BSA and 6 mM fatty acid in serum-free medium. After conjugation with BSA, the concentration of fatty acids in the solution is measured using a NEFA kit (Wako Chemicals). The reactions are performed in the sealed environment of the device at room temperature for 3 h. The reactions are terminated by injection of 250 μl of 1 M HCl into the cell containing wells to release CO2. This injection is performed through the pierceable septum using a syringe. The CO_2 is captured in 250 μl of 1 M NaOH in the adjacent connected well as room temperature overnight. The NaOH solution is removed into a scintillation vial and mixed with 4 ml of OptiPhase (HiSafe 3) scintillation cocktail and counted.

For measurement of fatty acid in isolated hepatocytes in suspension according to the procedure of Chang and coworkers (2006), cells (5 × 105) are incubated for 1 h in buffer containing carnitine, NAD, ATP, cytochrome c, $MgCl_2$, coenzyme A, and [^{14}C]palmitic acid complexed with fatty acid-free BSA. The reaction is stopped with 60 % perchloric acid. The released CO_2 is trapped by hyamine hydroxide at the top of a hanging center well and counted for radioactivity in a β-scintillation counter. Fatty acid oxidation is

calculated as percentage of the maximal value obtained by stimulation of the cells with epinephrine (1 μg/ml).

For measurement of fatty oxidation in native liver tissues according to the procedure introduced by Wu and coworkers (2006), liver slices (0.5 mm thick) are prepared using a tissue slicer and placed in 25-ml Erlenmeyer center-well flasks with 2 ml KRP buffer. [^{14}C]oleic acid bound to fatty acid-free BSA in KRP buffer is added to a final concentration of 0.5 mM and equilibrated for 30 s with a humidified 95 % O_2/5 % CO_2 gas mix. Flasks are then capped with a rubber stopper containing a center well enclosing a loosely folded filter paper moistened with 0.2 ml of 1 N NaOH solution. After incubation for 4 h at 37 °C, the reaction is stopped by injecting 0.2 ml of H_2SO_4 (1 M). The radioactivity trapped in the filter paper is determined by scintillation counting.

To improve the throughput, a device that accommodates four 48-well culture dishes in a single unit has been designed (Collins et al. 1998). Two adjacent wells form a single experimental unit by means of a connecting chamber, which is sealed from other experimental units and from the outside environment to prevent the release of radioactive material. In this design, one well of the experimental unit contains the cells and ^{14}C-labeled fatty acid. The [^{14}C]CO_2 produced by β-oxidation of the fatty acids is then released from the culture medium upon addition of HCl and is subsequently captured in the adjacent NaOH-containing well. The data published demonstrate that the use of this device, which has a simple design and is easy to use, allows highly reproducible evaluation of long-chain fatty acid oxidation in cultured cells with low variability; can be used with a variety of substrates, such as long- and medium-chain fatty acids, and cell lines, such as HepG2, primary rat hepatocytes, and HEK293; and unlike previously reported center-well capture methods for β-oxidation assays, can be run in medium throughput making it suitable for compound characterization and drug discovery. The ability to use primary hepatocytes opens the possibility to evaluate cells after exposure to drugs in vivo.

Release of Acid-Soluble Metabolites (ASM)

Procedure

Determination of fatty acid oxidation as the release of ASM, which predominantly consist of tritiated water and ketone bodies, follows the procedure described by Moon and Rhead (1987) with modifications introduced by Minnich and coworkers (2001). The reaction mixture is prepared by complete evaporation of the solvent from [9,10-^{3}H]palmitic acid with a 5 % CO_2/95 % air stream. The [3H]palmitate is resuspended in Hanks' basic salt solution (HBSS) containing 10 mg/ml BSA and brought to a final concentration of 22 μM [^{3}H]palmitate and 0.5 mg/ml BSA with HBSS. 0.2 ml of this mixture is applied to the wells of 24-well culture dishes containing HepG2 cells or L6 myotubes rinsed twice with 1.5 ml of Dulbecco's PBS. Blanks are prepared by applying 0.1 ml methanol for 30 s. After incubation (2 h, 37 °C) in humidified 5 % CO_2/95 % air, the reaction mixture is removed and added to a centrifuge tube containing 0.2 ml of 10 % TCA. Each well is rinsed with 0.1 ml of Dulbecco's PBS, which is added to the tube. After 2 min at room temperature, the reaction mixture is centrifuged (8,500 × g, 5 min). The supernatants are immediately removed, then supplemented with 70 μl of 6 N NaOH, and loaded onto a 1-ml Dowex-1 column in a Pasteur pipette. Columns are rinsed with 1 ml of distilled water. The eluate is collected in a scintillation vial containing 10 ml of scintillation cocktail and then counted for radioactivity.

Alternatively, for bypassing the removal of nonoxidized fatty acids by column adsorption, the ASM can be measured directly by transfer of the cells to plastic tubes according to the protocol introduced by Aas and coworkers (2004). The flasks are rinsed with 1.5 ml of perchloric acid (1 M) that is subsequently added to the same plastic tube. The tubes are then centrifuged (1,800 × g, 10 min) and 1.0 ml of the supernatant is counted by liquid scintillation. ASM are also measured in the growth media of cells grown on six-well plates that had been incubated with [1-^{14}C]oleic acid and [1-^{14}C]palmitic acid.

A 250-µl aliquot of the cell medium is precipitated with 100 µl of 6 % BSA and 1.0 ml of 1 M perchloric acid. After centrifugation (1,800 × g, 2 min), 500 µl of the supernatant is counted by liquid scintillation. No-cell controls have to be included.

Evaluation

The sensitivity of this assay critically depends on the substrate concentration, cell density, BSA concentration, and incubation time. Though the concentration of [^{3}H]palmitate employed routinely (22 µM) is not fully saturating, less than 2 % of the substrate is converted to ^3H_2O by L6 myotubes. The [^{3}H]palmitate concentration is thus virtually constant throughout the incubation period. ^3H_2O formation is linear to up to ~60 µg of cell protein per well, at which point the response levels off, and to up to 4 h of incubation. Optimal activity is typically observed in HBSS containing 0.5 mg/ml fatty acid-free BSA in comparison to other media (e.g., MEM). In conclusion, the ^3H_2O release assay combines the advantages of relatively low requirements of cells, radiolabeled substrate, and assay time.

ASM, a measure of ketone bodies in the liver and of tricarboxylic acid-cycle intermediates and acetyl esters in muscle, are assayed in supernatants of the acid precipitate. In myocytes, ASM routinely account for over 90 % of total oxidation products. Minimal increases in ^{14}C-radioactivity over background levels occur in media in which myocytes have been incubated, indicating that insignificant amounts (<15 % of the counts recovered as CO_2) of ASM are released into the medium.

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Carnitine Palmitoyltransferase I (CPTI) Activity

Purpose and Rationale

Carnitine palmitoyltransferase I (CPT I), which mediates the uptake of long-chain fatty acids into mitochondria, is the rate-limiting enzyme for mitochondrial fatty acid oxidation in mammalian cells. Pharmacological inhibition of CPT I in course of an increase in its physiological negative regulator, malonyl-CoA, as consequence of the inhibition of fatty acid synthase by a natural small molecule (C75) has been demonstrated to lead to appetite suppression. This apparent antiobesity therapeutic action is presumably based on an alteration in the metabolism of neurons in the hypothalamus, where an increase in malonyl-CoA serves as a secondary messenger of the nutrient status, thereby mediating satiety (Loftus et al. 2000; Hu et al. 2003). In addition, in peripheral tissues (Thupari et al. 2002), C75 has been postulated not only to increase the level of malonyl-CoA but also to act as a malonyl-CoA analog that antagonizes the inhibitory effect on CPT I (McGarry and Brown 1997). Both its central and the peripheral actions could reduce weight in lean and fat mice. Furthermore, both direct activation and direct inhibition of CPT I and the resulting increased and decreased fatty acid oxidation, respectively, have been implicated as putative target for the improvement of impaired insulin signaling and glucose metabolism in muscle and liver cells in course of reduced lipid accumulation (i.e., reduced lipotoxicity) and of enhanced glucose oxidation according to the inverse relationship between glucose and fatty acid oxidation (i.e., Randle cycle), respectively.

Mammalian tissues express three isoforms of CPT I, liver (L-CPT I), muscle (M-CPT I) (McGarry and Brown 1997), and brain (CPTI-C) (Price et al. 2002). The liver and muscle isoforms are tightly regulated by their physiological inhibitor malonyl-CoA, which allows CPT I to signal the availability of lipid and carbohydrate fuels to the cell. The malonyl-CoA sensitivity of L-CPT I in the adult rat depends on the physiological state. It is increased by renewed feeding of carbohydrates to fasted rats, by obesity, or by following administration of insulin to diabetic rats, whereas it is decreased by starvation and diabetes (Grantham and Zammit 1986, 1988). CPT I activity can be measured with mitochondria from cultured cells and tissues.

Procedure

Preparation of Mitochondrial Fractions

Mitochondria-enriched cell fractions from various cell types (INS, L6 myotubes, HEK293 cells) cultured in 15-cm dishes are obtained with a glass homogenizer as described by Rubi and coworkers (2002). The pellet, in which the mitochondria remain largely intact, is used directly for CPT I activity assays. Mitochondria-enriched fractions are obtained from rat and mouse muscle according to published procedures (Saggerson and Carpenter 1981) with minor modifications. Two soleus muscle samples of each animal are homogenized separately in 250 mM sucrose buffer using an omni-mixer and then centrifuged (1,000 × g, 15 min). The pellet is homogenized and centrifuged (600 × g, 10 min). The resulting supernatant is centrifuged (15,000 × g, 15 min) and the pellet is resuspended in 100 μl of a buffer containing 250 mM sucrose and 150 mM KCl. Mitochondria-enriched fractions from rat and mouse liver are obtained by homogenization in a buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris/HCl (pH 7.4). The liver suspension is centrifuged (560 × g, 15 min), and the

supernatant is further centrifuged (12,000 × g, 20 min). The pellet is resuspended in 2 mL of homogenization buffer, centrifuged (7,000 × g, 10 min), washed, and resuspended in 1 ml of the homogenization buffer. To obtain mitochondria-enriched fractions from mice pancreas, tissue is homogenized in a buffer containing 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 4 μg/ml aprotinin, 2 μg/ml pepstatin, and 100 μM PMSF. The homogenate is subjected to differential centrifugation (900 × g, 10 min, and 5,500 × g, 10 min). The pellet is resuspended with a Dounce homogenizer and centrifuged (2,000 × g, 2 min, and 4,000 × g, 8 min). Finally, the pellet is resuspended in 250 μl of 250 mM sucrose (Li et al. 1996). All the processes are performed at 4 °C, and fractions are assayed immediately for CPT I activity.

Determination of CPT I Activity

CPT I activity is measured in mitochondria-enriched fractions obtained from cultured cells or tissues. CPT I activity in 10–15 μg of mitochondria-enriched cell fractions or 20 μg of mitochondrial fractions from tissues is determined by the radiometric method as described by Morillas and coworkers (2001). Extracts are preincubated at 30 °C for different times in the presence or absence of compounds/drug candidates. Enzyme activity is assayed for 4 min at 30 °C in a total volume of 200 μl. The substrates are 50 μM palmitoyl-CoA and 400 or 1,000 μM L-[methyl-^{3}H]carnitine for L- and M-CPT I isoforms, respectively. In tissues and cell culture extracts, both CPT I (malonyl-CoA sensitive) and CPT II (insensitive to malonyl-CoA) are present. Thus, in these fractions, CPT I activity is determined as the malonyl-CoA-/etomoxiryl-CoA-sensitive CPT activity. CPT II activity, which is also present in mammalian mitochondrial extracts, is always subtracted from the total activity to calculate specific CPT I activity. The presence of CPT activity insensitive to malonyl-CoA (CPT II activity) in mitochondria obtained from cell cultures is typically less than 5 % and thus is not taken into consideration. Compounds/drug candidates are preincubated with the enzyme for 1–5 min depending on the assay. Compound/drug candidate concentrations ranging from 0.01 to 50 μM are used to estimate the IC50 value. Malonyl-CoA (50 μM) is used for malonyl-CoA inhibition assays. In all cases, the molar ratio of palmitoyl-CoA to BSA is kept at 5:1 to avoid the presence of free acyl-CoA and its deleterious detergent effects and to prevent the formation of micelles. Kinetic constants (Km and Vmax) are determined by Lineweaver-Burk analysis. Inhibition constants (KI and kinact) are determined at 20 μM palmitoyl-CoA by nonlinear parameter estimation (Maurer and Fung 2000) using SigmaPlot version 8.0. All protein concentrations are determined using the Bio-Rad protein assay with BSA as a standard.

Binding of Compounds/Drugs to CPT I

The binding of compounds/drugs to CPT I is assessed as described by Tutwiler and Ryzlak (1980) with some modifications. Mitochondria-enriched fractions are preincubated for 5 min at 30 °C with compounds/drugs at 50 μM. One aliquot is used directly for the CPT I activity assay (unwashed), and the other aliquot is centrifuged (13,000 × g, 5 min, 4 °C) and resuspended (washed) in 5 mM Tris/HCl (pH 7.2), 150 mM KCl, 2 μg/ml leupeptine, 0.5 μM benzamidine, 1 μg/ml pepstatin, and 1 mM PMSF before the activity assay.

To verify the reversibility of the interaction of compounds/drug candidates with CPT I, dialysis assays are performed. Mitochondria-enriched fractions (160 μg) are preincubated at 30 °C for 5 min (without the compound/drug) or with a final concentration of 50 μM compound and then dialyzed in buffer containing 10 mM HEPES (pH 7.4), 1 mM EDTA, and 10 % glycerol at 4 °C. Aliquots are taken before dialysis (0 h) and 24 and 36 h thereafter and assayed for CPT I activity. This method has been used by Bentebibel and coworkers (2006) to demonstrate novel effects of C75 on CP II activity and palmitate oxidation.

Respiratory Quotient (RQ)

Respiratory quotient changes during variable periods of fasting of rodents are monitored at 24 °C ambient temperature in an open respirometric system. Individual O_2 consumption and CO_2 production are recorded every 5 min at a flow rate of ~40 l/h. RQ equals volumes of CO_2 released/volumes of O_2 consumed. During the 18-h measurement period, mice are maintained in metabolic cuvettes (5 l9), which contained bedding material (~50 g) and provided access to water ad libitum. In each trial, typically four mice are investigated. Before and after the experiment, mice are weighed and body temperature is recorded by inserting a rectal probe.

Phosphorylation of Acetyl-CoA Carboxylase (ACC) and AMP-Dependent Protein Kinase (AMPK)

Purpose and Rationale

In recent years, a large body of evidence has been published showing that in mammals AMP-activated prokinase (AMPK) responds to hormonal and nutrient signals in the central nervous system and peripheral tissues, modulating food intake and whole-body energy homeostasis (for a review, see Carling 2005). AMPK is a heterotrimeric enzyme that has been proposed to function as a "fuel gauge" that monitors changes in the energy status of cells. When activated, AMPK shuts down anabolic pathways and promotes catabolism in response to an increase in the AMP/ATP ratio by downregulating the activity of key enzymes of intermediary metabolism. In its activated state, AMPK phosphorylates serine residues 79, 1,200, and 1,215 of acetyl-coenzyme A carboxylase (ACC), producing an 80–90 % decrease in the Vmax of the enzyme, suggesting that AMPK is the physiological ACC kinase. There is also evidence that long-chain fatty acids (LCFAs) act as potent feedback suppressors of lipogenesis by inhibiting ACC activity. ACC is a multifunctional enzyme that, when active (dephosphorylated form), catalyzes the conversion of acetyl-CoA to malonyl-CoA in the de novo lipid synthesis pathway. Malonyl-CoA is a potent inhibitor of CPT I, a rate-limiting step for the entry of LCFAs into mitochondria for oxidation (see above). When ACC is inactive (phosphorylated form), a decrease in malonyl-CoA occurs and disinhibits CPT I, thereby increasing the mitochondrial import and oxidation of LCFAs (for reviews, see Mc-Garry and Brown 1997; Carling 2005). Therefore, the AMPK/ACC system is thought to play a central role in the regulation of cellular lipid homeostasis (Spiegelman and Flier 2001). In certain metabolic disorders, such as obesity and type II diabetes, lipid metabolism is dysfunctional, causing fatty acids to increase in the circulation and also in intracellular compartments. High levels of fatty acids are toxic to the cells and may cause deleterious metabolic abnormalities (lipotoxicity). By increasing fatty acid oxidation in peripheral tissues, the AMPK/ACC system may play an important role by protecting the cells from these metabolic abnormalities. Of special interest are the mechanisms that regulate the AMPK/ACC system in skeletal muscle, because this tissue plays a major role in determining whole-body energy expenditure, accounts for 70 % of total-body glucose disposal, and may modify substrate utilization toward substantially increasing fatty acid oxidation.

The classical view is that AMPK is activated allosterically by an increase in the intracellular AMP/ATP ratio; by phosphorylation of threonine 172 (Thr-172) within the α-subunit, catalyzed by the upstream kinase LKB1 (the upstream kinase of AMPK); and by inhibition of the dephosphorylation of Thr-172 by protein phosphatases. To date, a wide range of physiological stressors, pharmacological agents, and hormones associated with increases in the intracellular AMP/ATP ratio have been demonstrated to activate AMPK (Corton et al. 1994; for a review, see Carling 2005). Fatty acids, another major cellular energy

source, may also regulate AMPK activity in skeletal muscle. It has been reported that in perfused rat cardiac muscle, palmitate and oleate significantly increase AMPK activity without causing any significant alteration in AMP/ATP ratio. Another study has reported that exposure to acetate, octanoate, or palmitate causes a significant reduction in AMP/ATP ratio followed by a significant increase in AMPK activity in primary rat hepatocytes (for reviews, see Kemp et al. 1999; Ruderman et al. 1999). In contrast to these observations are reports that the AMPK activity of rat liver purified LKB1/STRAD/MOM25 (the upstream kinase complex of AMPK) was inhibited by long-chain acyl-CoA esters in vitro (Lizcano et al. 2004; Hawley et al. 2003; Boudeau et al. 2003). Consequently, AMPK and ACC represent important targets for future antidiabetic and antiobesity therapy via activation of fatty acid oxidation in course of activation of the former and inhibition of the latter. The identification of activators of AMPK or inhibitors of ACC, which act directly at the enzyme level or affect the regulation of the enzymes (i.e., induce their phosphorylation), requires reliable cell-based assay systems for measurement of the activity and phosphorylation state of AMPK and ACC in cultured muscle and liver cells and tissues.

Procedure

Cells are grown on six-well plates and incubated for 60 min in the presence or absence of palmitic acid (1–10 μM). AICAR, a synthetic nucleotide precursor taken up by the cells and phosphorylated to the monophosphate form ZMP, which can accumulate in the cell, mimics the effect of AMP on AMPK phosphorylation and activation. Therefore, incubation with AICAR (2 mM, 60 min) is used as a positive control for AMPK and ACC phosphorylation in L6 myotubes. Because ACC is a substrate for AMPK, the determination of ACC phosphorylation also serves as an indicator of AMPK activity. Immediately after all treatments, cells are lysed in buffer containing 135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 20 mM Tris/HCl (pH 8.0), 1 % Triton X-100, 10 % glycerol, and protease and phosphatase inhibitors (0.5 mM Na_3VO_4, 10 mM NaF, 1 μM leupeptin, 1 μM pepstatin, 1 μM okadaic acid, and 0.2 mM PMSF), heated (65 °C, 5 min), and passed five times through a 25-gauge syringe. An aliquot of the cell lysates is used to determine the protein concentration in each sample by the Bradford method. Before loading onto SDS-PAGE gels, the samples are diluted 1:1 with 2 × Laemmli sample buffer (62.5 mM Tris/HCl, pH 6.8, 2 % SDS, 50 mM DTT, and 0.01 % bromophenol blue). Aliquots of cell lysates containing 30 μg of protein are subjected to SDS-PAGE (12 % and 7.5 % resolving gels for P-AMPK and P-ACC, respectively) and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The phosphorylation of AMPK is determined using phospho-AMPK(Thr172) antibody (1:1,000 dilution), which detects AMPK-α only when activated by phosphorylation at Thr-172 (Cell Signaling Technology). ACC phosphorylation is detected using phospho-ACC-specific antibody (1:500 dilution; Upstate Biotechnology), which recognizes ACC when phosphorylated at serine 79 (Ser-79). Equal loading of samples is also confirmed by Coomassie blue staining of all gels.

AMPK Activity

Regulating energy levels is a fundamental process in every living organism. At a cellular level, ATP must be maintained at relatively high levels (normally about tenfold above the concentration of ADP) in order to drive essential metabolic processes. At a whole-body level, maintaining a balance between energy supply and energy demand is also crucial. Within the Western world, the consequences of failing to regulate energy metabolism are ever more obvious as demonstrated by the huge increase in the prevalence of metabolic disorders, such as obesity and type II diabetes (Jönsson 2002). One system that is emerging as a key player in the overall regulation of energy balance is the AMP-activated protein kinase (AMPK) cascade. AMPK is activated in response to ATP depletion (Hardie et al. 1998, 2003;

Carling 2004, 2005), which causes a concomitant increase in the AMP/ATP ratio (Corton et al. 1994). Once activated, AMPK phosphorylates a number of downstream substrates, the overall effect of which is to switch off ATP-utilizing pathways, e.g., fatty acid synthesis, and switch on ATP-generating pathways, e.g., fatty acid oxidation (Hardie et al. 1998; Kemp et al. 1999). In addition to the acute effects of AMPK, activation of AMPK has longer-term effects, altering both gene expression (Foretz et al. 1998) and protein expression (Winder et al. 2000). Although the physiological consequence of these longer-term effects of AMPK are not fully understood, it seems likely that they are involved in the overall regulation of energy metabolism.

AMPK is activated allosterically by AMP and by phosphorylation. AMPK is phosphorylated on T172 which lies within the activation loop of the kinase catalytic domain, a region where many protein kinases require phosphorylation for their activation (Johnson et al. 1996). Phosphorylation of T172 is catalyzed by an upstream kinase, the molecular identity of which remained elusive until very recently. Through advances made in the investigation of the regulation by upstream kinases of SNF1, the yeast counterpart of AMPK, the identity of the upstream kinase appears finally to have been revealed. Three closely related yeast kinases, Elm1, Pak1, and Tos3, have been identified that phosphorylate and activate SNF1 in vitro (Hong et al. 2003; Sutherland et al. 2003). The amino acid sequences of the catalytic domains of Elm1, Pak1, and Tos3 are most closely related to members of the mammalian Ca^{2+}/calmodulin-dependent protein kinase kinase (CAMKK) subgroup. Another kinase sharing significant amino acid sequence identity with the three yeast kinases was LKB1 (also known as STK11). Recent studies have shown that LKB1 phosphorylates and activates a number of AMPK-related kinases (Lizcano et al. 2004), and although the physiological role of these kinases is largely unknown, it is possible that some of the tumor suppressor functions of LKB1 are mediated via these kinases rather than AMPK itself. The finding that LKB 1 lies upstream of AMPK, however, does provide the intriguing possibility that there may be a link between metabolic regulation and cell proliferation, mediated by AMPK (Carling 2005). The following assays enable the measurement of AMPK activity as a consequence of appropriate phosphorylation/dephosphorylation by upstream kinases or by direct activation/inhibition by compounds/drug candidates which may be helpful for future antidiabetic and antiobesity therapy, respectively.

Measurement with Recombinant AMPK

Purpose and Rationale

For analysis of direct effects of compounds/drug candidates on the activity of AMPK, the enzyme has to be purified (e.g., from rat liver, isoform mixture) or heterogeneously expressed (e.g., in *E. coli* or insect cells, specific isoforms) and subjected to kinase assay in the presence of the agents. These test systems may be used for the identification and characterization of activators or inhibitors for AMPK, which both might be helpful for future therapy of diabetes and obesity, respectively.

Procedure

Expression of Recombinant AMPK

A new polycistronic bacterial expression system has been introduced by Neumann and coworkers (2003), which yields milligram amounts of heterotrimeric and functional AMPK. For this, the cDNA sequences encoding the subunits of AMPK (GenBank accession nos. X95578, X95577, and U40819) are subcloned by PCR and inserted into individual pET-plasmids (NdeI/SpeI sites of pET3-ax, γ 1-subunit, resulting in pY 1; NcoI/SpeI sites of pET-3dx, β 1-subunit, resulting in pfi 1; NcoI/SpeI sites of pET14dx, α1-subunit, resulting in pHis-a1r), harboring an ampicillin resistance gene (ampR). Successive restriction and ligation of individual cistrons to the acceptor vector pγ 1 lead to a polycistronic gene consisting of a promoter, ribosome-binding sites (RBS), and the sequences of γ-, β-, and

His-tagged α-subunits of AMPK, as well as a transcriptional terminator.

Purification of Recombinant AMPK

The tricistronic plasmid pγ 1β 1 His-α1 is transformed into competent host cells (*E. coli* BL21-CodonPlus (DE3)-RIL) and incubated overnight at 37 °C on LB agar containing 150 μg/ml ampicillin and 25 μg/ml chloramphenicol. Resuspended cells are used to inoculate LB medium containing appropriate antibiotics. Cultures are grown in a shaker incubator at 37 °C. Protein expression is induced with 0.4 mM isopropyl β-D-thiogalactopyranoside final concentration at an O.D. (600 nm) of 0.6 and cultures were grown for additional 4 h. Cells are harvested, washed twice with 0.9 % NaCl, resuspended in lysis buffer (15 % sucrose, 50 mM sodium phosphate, pH 8.0, 50 mM NaCl, 10 mM imidazole, and 1 mM β-mercaptoethanol), and sonicated on ice in a Branson 250 sonifier (50 % duty, output 5, 2 min, three times). Insoluble material is removed by centrifugation and the supernatant is loaded onto Ni–NTA agarose (Qiagen). After washing (three column volumes with lysis buffer, 20 mM imidazole) and elution (lysis buffer, containing 250 mM imidazole), the protein is stored at –20 °C until use.

Gel Filtration Chromatography

For confirmation of complex formation of AMPK holoenzyme, the size of purified AMPK in solution is determined by gel filtration chromatography with a Superose 12 HR 10/30 column (Amersham-Pharmacia) connected to HPLC. Minor nonprotein contaminants in Ni^{2+}-purified AMPK are removed by batch affinity purification with Blue Sepharose (Amersham-Pharmacia), using elution with 50 mM sodium phosphate (pH 8.5), 1 M NaCl, 50 mM AMP, and 1 mM β-mercaptoethanol. One hundred micrograms of protein is then separated in 25 mM sodium phosphate (pH 7.0), 200 mM NaCl, and 1 mM β-mercaptoethanol at a flow rate of 0.75 ml/min and a temperature of 22 °C. The column is calibrated for Stokes radii with the following marker proteins: carbonic anhydrase (24.0 Å, 29 kDa), albumin (35.5 Å, 67 kDa), aldolase (48.1 Å, 158 kDa), catalase (52.2 Å, 232 kDa), ferritin (61 Å, 440 kDa), and thyroglobulin (70.0 Å, 669 kDa).

Alternatively, partially purified AMPK holoenzyme from rat liver (up to the Blue Sepharose step according to the protocol of Carling and coworkers 1989) can be used instead of the recombinant version. It is usually obtained in a partially phosphorylated state and therefore does not require the activation step by exogenous upstream kinases, such as LKB1.

Phosphorylation/Activation In Vitro

Ni^{2+}-purified recombinant AMPK is activated with a partially purified preparation of AMPKK (purified up to the Q Sepharose step (Hawley et al. 1996), with the exception that NaF and sodium pyrophosphate are omitted from the buffers) in the presence of 100 μM ATP, 5 mM $MgCl_2$, and 1 mM DTT in 50 mM HEPES (pH 7.4), for 30 min at 37 °C in a thermostated shaker. Samples are then diluted in HEPES assay buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 10 % glycerol, 50 mM NaF, 5 mM sodium pyrophosphate, and 1 mM DTT) and assayed for AMPK activity. For labeling of AMPK with [^{32}P]ATP, Ni^{2+}-purified recombinant AMPK is incubated in the presence or absence of AMPKK (Q Sepharose pure) in the presence of 100 μM [γ-^{32}P]ATP (approx. 400 cpm/pmol), 100 μM AMP, 5 mM $MgCl_2$, and 1 mM DTT for 30 min at 37 °C in a thermostated shaker. After dilution, AMPK is repurified on Ni–NTA magnetic agarose beads (Qiagen, Basel) before adding SDS sample buffer and running on 12 % acrylamide SDS gels. Radiolabeled AMPK is visualized by autoradiography or phosphorimaging.

Alternatively, for phospho-AMPK (Thr172) detection, the 35 % ammonium sulfate precipitate from treated myotubes or hepatocytes can be used for immunoblotting with polyclonal antibodies to AMPK phosphorylated at Thr172 (Cell Signaling Technology, USA) according to the protocol of Zhou and coworkers (2001).

Radioactive Assay Method

The radioactive AMPK filter assay with [γ-^{33}P]ATP and a consensus substrate, the so-called

SAMS peptide (HMRSAMSGLHLVKRR) (Dale et al. 1995), is conducted as described by Zhou and coworkers (2001) and Davies and coworkers (1989). The 100-µl reaction mixture contained 100 µM AMP, 100 µM ATP (0.5 µCi ^{33}P-ATP per reaction), and 50 µM SAMS in a buffer (40 mM HEPES, pH 7.0, 80 mM NaCl, 0.8 mM EDTA, 5 mM $MgCl_2$, 0.025 % BSA, and 0.8 mM DTT). The reaction is initiated with addition of the enzyme. After 30-min incubation at 30 °C, the reaction is stopped by addition of 80 µl 1 % H_3PO_4. Aliquots (100 µl) are transferred to 96-well MultiScreen plates (MAPH-NOB50; Millipore Corp., USA). The plate is washed three times with 1 % H_3PO_4 followed by radioactivity detection in a microtiter plate TopCount apparatus.

AlphaScreen Assay Method

The enzymatic reactions for the AlphaScreen are carried out according to Li and coworkers (2003) in white round-bottomed 96-well plates (Packard). Each reaction mixture contained (in a total volume of 0.015 ml) ACC-CREBp (100 nM), ATP (200 µM), buffer A (50 mM HEPES, pH 7.0, 100 mM NaCl, 0.01 % BSA (w/v), 5 mM $MgCl_2$, 0.8 mM DTTl), and AMPK (0.74 mU, 1 mU = 1 pmol phosphate incorporated into 200 µM SAMS per min at 30 °C in the radioactive filter assay). The reaction is initiated by the addition of the enzyme. Incubation is at 30 °C for 30 min. The reaction is terminated by the addition of the detection mixture which contained donor and acceptor beads (40 µg/ml each), anti-pS133-CREB antibody (4 nM), and EDTA (16 mM) in buffer A (0.015 ml) without $MgCl_2$. The plate is read on an ALPHAQuest instrument (Packard Biosignal) after incubation at room temperature in the dark for 2–24 h.

HTRF Assay Method

The enzymatic reactions for HTRF are identical to those for the AlphaScreen but are carried out in black round-bottomed 96-well Optiplates (Packard) as described by Li and coworkers (2003). The reaction is terminated by the addition of detection mixture (0.035 ml) which contained SA-XL665 (14 nM), LANCE Eu-W1024-labeled anti-pS133-CREB antibody (140 µM), and EDTA (3 mM) in buffer A without $MgCl_2$. The plate is incubated for 1 h at room temperature followed by determination of fluorescence with a Discovery instrument (Packard). The 620- and 665-nm fluorescence signals are counted simultaneously and the ratio (665 nm/620 nm × 10,000) is recorded for each well.

Evaluation

Inhibition

The in vitro AMPK inhibition data are fit to the following equation for competitive inhibition by nonlinear regression using a least-squares Marquardt algorithm in a computer program: $Vi/V0 = (Km + S)/[S + Km \times (1 + I/Ki)]$, where Vi is the inhibited velocity, Vo is the initial velocity, S is the substrate (ATP) concentration, Km is the Michaelis constant for ATP, I is the inhibitor concentration, and Ki is the dissociation constant for the inhibitor. For determination of Km, AMPK is assayed at varying concentrations of SAMS peptide in the presence of 200 µM AMP. Kinetic analysis is performed using GraphPad Prism software as has been introduced for AMPK analysis by Stein and coworkers (2000).

Activation

The availability of ultrafast, ultrasensitive, and robust assays suited to high-throughput screening is key to obtaining small-molecule AMPK activators. In the absence of high-affinity and selective anti-phospho-Ser/Thr antibodies for AMPK substrates, two homogeneous AMPK assays have been developed with the commercially available antibody anti-pS133-CREB and an engineered peptide ACC-CREBp. Anti-pS133-CREB antibody was raised against the phospho-CREB peptide derived from cAMP response element-binding protein (CREB). ACC-CREBp is a variant (Arg to Pro) of ACC-CREB, a hybrid peptide consisting of a 9-amino acid peptide from rat ACC, CREB peptide, and the addition of two hydrophobic Leu residues. ACC-CREBp shows increased suitability as a substrate for AMPK, eliminates phosphorylation by PKA, and preserves antibody binding. The homogeneous

time-resolved fluorescence and AlphaScreen AMPK assays are developed using both anti-pS133-CREB antibody and ACC-CREBp that are either labeled with a fluorescent probe or linked to a photoactivated bead, respectively. Very similar biphasic ATP titration curves are generated by both the filter assay and the AlphaScreen. The inhibitory effect observed at higher ATP concentrations most likely results from ATP competing with AMP binding at the allosteric site. Consistent with this hypothesis, an increase in the AMP concentration (from 30 to 200 μM) shifted the curves to the right, suggesting that more ATP is required to compete with AMP. Nearly identical AMP titration curves are generated by the filter assay and the AlphaScreen.

A small-molecule AMPK activator of the thienopyridine class has been identified in a high-throughput screening effort (Anderson et al. 2004; Cool et al. 2006), which directly stimulates partially purified rat liver and recombinant human AMPK (EC50 ~ 1 μM) and inhibits fatty acid synthesis in primary rat hepatocytes (IC50 ~3 μM) and is lower plasma glucose in ob/ob mice. These results demonstrate that small-molecule-mediated activation of AMPK in vivo is feasible and represents a promising approach for the treatment of type II diabetes.

Immune Complex Kinase Assay

Purpose and Rationale

For analysis of the AMPK activity and its regulation ex vivo in (muscle and liver) tissues or cells upon treatment of rodents or cultured cells with putative AMPK modulators, AMPK is immunoprecipitated from total cell or tissue homogenates and then subjected to immune complex kinase assay using a consensus substrate peptide.

Procedure

Cultured myotubes or hepatocytes, incubated with AICAR or compounds/drug candidates in KRH containing 20 mM glucose for 15 min at 37 °C, or alternatively, muscle and liver tissues from rodents, treated with compounds/drug candidates, are homogenized by direct addition of an equivalent volume of ice-cold homogenization buffer (50 mM Tris/HCl, pH 7.4, 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM PMSF, 1 DTT and 10 % glycerol, 0.1 % Triton X-100,1 μl/ml protease inhibitor cocktail [Sigma]) before snap freezing in liquid nitrogen. The crude homogenate is sonicated with four pulses of 3 s each before centrifugation (18,000 × g, 3 min). Alternatively, Witters and Kemp (1992) lysed treated hepatocytes directly in digitonin-containing and phosphatase-containing buffer, followed by precipitation with ammonium sulfate at 35 % saturation.

To 200 μl of the sample, 2 μl of α-AMPK antibody (Cell Signaling Technology) is added, and the immunoprecipitation is incubated overnight at 4 °C with gentle mixing. After 12 h immunoprecipitation, 30 μl of protein A beads (50 % slurry) is added to each sample and incubated for 2 h at 4 °C with gentle mixing. The assay is started by the addition of the immune complexes to assay buffer (80 mM HEPES, pH 7.4, 160 mM NaCl, 1.6 mM EDTA, 200 μM SAMS peptide, 200 μM AMP, 200 μM ATP, 16 % glycerol, 0.1 % Triton X-100) containing [γ-^{32}P]ATP (0.5 μCi per sample). After the addition of enzyme to the reaction tube, samples are vortex mixed for 5 s and incubated for 10 min at 30 °C. Following the incubation, the reaction mixture is vortex mixed and spotted on P81 Whatman filter paper, briefly allowed to dry, and washed three times in 1 % $HClO_4$ before a single wash in acetone. After sufficient time to allow the filter papers to air dry, they are immersed in a scintillant-fluor cocktail and the activity of each sample is measured in a scintillation counter.

Selectivity Versus Glycogen Phosphorylase (GP) and Fructose 1,6-Bisphosphatase (FBP)

Rationale and Purpose

The enzymes glycogen phosphorylase (GP) and fructose 1,6-bisphosphatase(FBP) are allosterically modulated by AMP and ZMP (Longus

et al. 2003; Vincent et al. 1991). AMP activates GP, whereas it inhibits FBP. To assess the specificity of small molecules directly activating AMPK, it may be useful to study the effects of the compounds on the activity of GP and FBP. Lack of increase or decrease, respectively, in their activity indicates that unlike AICAR/ZMP these compounds do not act as general AMP mimetics but selectively activates AMPK. The possibilities of direct interaction with the AMP-binding site of AMPK (but apparently not with those of GP and FBP) or with a distinct allosteric site of AMPK can then be tackled by investigating the putative additive, subadditive, or potentiating effects on AMPK activity of combinations of maximally efficacious concentrations of compounds and increasing concentrations of AMP (and vice versa). On basis of additive effects obtained by following this strategy between AMP and a newly identified small-molecule AMPK activator, which inhibited fatty acid synthesis in primary rat hepatocytes and increases whole-body fatty acid oxidation in normal rats, Cool and coworkers (2006) suggested its binding to a unique site that differs from that of AMP binding.

GP Activity Assay

To assay GP activity according to Kaiser and coworkers (2001), 1.5 μg/ml of rabbit muscle GPb (Sigma) is added to a reaction mix containing 20 mM Na_2HPO_4 (pH 7.2), 2 mM $MgSO_4$, 1 mM β-NADP (β-nicotinamide adenine dinucleotide phosphate), 1.4 U/ml G-6-PDH (glucose-6-phosphate-de-hydrogenase), and 3 U/ml PGM (phosphoglucomutase). AMP or compounds/drug candidates are added to the assay medium followed by the addition of glycogen (final concentration 1 mg/ml) to initiate the reaction. After incubating 10 min at 25 °C, GPb activity is assessed by measuring absorbance at 340 nm.

FBPase Activity Assay

FBPase (0.01 U/ml final concentration) in 2 × assay buffer (100 mM Tris/HCl, pH 7, 4 mM $MgCl_2$, 300 mM NaCl, 0.2 mg/ml BSA, and 6 mM DTT) is added to the compounds/drug candidates to result in the final desired concentrations. Substrate, D-fructose-1,6-diphosphate, at a final concentration of 0.1 mM is added to the reaction. The reaction mix is incubated at 30 °C for 20 min. Following the incubation, two volumes of Malachite Green solution (Upstate Biotechnology, USA) containing 0.001 % Tween 20 are added and absorbance at 640 nm is read immediately.

AMP/ATP Levels

Purpose and Rationale

To confirm that effects elicited by small-molecule AMPK activators (e.g., inhibition of fatty acid synthesis, stimulation of fatty acid oxidation) are mediated by direct upregulation of AMPK activity rather than by cytotoxic effects (e.g., uncoupling of mitochondria, blockade of mitochondrial respiration) ultimately leading to decreased levels of ATP and increased levels of AMP, the AMP/ATP ratio of cells can be measured upon challenge with the putative AMPK activator. Lack of effect on AMP/ATP is compatible with the compound exerting its cellular effects by direct activation of AMPK and not as a consequence of cellular stress, toxicity, or other means of increasing the AMP/ATP.

Procedure

Primary rat hepatocytes are plated and treated as described above. Following the 4-h incubation with compounds/drug candidates, cells are washed with ice-cold PBS and the adenine nucleotides are extracted by Corton and coworkers (1994) with 500 μl/well $HClO_3$. The acid is extracted with three treatments of 1.1 volumes of 1/1 tri-*n*-octylamine:1,1,2-trichlorotrifluoroethane. 50–100 μl of the aqueous sample are injected in a Whatman Partisil 10 SAX column preequilibrated in buffer A (10 mM K_2PO_4, pH 6.75) according to the procedure of Cool and coworkers (2006). For elution, sample columns are run for 5 min in buffer A, then for 15 min in a linear gradient to buffer B (500 mM K_2HPO_4, pH 5), and finally for 35 min in buffer B at a flow rate of 2 ml/min. To measure nucleotide concentration, absorbance is read at 254 nm.

Malonyl-CoA Levels

Purpose and Rationale

Since AMPK activation inhibits ACC activity, one expected in vivo consequence is a decrease in cellular malonyl-CoA levels, leading to reduced lipid synthesis and elevated fatty acid oxidation (see "Fatty Acid Oxidation"). Thus, in conjunction with increased ACC phosphorylation in primary rat hepatocytes, a reduction in malonyl-CoA levels may be anticipated in the liver of rats following treatment with compounds/drug candidates.

Procedure

Malonyl-CoA levels in rat tissues from animals treated with the appropriate feeding paradigm and sacrificed 1.5 h after treatment with compounds/drug candidates are measured according to the procedure of Cool and coworkers (2006). Tissues are harvested, immediately frozen in liquid nitrogen, and then homogenized in a 1:10 volume (w/v) of ice-cold 5 % sulfosalicylic acid containing 50 µM dithioerythritol. Homogenates are centrifuged (15,000 × g, 60 min, 2 °C) and the supernatants filtered through a 0.22-µm filter (Ultrafree-MC, Millipore, USA). Samples are stored at –80 °C prior to liquid chromatography mass spectrometry analysis. HPLC is run at binary mode (A: 5 mM dimethylbutylamine and 6 mM HOAc; B: 0.1 % formic acid in CH_3CN) at 200 µl/min with the third pump to deliver post-column mixing solvent CH_3CN. The data are acquired on a TOF mass spectrometer using positive ion TOF-MS mode.

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Acetyl-CoA Carboxylase (ACC) Activity

General Considerations

Activation of AMPK increases phosphorylation of various downstream target proteins including ACC, a key component in the regulation and coordination of fatty acid metabolism and catabolism (Carling 2004). ACC, the rate-limiting enzyme for fatty acid biosynthesis, produces malonyl-CoA via the catalysis of the carboxylation of acetyl-CoA which acts as inhibitor of CPT 1 (Ruderman et al. 2003). Thereby, ACC controls mitochondrial β-oxidation in negative fashion coordinating fatty acid synthesis and degradation in reciprocal fashion. Inhibition of ACC may lead to stimulation of fatty acid oxidation in both muscle and liver with beneficial effects for the therapy of type II diabetes and obesity.

In theory, any component of ACC could be a potential target for drug discovery. In fact, a high-throughput screening assay that screens for inhibitors of the holoenzyme has been described by Soriano and coworkers (2006). However, a more robust assay could increase the potential for finding inhibitors. One assay commonly used to measure CT activity is an enzyme-coupled assay that relies on the absorbance of NADH (Guchhait et al. 1974). High-throughput assay formats based on the UV detection of NADH are problematic because the screening libraries often

contain many compounds that absorb strongly at 340 nm, thereby generating false negatives.

DTNB Method

Purpose and Rationale

A modification of the coupled enzyme assay for carboxyltransferase that avoids the use of NADH detection and therefore offers a potential advantage in a typical HTS environment has been introduced by Santoro and coworkers (2006). The CT reaction is assayed in the reverse direction in which malonyl-CoA reacts with biocytin (an analog of the biotin carboxyl carrier protein) to form acetyl-CoA and carboxybiotin. The production of acetyl-CoA is coupled to citrate synthase, which produced citrate and coenzyme A. The amount of coenzyme A formed is detected using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB = Ellman's reagent). The assay has been developed for use in both 96- and 384-well microplate formats and validated using a known bisubstrate analog inhibitor of CT. The spectrophotometric readout in the visible absorbance range used in this assay does not generate the number of false negatives associated with frequently used NAD/NADH assay systems that rely on detection of NADH using UV absorbance.

Procedure

The assay buffer consists of 100 mM potassium phosphate (pH 7.6) with 0.1 % Tween 20. In preparing the reagent stock solutions, 10 mM malonyl-CoA is dissolved in water, 100 mM OAA is dissolved in assay buffer, 1.2 mM DTNB is dissolved in assay buffer, 100 mM biocytin is sonicated in assay buffer, and ACC is dissolved in assay buffer. Commercially obtained citrate synthase solution is diluted 1:10 in assay buffer. Using the above stock solutions, assay solutions are prepared. "CT solution" contains 30 ml of OAA stock, 3 ml of ACC, and 67 ml of assay buffer, and "CS solution" contains 6 ml of malonyl-CoA stock solution, 36 ml of biocytin stock solution, 6 ml of citrate synthase solution, and 52 ml of assay buffer. Final concentrations of reagents in the assay are as follows: 200 μM malonyl-CoA, 12 mM biocytin, 10 mM OAA, 200 μM DTNB, 21.9 nM ACC, and 104 nM citrate synthase.

Automated screening is performed on an integrated core system (e.g., Beckman SAGIAN). The assay plates used are prespotted with 1 μl of each test compound/drug candidate per well. Using an automated liquid dispenser, 30 μl of CT solution is added to each assay well. The plates are incubated at room temperature for 5 min 40 s to allow for association of potential slow-binding inhibitors with ACC. An automated liquid handler with 384-tip pod (e.g., Beckman Biomek FX) is used to add 9 μl of DTNB to each well. The reaction is then initiated by the addition of 20 μl of CS solution using the liquid handler (with gentle aspiration mixing). The plate is promptly transferred (40 s after the beginning of the CS addition step) to an automated 384-detector (e.g., Molecular Devices SPECTRAmax Plus), and the absorbance at 412 nm is recorded. A second reading is taken 5 min 40 s later. The HTS assay is carried out at room temperature.

Evaluation

The quality of this assay for throughput assay for inhibitors to the CT subunit of ACC is demonstrated by a large assay signal window, low data variation among controls, and strong Z′ factors. The assay is reliable for many hours and reproducible from day to day. The screening assay described here will be a useful tool in the discovery of potential antidiabetic and antiobesity drugs targeting human ACC.

Phosphate Measurement

Colorimetric Method

All assays are performed using clear 96-well plates (e.g., Costar). The order of addition of assay components depended on the type of experiment performed. For initial velocity measurements, all assay components except the substrate with variable concentration are premixed in 200 mM HEPES/KOH (pH 8). The reaction is initiated by adding an equal volume of the missing substrate. Solutions of varying ATP and

acetyl-CoA concentrations are prepared in doubly distilled/deionized water; holo-biotin carboxyl carrier protein solutions are prepared in 50 mM HEPES (pH 7.3) and 10 % glycerol. Aliquots (50 µl) are removed from the reaction mix at fixed time points and placed in a 96-well plate preloaded with 100 µl BioMol Green reagent (BIOMOL) to quench the reaction. Incubation time for color development, typically 30 min, is the same for all mixtures. Absorbance is read at 620 nm using a microplate spectrophotometer. Initial rates are measured within a 10 % turnover range of the limiting substrate. Phosphate concentration is determined using a standard curve.

Charcoal Adsorption–Scintillation Counting Method

Alternatively, phosphate can be measured using the charcoal adsorption–scintillation counting method adapted from Stitt and Xu (1988). All assay solutions included a trace of [γ-^{33}P]-labeled ATP. Initial velocity measurements are as described for the colorimetric method. At various time points, a 30-µl aliquot is transferred to an Eppendorf tube, fitted with a 0.45-pm filter, containing 120 µl of 5 % TCA and 50 µl of 80 mg/ml activated charcoal. After vigorous mixing, the tube is spun in a microcentrifuge (11,000 × g, 30 s) to filter out the charcoal. A 50-µl aliquot of the filtrate is added to 7 ml of aqueous scintillation fluid and counted for 1 min in a scintillation counter. To obtain the 100 % turnover count (plus background), a 30-µl reaction mix is placed in 170 µl of 5 % TCA without charcoal and a 50-µl aliquot is counted as above. Background counts, measured by repeating the assay adding water instead of enzyme, are subtracted from all measurements to determine percentage turnover.

Scintillation Proximity Assay (SPA) Method

Assay solutions (33 µl) containing 5 (or 10) µM ATP, 1 (or 2) nCi/pl [γ-^{33}P]-labeled ATP, 20 µM acetyl-CoA, 40 nM AccC, 40 nM AccA/D, and 80 nM BCCP in buffer A (100 mM HEPES/KOH, pH 8, 150 mM NaCl, 10 % glycerol, and 1 mM DTT) are incubated for 1 h. The reaction is quenched by the addition of 10 µl of freshly made solution A (two volumes of solution B [30 mg/ml wheat germ agglutinin-coated polystyrene imaging beads or 30 mg/ml streptavidin-coated SPA scintillation beads, Amersham-Pharmacia Biosciences, in 50 mM HEPES/KOH, pH 7.3] mixed with one volume of 16 mM ammonium heptamolybdate in 2.4 N HCl solution). Shortly thereafter (but no longer than 40 min after), 10 µl of 256 mM citrate solution is added. The beads are spun down (2,000–3,000 rpm, 5 min). For experiments using SPA imaging beads, the plate is kept in the dark for at least 1 h before reading the signal using the LEADseeker instrument (Amersham-Pharmacia Biosciences). For experiments using SPA scintillation beads, radioactivity is determined in a TopCount instrument (e.g., Packard) after the plate has been centrifuged.

Evaluation

Initial velocity measurements as a function of substrate concentration are carried out using either the charcoal adsorption–scintillation counting method, the colorimetric method, or the SPA method. Data are fitted to the Michaelis–Menten equation (using, e.g., the GraphPad Prism software).

In general, phosphate determination is a simple and robust method for the measurement of the overall activity of ACC. The monitoring of phosphate production, at low (nanomolar) protein concentrations, is demonstrated to represent the overall activity of ACC due to the coupled activities of biotin carboxylase and CT and the cycling of biotin cofactor attached to BCCP between the two half-reactions. The method has been adapted for a 384-well screening assay format and validated to monitor competitive inhibitors of either biotin carboxylase or CT. The use of holo-BCCP instead of free biotin, as well as the use of physiologically relevant concentrations of the protein components, makes this assay a good model for the in vivo ACC system and will facilitate the identification of physiologically relevant inhibitors.

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Gluconeogenesis, Ketone Body Formation, and TCA Cycle

Purpose and Rationale

De novo synthesis of glucose in the liver from precursors such as fructose, pyruvate, lactate, gluconeogenic amino acids, and glycerol is a central mechanism to provide the organism with glucose in times of starvation (Cherrington 1997; Nordlie et al. 1999). On the other hand, when glucose is directly available from external resources, gluconeogenesis is dispensable and consequently needs to be shut off. Integration of these events is complex and occurs through various hormonal and nutritional factors (Barthel et al. 2005). The principal parameters affecting hepatic glucose output are the concentrations of the available glucogenic substrates and the activity of a few regulatory enzymes. The activity of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) is regulated by transcriptional and non-transcriptional mechanisms, whereas the third key enzyme, fructose-1,6-bisphosphatase (FBPase), is also regulated through competitive inhibition by fructose 2,6-bisphosphate. Insulin is the most important hormone that inhibits gluconeogenesis. It acts predominantly by suppressing the expression of the genes for the key gluconeogenic enzymes, PEPCK and G-6-Pase (Barthel and Schmoll 2003).

Over the recent years, considerable progress has been made in understanding the mechanisms and molecular details involved in the regulation of hepatic glucose production and its impairment in diabetes. A spectrum of different experimental approaches, ranging from genetic manipulations like knockout models and use of gain or loss of function-mutated constructs in cellular models to the development and application of selective pharmacological compounds, has provided evidence for the convergence of multiple signaling pathways in particular on the level of the transcriptional regulation of PEPCK and G-6-Pase gene expression, the key gluconeogenic genes in liver cells. Analogous to multiple braking systems in cars or motorbikes, the redundancy in the regulation of hepatic glucose production emphasizes the critical importance of this process in the glucose homeostasis of the organism. On the other hand, our increasing knowledge in this field has made it possible to identify a group of promising pharmacological targets, therefore providing a solid basis for the development of new and more potent antidiabetic drugs.

Hepatocytes

Purpose and Rationale

Isolated hepatocytes can be used to study the effect of drugs on hepatic gluconeogenesis and other hepatic metabolic reactions such as ketone body formation and the tricarboxylic acid cycle.

Procedure

Male Wistar rats weighing 200–300 g are used. The hepatocytes are isolated by perfusion of the liver with collagenase (Berry and Friend 1969; Seglen 1976; Alvarez et al. 1987). The viability of the isolated hepatocytes can be evaluated by the Trypan blue test. Usually 90–95 % of the cells exclude the stain and can therefore be regarded as intact. The isolated hepatocytes (20×106 cells/vial) are suspended in 3.0 ml of KRB containing 4 % BSA. The cell suspension is preincubated for 15 min at 37 °C in a Dubnoff metabolic shaking incubator gassed with carbogen. The following substrates are added in various combinations, and each sample is incubated for 60 min with 1 mM each of alanine, fructose, glycerol, lactate, and 10 mM pyruvate or 2 mM sodium palmitate bound to albumin. Compounds/drug candidates are added in concentrations between 0.05 and 5.0 mM. At the end of the 60-min incubation period, 0.2 ml of 70 % $HClO_4$ is added into the medium to stop the reaction. The reaction mixture is then centrifuged, and the supernatant obtained is used to determine the intermediate metabolites. Glucose is assayed by the glucose oxidase method, lactate (Gutmann and Wahlefeld 1974), pyruvate (Czok and Lamprecht 1974), acetoacetate (Mellanby and Williamson 1974), and β-hydroxybutyrate (Williamson and Melanby 1974) by appropriate enzymatic methods.

In order to measure the influence on the tricarboxylic acid cycle, (U-^{14}C) alanine, (1-^{14}C) glutamate, (1-^{14}C) pyruvate, (1-^{14}C) palmitate, or (1-^{14}C) glucose is added after preincubation together with various combinations of compounds and substrates and incubated for 60 min. The radioactivity is measured by the $^{14}CO_2$-capturing method (Gliemann 1965).

Perfused Isolated Rat Liver

Purpose and Rationale

The technique of liver perfusion has been described extensively by Ross (1972), mentioning in his introductory remarks that the isolated liver was first used by Claude Bernard in 1885. Derived from several modifications, the perfusion of rat liver from the portal vein is the most widely used technique.

Evaluation

Several parameters can be determined in the effluate, such as net glucose production from lactate and net lactate utilization. The values are plotted against time before and after addition of the compounds and drug candidates, such as insulin or sulfonylureas.

Modifications of the Method

This method has been widely used for studying carbohydrate and lipid intermediary metabolism (Herling et al. 1998) as well as drug metabolism (Milne et al. 1997, 2000; Vuppugalla 2004). Many variations have been reported predominantly with respect to the animal species used. Chaib et al. (2004) compared isolated perfused livers of rats with those of guinea pigs. den Butter et al. (1994) used livers from rabbits. Further modifications are related to the direction of perfusion via hepatic artery or portal vein or both simultaneously or in connection with the isolated jointly perfused small intestine (Stumpel et al. (1997, 2000) as well as the continuous perfusion in a recirculated (see above) or open (non-recirculated) manner (Lopez et al. 1998).

Alexander and coworkers (1992) studied hepatic blood flow regulation in an isolated dual-perfused rabbit liver preparation. Alexander and coworkers (1995) described a miniaturized perfusion circuit using a novel design of organ bath, to maintain a buoyant preparation, and a high-efficiency miniaturized membrane tubing oxygenator for testing hepatic function during prolonged isolated rat liver perfusion. Kobayashi and coworkers (1991) found inhibition of gluconeogenesis in isolated hepatocytes from normal, fasted rats by the sulfonylurea, glimepiride.

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Phosphoenolpyruvate Carboxykinase (PEPCK) Activity

Radioactive Method

PEPCK is measured using the $NaH^{14}CO_3$ fixation assay as described by Noce and Utter (1975) and Burcelin and coworkers (1995) with some modifications. Four hundred ninety microliters of rat liver cytosol is added to 500 µl of reaction buffer containing 150 µmol Tris/acetate (pH 7.2), 5 µmol sodium IDP, 10 µmol $MnCl_2$, 250 µmol KCl, 10 mM DTT, 2 mM GSH, 400/150 µmol $KHCO_3$, and 15 µCi $NaH[^{14}C]O_3^-$ (10 µmol, Amersham-Pharmacia). The reaction is started by the addition of 10 µl of 0.4 M phosphoenolpyruvate and terminated after 10 min incubation at 25 °C by addition of 1 ml of 6 N HCl and placing the tube on ice. After dilution with 1 ml of deionized water, unreacted CO_2 ($H[^{14}C]O_3^-$) is removed by bubbling with N_2 and CO_2 for 30 min each. The reaction mixture is supplemented with 10 ml of aqueous scintillation cocktail (Beckman ReadySafe) and measured for radioactivity by liquid scintillation counting. From each value, appropriate blanks are subtracted containing the same ingredients but lacking either cytosol or IDP. Under these conditions and up to the maximal amount of cytosol used, the incorporation rates are linear with both cytosol concentration and time during the first 15 min at least.

Bioluminescent Method

Alternatively, PEPCK is determined using nonradioactive bioluminescent microassay, also suitable for frozen tissue samples (Wimmer 1988). For preparation of frozen tissue samples, weighed frozen (e.g., liver) samples were homogenized (under liquid nitrogen) in nine volumes of ice-cold 25 mM HEPES/KOH (pH 7.5) by using a Teflon-in-glass homogenizer. Activity is measured on basis of a three-step reaction sequence. First, phosphoenolpyruvate is formed in the PEPCK reaction in a total reaction volume of 1.04 µl reaction buffer I containing 50 mM potassium phosphate (pH 7.5), 10 mM $MgSO_4$, 0.1 mM $MnSO_4$, 0.1 mM EGTA, 1 mM mercaptoethanol, 2.5 mM oxaloacetate, and 0.05 % BSA. The reaction is started by addition of 0.69 µl of 5 mM inosine-5′-triphosphate. After incubation for 20 min at 25 °C, the reaction is terminated by the addition of 0.69 µl of 250 mM Na_3PO_4/K_2HPO_4 (pH 12.0) and heating to 70 °C for 10 min. Second, ATP is formed from phosphoenolpyruvate and ADP after neutralization by the addition of 0.69 µl of 0.25 N HCl and start of the reaction by addition of 0.69 µl of reaction buffer II containing 50 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 2 mM ADP, 2.5 mM KCl, 5 mM $MgCl_2$, 0.05 U pyruvate kinase, and 0.05 % BSA. After incubation for 45 min at 25 °C, the reaction is terminated by the addition of 0.69 µl of 0.7 N NaOH. Third, ATP is determined by luminometry. For this, 1 µl of assay mixture is transferred into a cuvette (Hamilton Microlab 1000) and then supplemented with 199 µl of redistilled H_2O. After addition of 50 µl of reagent solution from the ATP

luminescence CLS kit (Roche Biochemicals), the peak luminescent signal is recorded by luminometry (LKB Wallac luminometer). Distinct lots of reagent solution have to be standardized using known concentrations of phosphoenolpyruvate and ATP. Standards consisting of phosphoenolpyruvate and ATP are added to the first step reaction mixture. To obtain tissue blanks, reactions performed in the presence of the reaction mixture are incubated with and without substrate. To obtain controls, samples are incubated without substrate. Tissue blank values remain constant for about 2 h. Control values are variable depending on the remaining ATP content. Typical standard deviations for blank measurements of the same sample are below 10 %. As specificity test, 0.5 mM 3-mercaptopicolinic acid (inhibitor of PEPCK) is added to the reaction buffer for the first step. Typically, inhibition is higher than 98 %. Non-inhibitable values may be subtracted from total values. Upscaling by tenfold, at least, is feasible but may require dilution prior to the ATP determination step, i.e., addition of the reagent solution.

Activity Staining Method

Enzyme activity staining after native polyacrylamide gel electrophoresis (PAGE) is a widely used technique for isozyme analysis. It allows the detection and visualization of multiple isoforms of a specific enzyme present in a small amount of extract. The study of isoforms by native PAGE, however, is often limited by the availability of a specific and sensitive detection method. A number of glycolytic enzymes can be linked to a dehydrogenase and to NAD(P) reduction (Rivoal et al. 1989). Using this approach, NAD(P)H generation can be visualized by reduction of electron acceptors, phenazine methosulfate and nitroblue tetrazolium (which forms an insoluble purple precipitate). This type of activity stain is widely used. However, this approach cannot be used with some enzymes. This is the case for PEPCK, which catalyzes an irreversible reaction. In spectrophotometric assays, PEPCK activity is measured by a coupled assay linked to malate dehydrogenase in the oxidizing (NADH-consuming) direction, precluding the use of nitroblue tetrazolium detection for staining after native PAGE. PEPCK can be detected after native PAGE using an activity stain (Karn et al. 1973; Law and Plaxton 1995; Rivoal et al. 1998). This stain is based on the detection of PEPCK reaction product oxaloacetate by reaction with Fast Violet B. However, this method has several drawbacks such as long incubation times and lack of sensitivity and therefore can only be used reliably with purified enzymes (Law and Plaxton 1995; Rivoal et al. 1998). In contrast, a staining technique is described here that allows the sensitive detection of PEPCK. The method is very specific since it is based on the same reaction used for the spectrophotometric determination of the enzyme. Also a native PAGE stain is described for pyruvate kinase (PK) and PPi-dependent phosphofructokinase (PFP) based on the same principle. This serves to illustrate that the method can probably be generalized to assay all enzymes that can be linked to an NAD(P)H-consuming coupled enzyme assay.

Native PAGE and Activity Stains Using Fluorescence Detection

Native PAGE is performed on a Bio-Rad Mini Protean II system using a discontinuous system (Doucet et al. 1990) with the modifications described (Law and Plaxton 1995) except that gels are run for 4 h at 150 V and 4 °C. Enzyme activity staining is carried out at room temperature. For all the coupled enzyme assay/fluorescence detection methods, immediately after electrophoresis, gels are incubated at room temperature in the indicated equilibration buffer (10 ml) for 15 min. This solution is removed and replaced by the indicated development buffer. Staining developed over 5–10 min and is visualized on a UV transilluminator. Activity bands (sites of NADH oxidation in the gel) appear as dark bands over a fluorescent background. In all cases, control experiments are carried out in order to verify that enzyme activity staining is substrate dependent and therefore specific for the activity tested. The gels are routinely photographed using a Polaroid T-667 film (3000ISO/36DIN) and an exposure time of 3 s. Image analysis and

quantification are done with ImageJ, a public domain program available from http://rsb.info.nih.gov/ij/. The solutions used in equilibration and development are as follows: PEPC equilibration buffer (50 mM Bis/Tris propane (pH 8.4), 2.5 mM $KHCO_3$, 10 mM $MgCl_2$, 5 mM DTT, and 15 % (v/v) glycerol), PEPCK development buffer (PEPCK equilibration buffer plus 1.5 mM PEP, 0.15 mM NADH, and 2 U/ml rabbit muscle MDH), PK equilibration buffer (50 mM MES/50 mM Bis/Tris propane (pH 6.8), 1 mM DTT, 5 % PEG (Mr 8000), 50 mM KCl, and 10 mM $MgCl_2$), PK development buffer (PK equilibration buffer plus 2 mM PEP, 1 mM ADP, 0.15 mM NADH, and 2 U/ml rabbit muscle LDH), PFP equilibration buffer (50 mM HEPES/KOH (pH 6.8) and 5 mM $MgCl_2$), PFP development buffer (PFP equilibration buffer plus 5 mM fructose 6-phosphate, 5 mM fructose 2,6-bisphosphate, 0.4 mM PPi, 0.15 mM NADH, 1 U/ml aldolase, 10 U/ml triose-phosphate isomerase, and 1 U/ml glycerol-3-phosphate dehydrogenase), and PEPC activity stain using the Fast Violet B method. Detection of PEPCK by the Fast Violet B method is adapted (Karn et al. 1973). Immediately after electrophoresis, the gel is soaked for 15 min in 10 ml of 50 mM Bis/Tris propane (pH 8.4), 10 mM $MgCl_2$, 5 mM DTT, and 15 % (v/v) glycerol. This solution is then replaced by 10 ml of 50 mM Bis/Tris propane (pH 8.4) containing 2.5 mM $KHCO_3$, 1.5 mM PEP, 10 mM $MgCl_2$, 5 mM DTT, 15 % (v/v) glycerol, and 3 mg/ml freshly prepared Fast Violet B. PEPCK activity appears as red bands. After 30 min staining, the reaction is stopped by soaking the gel in 50 ml of 5 % (v/v) acetic acid.

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Glucose Transport

General Considerations

Skeletal muscle contains several glucose transporters. Evidence indicates that GLUT4 translocation to the cell surface is the major mechanism responsible for regulated glucose uptake in this tissue. Insulin and muscle contractions are the most important stimuli for GLUT4 mobilization from the intracellular storage compartment to the plasma membrane. Nevertheless, other factors like hypoxia, catecholamines, and glucocorticoids can alter this mobilization. Contraction and insulin cause increases in the maximal velocity of glucose transport (Nesher et al. 1985) through three mechanisms: (1) a conformational alteration in cell surface glucose transporter leading to increased transport activity, (2) increase in the

number of GLUT4 in the plasma membrane, and (3) rapid synthesis of new transporters (Hayashi et al. 1997, Rea and James 1997). The current knowledge on the differential effects of insulin and contraction on glucose transport in skeletal muscle has recently been reviewed by Pereira and Lancha (2004).

Purpose and Rationale

Some studies have been developed with the purpose of explaining the two different mechanisms that stimulate glucose transport due to the action of insulin and muscle contraction. One hypothesis is that there are two separate pools of GLUT4 in the skeletal muscle. GLUT4 from one pool is thus translocated by the action of insulin but not of contraction, whereas GLUT4 from another pool is translocated by contraction stimulation but not by insulin. In any case, on basis of the major contribution of the skeletal muscle in the insulin-stimulated whole-body glucose disposal in humans, compounds/drug candidates which activate the muscle glucose transport by engagement of either the insulin- or contraction-induced signaling pathway may be helpful for the future antidiabetic therapy. The following tissue- and cell-based assays can be used for their identification and characterization.

Method Based on Diaphragms

Procedure

Intact washed rat diaphragms are incubated (30 min, 37 °C) in HEPES-buffered saline (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 0.5 mM sodium pyruvate, 1.5 mM KH_2PO_4, pH 7.4) under constant bubbling with 95 % O_2/5 % CO_2. The diaphragms are then washed two times with the same buffer lacking glucose and further incubated (30 min) in 5 ml of glucose-free buffer in the presence of insulin/compounds/drug candidates. Glucose transport is initiated by addition of 50 μl of 10 μM 2-[1-^{3}H] H]deoxyglucose (10 μCi/ml) in the absence or presence of 25 μM cytochalasin B (control). After 15 min, the diaphragms are rinsed four times with ice-cold buffer containing 10 mM glucose and 25 μM cytochalasin B, blotted with filter paper, and homogenized. Portions of the homogenate are used for protein determination (BCA method).

Evaluation

One-milliliter portions of the supernatant of a centrifugation (10,000 × g, 15 min) are mixed with 10 ml scintillation cocktail and counted for radioactivity.

Method Based on Myocytes

Procedure

Transport studies are carried out using six-well Falcon plates. Cells are routinely plated at a density of 1.5 × 105 cells/well. Medium is aspirated and each well is washed with 10 ml of PBS. Nine hundred microliters of uptake buffer (PBS containing 1 mg/ml BSA) are added to each well. Transport studies are carried out at 23 °C and are initiated by adding 100 μl of 2-[1-^{3}H] deoxyglucose (10 μCi/ml) to the desired final concentration (up to 500 μM). At appropriate times, uptake is terminated by rapidly washing the cells twice (less than 15 s) with 10 ml of ice-cold PBS. In the case of 3MG uptake, cells are washed with cold PBS containing 1 mM mercuric chloride. Samples are taken at 15, 30, 45, and 60 s after the addition of radioactive substrate. Cells are solubilized with 1 ml of 0.1 % Triton X-100, and 0.8 ml aliquots are counted in 10 ml of scintillation fluid. Under these conditions, the uptake of 3MG and 2DG is linear with time, and over 95 % of the internalized 2DG are phosphorylated. Cells in two wells from each plate are detached with 0.1 % trypsin and counted using a Coulter counter. Studies are carried out in duplicate and each experiment is repeated at least twice. Results are consistent in all cases. Data are analyzed by a linear least-squares regression fit program (e.g., SlideWrite Plus, version 4.0, Advanced Graphics Software, CA) and by a nonlinear regression data analysis program (e.g., EnzFitter program, Biosoft, UK).

Evaluation

Specific glucose transport (dpm/mg of protein) is calculated as the difference between diaphragm-associated radioactivity measured in the absence (total uptake) and presence of cytochalasin B (nonspecific uptake). Under these experimental conditions, transport of 2DG is linear for up to 30 min.

GLUT4 Translocation in Myocytes

General Considerations

Understanding the molecular mechanisms of insulin action has been of major interest since the discovery, several decades ago, that insulin stimulates glucose transport in vivo by inducing GLUT4 translocation (see above). GLUT4 translocation is a complex vesicular traffic process which includes fusion and docking steps. Herbst and coworkers (1995) showed previously that introduction of a peptide corresponding to tyrosine-phosphorylated IRS-1 motifs into 3T3-L1 adipocytes resulted in a significant increase in the translocation of GLUT4 to the plasma membrane by immunofluorescence staining which mimicked the response to insulin. However, no relative increase in the rate of glucose uptake was observed in those cells. This led to the speculation that some stimulus may bring GLUT4 vesicles to the plasma membrane (docking) but is not sufficient to trigger fusion with the membrane. A converse phenomenon was observed by others upon introducing isoproterenol to rat adipocytes whereby the insulin-dependent gain in glucose transporter detected in isolated plasma membrane was not changed while glucose transport was decreased. It is therefore conceivable that immunostaining of isolated membranes or of permeabilized cells cannot distinguish GLUT4 proteins docked on the plasma membrane from those which are fully fused with it. It is well conceivable that compounds/drug candidates provoke GLUT4 translocation without concomitant glucose transport activation and, vice versa, transport activation in the absence of translocation.

Purpose and Rationale

Subcellular fractionation, cell photolabeling coupled to immunoprecipitation and immunofluorescence, or immunoelectron microscopy has been used to detect translocation of GLUT4 to the cell surface. All of these methods are laborious and suffer from methodological inaccuracies. Subcellular fractionation is cumbersome and produces membranes that are rarely pure. Moreover, quantitative recovery of all membrane compartments is difficult or impossible. Affinity labeling of surface GLUT4/1 followed by immunoprecipitation depends on the ability to obtain quantitative immunoprecipitation and recovery upon SDS-PAGE. This technique results in the incorporation of about 1/10,000 of the label added; thus the signal-to-noise ratio is low. Moreover, the reactivity of the photolabel can depend on the level of activity of the GLUT in addition to the amount of GLUT exposed at the cell surface. Immunofluorescence detection does not distinguish the native GLUT4 molecules incorporated into the cell membrane from molecules in subplasmalemmal vesicles and is not a quantitative technique. Immunogold electron microscopy detects antigens at the plasma membrane accurately but has not been successfully used in a quantitative fashion. None of the above techniques is suitable for large numbers of experiments, as would be required for throughput screening and even characterization of antidiabetic drugs or of agents interfering with insulin signaling or intracellular trafficking. Therefore, a fast, sensitive, and quantitative technique is needed to measure GLUT4 translocation in intact cultured myocytes, which does not rely on immunoprecipitation, SDS-PAGE, or use of radioactivity, as required by the photolabeling technique, and does not require large amounts of cells or laborious subcellular fractionation.

Previous studies have shown that in CHO cells or other fibroblasts stably expressing heterologous GLUT4, insulin-stimulated translocation of the transfected GLUT4 is not detectable by immunoblot analysis of subcellular fractions nor by immunofluorescence microscopy (Kanai et al. 1993). However, transfection of GLUT4myc allowed for detection of the exofacial epitope by

125I-labeled secondary antibody. By this approach, insulin-induced GLUT4myc translocation was observed in CHO cells (Wang et al. 2000). For study of GLUT4 translocation in L6 myocytes, GLUT4myc has to be introduced into L6 insulin-sensitive muscle cells which express endogenous GLUT4 as well as GLUT1 and GLUT3. The amount of GLUT4myc expressed at the surface of basal cells (5 fmol/mg protein) is usually in the range of that of native GLUT4 (about 12 fmol/mg). This discrete level of expression of GLUT4myc likely allows for its correct localization without saturation of the proteins determining its intracellular sorting.

Procedure

Construction and Culture of GLUT4 Ectopically Expressing L6 Myocytes

For the construction of GLUT4myc-expressing L6 myocytes, the human c-myc epitope (14 amino acids) is inserted into the first ectodomain of GLUT4 as described by Kanai and coworkers (1993). The epitope does not affect GLUT4 activity. GLUT4myc cDNA is subcloned into the mammalian expression vector pCXN (pCXN-GLUT4myc). L6 myocytes are transfected with pCXN-GLUT4myc and pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride. L6-GLUT4myc myocytes in cell monolayers are maintained in α-MEM supplemented with 10 % FBS in a humidified atmosphere containing 10 % CO_2/90 % air at 37 °C. Cells are grown in 12- or 24-well plates for immunofluorescence and on glass coverslips for fixation. Prior to experiments, cells are incubated with serum-free α-MEM supplemented with 25 mM glucose for 5 h.

Method Based on Indirect Immunofluorescence

Quiescent L6-GLUT4myc cells grown on glass coverslips are treated with insulin/compounds/drugs, then rinsed once with PBS, fixed with 3 % paraformaldehyde in PBS for 3 min at room temperature, and neutralized with 1 % glycine in PBS at 4 °C for 10 min. The cells are incubated with PBS containing 10 % goat serum and 3 % BSA at 4 °C for at least 30 min. Primary antibody (anti-c-myc 9E10) is added at a dilution of 1:100 and maintained at 4 °C for 30 min. The cells are extensively washed with cold PBS before introducing the secondary antibody (Cy3-IgG, 1:1,000) for 30 min at 4 °C. The cells are washed, then mounted, and immediately examined by immunofluorescence microscopy.

Method Based on Cell Fixation

Procedure

Quiescent L6-GLUT4myc cells treated with insulin/compounds/drugs are washed once with PBS, fixed in 3 % paraformaldehyde in PBS for 3 min at room temperature. Thereafter, the fixative is immediately neutralized by incubation with 1 % glycine in PBS at 4 °C for 10 min. The cells are blocked with 10 % goat serum and 3 % BSA in PBS at 4 °C for at least 30 min. Primary antibody (anti-c-myc, 9E10) is then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4 °C. The cells are extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1,000). After 30 min at 4 °C, the cells are extensively washed and 1 ml OPD reagent is added to each well. The colorimetric reaction is stopped by addition of 0.25 ml of 3 N HCl for 10 min at room temperature. The supernatant is collected and the optical absorbance is measured at 492 nm. Standard curves are generated using either peroxidase conjugated anti-mouse IgG alone or myc tag peptide. The myc tag peptide at various concentrations is coated onto 24-well plates by incubation at 4 °C for 24 h then allowed to dry. The plates are rinsed with PBS to remove excess salt and the uncoated spaces are blocked with 10 % goat serum and 3 % BSA.

Evaluation

Introduction of a tag c-myc epitope (14 amino acids) into the first exofacial loop of GLUT4 cDNA allows for the direct detection of GLUT4myc molecules translocated to the plasma membrane upon fixation of the intact myocytes under conditions which do not allow access of the anti-myc antibodies to intracellular structures,

such as the GLUT4 vesicles. The efficacy of this method has been compared with that of the conventionally used subcellular fractionation and immunofluorescence approaches. Virtually similar effects of insulin on GLUT4 translocation are detected by all three approaches.

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Glycogen Synthesis

General Considerations

Insulin and contractile activity are major regulators of glycogen metabolism in skeletal muscle (Pederson et al. 2005). Insulin stimulates glycogen synthesis, and postprandially, ~80 % of ingested glucose is taken up by skeletal muscle and converted to glycogen (Jue et al. 1989a, b; Shulman et al. 1990). Under these conditions, insulin activates glycogen synthase (GS), as well as glucose transport, via translocation of the GLUT4 transporter. Glycogen is a major fuel for the contractile activity of skeletal muscle. During contraction, glycogen is utilized as a source of energy, and it has been demonstrated that, perhaps paradoxically, glycogen resynthesis occurs while glycogen is being broken down (Price et al. 1991, 1994). Presumably, this represents a mechanism for the rapid replenishment of glycogen stores when exercise ceases (Bloch et al. 1994; Ivy and Kuo 1998). The period following exercise is characterized by increased glucose uptake and net glycogen synthesis in skeletal muscle, a scenario similar to insulin stimulation of muscle. Despite the fact that the mechanism of GS activation in response to insulin has been extensively studied, the molecular details of both insulin- and contraction-induced activation remain mostly unknown. The following assays enable the demonstration of stimulatory effects on glycogen synthesis in muscle cells of compounds with insulin-like activity which may contribute significantly to their blood glucose-decreasing potency.

Method Based on Diaphragms

Procedure

Male Sprague Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia and the diaphragms are carefully removed, spread out, and divided into two equal pieces. The hemidiaphragms are incubated in Krebs–Henseleit buffer gassed with carbogen (95 % O_2/5 % CO_2) with 5 mM [U-^{14}C]glucose

(0.5 μCi/ml) in the presence of insulin/compounds/drugs. After 30 min incubation at 37 °C, the hemidiaphragms are blotted on tissue, frozen in liquid nitrogen, and ground in a porcelain mortar and pestle chilled with liquid nitrogen. Samples of the powdered tissue are weighed and dissolved by heating for 45 min at 100 °C in 30 % KOH (1 ml/100 mg tissue) before ethanol is added to a final concentration of 70 %. After 4 h at –20 °C, the samples are centrifuged (2,000 × g, 10 min). The glycogen pellets are washed three times with 70 % ethanol before the amount of 14C-labeled glycogen is determined by liquid scintillation counting. Total glycogen is determined according to Lowry and Passoneau (1972) after hydrolysis to glucose (1N HCl at 100 °C for 3 h). The incorporation of [^{14}C]glucose into radiolabeled glycogen is normalized for the total amount of glycogen.

Evaluation

The concentration dependence of [U-^{14}C]glucose uptake and conversion into glycogen by insulin/compounds/drug candidates is determined. The isolated diaphragm of rats or mice is the preferable organ to study the effect of insulin and substances with insulin-like effects, such as sulfonylureas, on muscle tissue or the influence of denervation (Standing and Foy 1970; Smith and Lawrence 1984; Ishizuka et al. 1990; Hothersall et al. 1990).

Method Based on Myotubes

L6 myotubes are washed three times with Krebs-Ringer phosphate HEPES (KRPH) buffer (150 mM NaCl, 5 mM KCl, 2.9 mM Na_2HPO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4) supplemented with 0.1 % BSA. The cells are then incubated for 30 min to up to 2 h with 25 μl of reaction medium (glucose-free α-MEM containing 20 mM HEPES, pH 7.4, 0.1 % BSA, 100–500 μM [^{14}C]glucose [0.1–0.5 μCi]) in the presence of increasing concentrations of insulin/compounds/drug candidates at 37 °C in a humidified atmosphere of 8.5 % CO_2. Subsequently, the reaction medium is removed by aspiration and the cells are washed with 100 μl of ice-cold PBS. The cells are then disrupted by incubation in 50 μl of 1 N NaOH for 10 min at 60 °C. The cell homogenates are cooled to room temperature and transferred to 96-well FC/DV filter plates (Millipore) containing 100 μl of ice-cold ethanol. The plates are then incubated for 2–3 h at 4 °C. The precipitate is filtered under vacuum and washed three times with 250 ml of ice-cold 66 % ethanol. The filters are dried under a 100-W incandescent lamp. 50 μl of scintillation cocktail (e.g., MicroScint 20, Packard) are added and the wells are sealed. The [^{14}C]glucose incorporated into cellular glycogen is quantified in a TopCount 96-well liquid scintillation counter.

Evaluation

In order to identify hypoglycemic drugs with insulin-like or insulin-sensitizing activity, Berger and Hayes (1998) developed an assay for activators of glucose incorporation into glycogen-utilizing differentiating L6 muscle cells in 96-well plates. In general, in vitro assays of glucose incorporation into glycogen in insulin-sensitive tissue have been used in culture media containing the normal physiological concentration, ~5.5 mM, of unlabeled glucose and tracer levels of radiolabeled glucose (e.g., Chou et al. 1987; Robinson et al. 1993). Similarly, using this assay design and L6 myotubes, Berger and Hayes (1998) observed that such conditions do not produce glycogen of a high enough specific activity to be accurately quantified in single-well extracts from a 96-well plate. In contrast, use of glucose-free α-MEM as the reaction media increases the signal by more than one order of magnitude, thereby allowing quantification of single wells in the 96-well format. Consequently, addition of exogenous (nonradioactive) glucose is omitted, which apparently does not lead to physiological alterations in course of the brief incubation with compounds/drug candidates and the subsequent assay period. Glucose incorporation follows in a time- and concentration-dependent fashion and is blocked by typical inhibitors of glucose transport. Both insulin and pervanadate exerting insulin-like activity increase glycogen synthesis in concentration-dependent manner. This assay may serve as a high-capacity

screen to identify novel compounds that upregulate glucose anabolic metabolism in skeletal muscle.

Glycogen Synthase (GS) Activity

General Considerations

Glycogen metabolism is controlled largely by the coordinated action of the two enzymes glycogen synthase (GS) and glycogen phosphorylase (GP). Both enzymes are controlled by covalent phosphorylation and by allosteric effectors (Cohen 1986; Roach 1991; Roach et al. 2001). GS undergoes a complex multisite phosphorylation at nine sites by several protein kinases (Roach et al. 2001), most notably PKA, casein kinase I, casein kinase II, GSK-3, and AMPK (Carling and Hardie 1989), which generally lead to inactivation. Important regulatory phosphorylation sites are distributed between the amino- (sites 2 and 2a) and the carboxyl-termini (sites 3a and 3b) of the GS molecule (Lawrence et al. 1983; Skurat et al. 1994, 2000). Full activity can be restored to phosphorylated enzyme by the presence of the allosteric activator glucose-6-phosphate (G-6P). GP is activated by phosphorylation of a single site (GP a) by phosphorylase kinase (Cohen 1987). The less active, dephosphorylated form (GP b) acquires full activity in the presence of the allosteric effector AMP. Dephosphorylation of all three of these key regulatory proteins, GS, GP, and phosphorylase kinase, is believed to be catalyzed primarily by glycogen-associated phosphatases (PP1G) (Hubbard and Cohen 1989a, b).

Method Based on Diaphragms

Procedure

GS is assayed according to Oron and Larner (1979), Guinovart and coworkers (1979), and Altan and coworkers (1985) with the following modifications: Intact hemidiaphragms are dissected from male Wistar rats and incubated in DMEM (10 ml/hemidiaphragm) with constant bubbling of 95 % O_2/5 % CO_2. For treatment with insulin/compounds/drug candidates, the hemidiaphragms are incubated (37 °C) in the same medium containing 5 mM glucose. For preparation of homogenates, the diaphragms are blotted and frozen in liquid nitrogen. The frozen diaphragms (pool of four) are manually ground in a porcelain mortar and then homogenized at 0 °C in ten vol. of 25 mM Tris/HCl (pH 7.4), 100 mM NaF, 5 mM EDTA, and 0.1 mM PMSF. The homogenate is centrifuged (10,000 × g, 20 min). The supernatant is used for the GS assay. After addition of 10 µl of diaphragm homogenate to 200 µl of 25 mM Tris/HCl (pH 7.4), 50 mM NaF, and 10 mM EDTA (preincubated at 30 °C) containing either 0.1 or 10 mM G6P, the reaction is initiated by supplementing 0.2 mM [U-14C]UDP-glucose (10 µCi) and terminated after 15 min by the addition of 2.5 ml of ice-cold 66 % ethanol and filtration over pre-wetted Whatman GF/C glass fiber disks. The filters are washed, dried, and counted for radioactivity. Blanks are assayed by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol.

Evaluation

The fractional velocity as parameter for the portion of GS active in vivo (l-form) toward the total enzyme contents (l-+d-forms) at the time point of homogenization is calculated as ratio between the activities measured at 0.1 (l-form) and 10 mM G-6P (l-+d-forms).

The concentration dependence of sulfonylurea-stimulated GS and its potentiation by insulin in the isolated rat diaphragm were studied by Müller and coworkers (1994).

Method Based on Myotubes/ Hepatocytes

Procedure

GS activity is assayed by measuring the incorporation of D-[14C]glucose from UDP-[^{14}C]glucose

into glycogen. L6 myotubes or HepG2 cells cultured in 6- or 12-well plates and serum-deprived overnight are incubated with insulin/compounds/drugs in serum-free α-MEM under an atmosphere of 5 % CO_2 for 30 min at 37 °C. After removal of the incubation medium and washing, the cells are scraped into 0.2–0.5 ml of 10 mM Tris/HCl (pH 7.5) containing 10 mM EDTA, 150 mM KF, and 5 mM DTT. The cell lysates are centrifuged (5,500 × g, 2 min, 10 °C). The infranatant below the lipid layer is cleared from residual lipids by two additional centrifugations (defatted homogenate) and served as source for GS. The reaction is started by adding 30 µl of the homogenate to 60 µl of a reaction mixture (prewarmed at 30 °C) containing 33 mM Tris/HCl (pH 7.8), 0.2 mM UDP-[U-^{14}C]glucose (4 µCi), 6.7 mg glycogen, 150 mM KF, and 0.1 mM/10 mM G-6P. After incubation for 20 min at 30 °C, the reaction is terminated by addition of 2 ml of 66 % ethanol and 10 mM LiBr (–20 °C), rapid mixing, and filtration over pre-wetted Whatman GF/C glass fiber disks. The filters are washed five times with 5 ml of 66 % ethanol each at 25 °C, dried, and measured for radioactivity. Blank values determined by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol are subtracted from the total values each.

Evaluation

The fractional velocity is calculated as the ratio of GS activity in the presence of 0.1 mM and 10 mM G6P. Measurements of GS activity in defatted homogenates from myotubes/hepatocytes which had been incubated in the presence of 0.1 mM glucose instead of 5 mM glucose (the concentration routinely used) did not reveal significant differences with respect to both the activity ratio and the effect of insulin. Presumably, the dilution of the limited amount of G6P, which accumulates during incubation with 5 mM extracellular glucose, during the subsequent preparation of the homogenate and the GS assay is high enough to prevent allosteric activation of GS. According to our experience, incubation of the isolated rat adipocytes in the presence of 5 mM glucose has a positive impact on their viability, in general, and insulin sensitivity (glucose transport, glycogen synthesis), in particular.

Phosphorylation State of GS

Purpose and Rationale

A large body of evidence suggests that insulin activation of GS proceeds via the PI-3K/Akt pathway that leads to phosphorylation and inhibition of GSK-3 (Shepherd et al. 1995; Cross et al. 1995). However, GSK-3 alone is not sufficient to account for GS dephosphorylation and activation by insulin (Lawrence and Roach 1997; Skurat et al. 2000). The mTOR, mammalian target for the immunosuppressant drug rapamycin, pathway is also activated by insulin. Rapamycin has been shown to block insulin-mediated activation of GS in muscle and 3T3-L1 adipocytes (Azpiazu et al. 1996, Shepherd et al. 1995) without affecting insulin-induced inactivation of GSK-3 (Cross et al. 1997), opening the possibility that mTOR could control GS phosphorylation via a phosphatase. Therefore, insulin may promote glycogen synthesis both via inhibition of GSK-3 and stimulation of a glycogen-associated type 1 serine/threonine protein phosphatase, PP1G. Studies with mice deficient in the regulatory subunit of PP1G indicate that although PP1G is not necessary for activation of GS by insulin, it is essential for regulation of glycogen metabolism under basal conditions and in response to contractile activity and may explain the reduced muscle glycogen content in the KO mice, despite the normal activation of GS (Aschenbach et al. 2001). Contractions may utilize a separate signaling pathway from insulin to activate GS in response to contractions. Changes in GS activity in human muscle biopsy samples obtained during isometric contractions are associated with changes in protein phosphatase activity (Katz and Raz 1995), but the identity of this enzyme has not been determined.

The phosphorylation state of GS in muscle and liver cells and the impact of compounds/drug candidates can be monitored by 32P labeling and subsequent immunoprecipitation of GS.

Procedure

Myotubes/hepatocytes are washed twice by flotation in low-phosphate medium composed of KRP-HEPES modified to contain 50 μM KH_2PO_4, 2 mM glucose, and 2 % BSA and then suspended in the same medium (7.5 × 105 cells/ml). Five milliliter portions are incubated with [^{32}P]phosphate (0.2 mCi/ml) for 2 h prior to addition of insulin/compounds/drug candidates and of 0.5 ml of 50 mM glucose. After incubation (20 min, 37 °C), the cells are floated by centrifugation (1,000 × g, 1 min) and the infranatant is aspirated. The cells are suspended in 1.5 ml of cold 50 mM Tris/HCl (pH 7.6), 100 mM KF, 20 mM glycerol-3-phosphate, 10 mM $K_4P_2O_7$, 10 mM EDTA, 1 mM benzamidine, and 0.2 mM PMSF and homogenized by ten strokes using a tight-fitting Teflon-in-glass homogenizer in the same Eppendorf cup. The homogenate is centrifuged (18,000 × g, 30 min, 4 °C). One milliliter of the post-mitochondrial supernatant is incubated with 10 μl of GS antiserum raised in guinea pigs by immunization with purified GS from rabbit skeletal muscle. After incubation for 2 h at 4 °C, 50 μl of protein A-Sepharose (10 % w/v in TES; see below) is added and the incubation continued overnight. The immunoprecipitates are collected by centrifugation (12,000 × g, 2 min, 4 °C) and washed twice with 1 ml each of TES (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl) containing 1 % TX-100, twice with TES, and finally once with 50 mM Tris/HCl (pH 7.4). The beads are suspended in 25 μl of 2 × Laemmli sample buffer, heated (95 °C, 5 min), and centrifuged. The supernatant is subjected to SDS-PAGE (8 % resolving gel). The amount of [^{32}P] contained in GS is determined by excising the corresponding gel pieces and measuring their radioactivity by liquid scintillation counting.

Protein Phosphatase 1G (PP1G) Activity and Phosphorylation

PP1 Activity

Muscle PP1 activity is measured according to a protocol from Begum and Ragolia (1996). L6 myotubes treated with insulin/compounds/drug candidates are scraped off the 24-well dishes with 0.3 ml of phosphatase extraction buffer containing 20 mM Tris/HCl (pH 7.2), 2 mM EDTA, and 2 mM EGTA with 10 μg/ml each of aprotinin, leupeptin, antipain, soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM PMSF. The cells are sonicated for 10 s and centrifuged (2,000 × g, 5 min) and the supernatants are used for the assay of PP1G activity in the presence and absence of 2 nM okadaic acid to inhibit PP2A activity. The conditions of the assay allow measurement of PP1 and PP2A activities and not PP2B and PP2C enzymes, which require divalent ions. Okadaic acid at 2 nM inhibits PP2A only, and the PP1 activity remaining in the assay is comparable with the activity inhibited by inhibitor 2 (Srinivasan an Begum 1994). Purified [^{32}P]-labeled GP b is used as a substrate. [^{32}P]-labeled GP b is prepared by reacting γ[^{32}P]ATP with purified phosphorylase kinase and GP a (Cohen 1983).

In Vivo Phosphorylation and Immunoprecipitation of PPI

Differentiated L6 myotubes are serum starved overnight. The next day, the medium is removed and replaced by 1 ml of phosphate-free DMEM and incubation is continued for 1 h. [^{32}P]-orthophosphate is added (0.5 mCi/ml), and the cells are incubated for 4 h followed by incubation with compound/drug candidate for up to 1 h. Subsequently, the cells were rinsed four times with 1 ml of ice-cold PBS containing phosphatase and protease inhibitors and harvested in 0.5 ml of lysis buffer (20 mM triethanolamine, pH 7.2; 0.5 mM EGTA; 1 mM EDTA; 2 mM

sodium vanadate; 100 mM sodium pyrophosphate; 100 mM sodium fluoride; 40 mM glycerol-3-phosphate; 1 mM benzamidine; 0.1 mM PMSF; 10 μg/ml each of leupeptin, aprotinin, antipain, trypsin inhibitor, and pepstatin A; 140 mM NaCl; and 1 % Triton X-100. The cell lysates are centrifuged (16,000 rpm, 10 min) to remove cell debris. One hundred micrograms of cell lysate protein are diluted to 1 ml with lysis buffer and precleared by incubation with rat IgG (5 μg/ml, coupled to protein A-Sepharose) at 4 °C for 1 h. The supernatants are immunoprecipitated with PP1G subunit antibody (10 μg/ml) for 1 h at 4 °C followed by treatment with 50 μl protein A-Sepharose CL6B (50 %, by vol.). The pellets are washed four times with 1 ml of lysis buffer and resuspended in 40 ml of twofold SDS-sample buffer. The samples are incubated (37 °C, 10 min) followed by centrifugation (10,000 × g, 1 min) to pellet the Sepharose beads. Electrophoresis of the immunoprecipitates is performed in 7.5 % SDS-polyacrylamide gels followed by autoradiography. The protein contents of PP1G regulatory subunit are determined by immunoprecipitating unlabeled cell lysates with anti-G subunit antibody, followed by separation of immunoprecipitated proteins on SDS-PAGE. The proteins are transferred to polyvinylidene difluoride membrane and probed with PP1G subunit antibody. The regulatory subunit of PP1 is identified by incubating with [125I]protein A (0.2 μCi/ml) followed by autoradiography. The intensity of the signal is evaluated by densitometric analysis of the autoradiograms as well as by radioactivity scanning.

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Lipid Metabolism in Muscle and Liver Cells

General Considerations

Type II diabetes is characterized by hyperglycemia, hyperinsulinemia, reduced ability to oxidize fat, and TAG accumulation in skeletal muscle fibers. Impaired glucose transport and glycogen synthesis are well documented in insulin-resistant subjects (Richter et al. 1988; Hansen et al. 1992; Boden et al. 1996; Kawanaka et al. 2001), but lipid metabolism is less clearly understood (Goodpaster and Kelley 1998). Especially, focus has been put on the increased intramyocellular TAG content, since an inverse correlation between insulin resistance and intramyocellular TAG has been demonstrated (Pan et al. 1997; Koyama et al. 1997; Simoneau et al. 1995; Phillips et al. 1996; Boesch et al. 1997; Szczepaniak et al. 1999). The mechanism responsible for TAG accumulation remains unclear, but it may depend on either an increased lipid uptake or a decreased lipid oxidation as de novo lipogenesis within skeletal muscle is low. Increased plasma-free fatty acid (FFA) availability induces insulin resistance within hours by lipid infusion or within weeks by feeding rats a high-fat diet or in massively obese humans accompanied with intramyocellular TAG accumulation (Storlien et al. 1991; Oakes et al. 1997; Griffin et al. 1999; Boden et al. 2001; Perseghin et al. 1999; Chalkley et al. 1998). Weight loss and lowering of plasma free fatty acids reduced insulin resistance and the TAG content (Roden et al. 1996; Boden et al. 2001). Several studies have shown an association between obesity, insulin resistance, and reduced oxidative capacity in skeletal muscle (Bachmann et al. 2001; for reviews, see Guo 2001; Saloranta and Groop 1996; Boden 1997), as demonstrated by the finding of decreased mitochondrial number, altered mitochondrial morphology, and decreased expression of mitochondrial genes of the oxidative metabolism (Morino et al. 2005; Petersen et al. 2004; Mootha et al. 2003; Patti et al. 2003). Deficiency of CPT leads to pathological TAG accumulation in

muscle tissue further emphasizing the role of mitochondria for lipid homeostasis. Our current knowledge on TAG accumulation in relation to insulin resistance and type II diabetes originates mainly from in vivo studies rendering it difficult to determine the contribution of genetic and environmental factors to TAG accumulation in type II diabetes and obesity. Cultured myotubes offer a unique model for separating the genetic influence on insulin resistance and type II diabetes from environmental factors. The following assays can be used for the analysis of lipid metabolism in cultured muscle cells and its modulation by compounds and drug candidates.

Incubation with Fatty Acids

Solutions containing fatty acids are prepared according to the procedure described by Montell and coworkers (2002). Sodium salt of oleic acid is prepared immediately prior to utilization by dissolving the fatty acid in deionized water containing 1.2 eq. of NaOH at 70 °C with stirring until an optically clear dispersion is obtained. The fatty acid salt solution is immediately added to DMEM containing fatty acid-free BSA with continuous agitation to avoid precipitation. Typically, the fatty acid:BSA molar ratio is 5:1. Myocyte cultures are grown in a DMEM/M199 medium (3/1) supplemented with 10 % fetal bovine serum, 10 μg/ml insulin, 2 mM glutamine, 25 ng/ml fibroblast growth factor, and 10 ng/ml epidermal growth factor. Immediately after myotube differentiation, cells are rinsed in Hanks' balanced salt solution, and a medium devoid of fibroblast growth factor, epidermal growth factor, and glutamine is added. Myotube cultures are maintained in this medium for up to 2 weeks.

Lipid Synthesis

Incorporation of [14C]Glycerol into Lipids

For qualitative analysis of esterification of glycerol into AG, cells are incubated with 5 mM [U^{14}C]glycerol (100 μCi/mmol), 5 mM glucose, and 1 mM sodium oleate (BSA:oleate molar ratio of 1:5) for 15 h (Montell et al. 2002). The cell monolayers are then washed three times in Hanks' balanced salt solution, and the lipids are extracted twice with hexane/isopropanol (3/2). After drying under nitrogen, the residual lipid extract is redissolved in chloroform/methanol (2/1) as described (Folch et al. 1957) and separated by TLC by use of hexane/diethyl ether/acetic acid (70/30/1). The lipid spots are identified by iodine vapor and counted in a phosphorimager.

Incorporation of [^{14}C] Glucose into Lipids

The synthesis of neutral lipids starting from glucose can be followed according to the protocol of Aas and coworkers (2004). Myotubes are incubated with either [1-^{14}C]oleic acid (18.5 kBq/ml, 0.6 mM) or [1-^{14}C]palmitic acid (18.5 kBq/ml, 0.6 mM) in the absence or presence of insulin/compound/drug candidate for 4 h before they are harvested into ice-cold PBS, centrifuged (1,000 × g, 5 min), resuspended in distilled water, and sonicated. Cell-associated lipids are extracted with chloroform/methanol as described (Folch et al. 1957). Briefly, 400 μl of cell homogenate is mixed with 8 ml of chloroform/methanol (2/1, by vol.), and FCS (30 μl) was added as a carrier. After 30 min, 1.6 ml of 0.9 % NaCl (pH 2) is added and the mixture is centrifuged (1,000 × g, 5 min). The organic phase is evaporated under a steam of nitrogen at 40 °C. The residual lipid extract is redissolved in 200 μl of n-hexane and separated by TLC using hexane/diethylether/acetic acid (65/35/1) as the mobile phase. The bands are visualized with iodine, excised, and counted by liquid scintillation. Lipids are also extracted after incubation of myotubes with D-[^{14}C(U)]glucose (74 and 111 kBq/ml, 5.5 mM or 20 mM) for 24 h.

Evaluation

Extraction of lipids is carried out after incubation of myotubes with D-[^{14}C(U)]glucose for 4 h. Myotubes are exposed to DMEM supplemented with 0.24 mM fatty acid-free BSA, 0.5 mM L-carnitine, 20 mM HEPES, 5.5 or 20 mM [D-^{14}C(U)]glucose [(2.0 μCi/ml), and

insulin/compound/drug candidate. On the basis of the typically very low incorporation, the overall glucose incorporation into lipids is determined by counting the total lipid extract washed with a water phase that contained 0.5 M glucose to displace all glucose labeled.

Incorporation of [^{14}C] Palmitate into Lipids

For analysis of lipid synthesis starting with fatty acids according to the protocol of Busch and coworkers (2005), cells are treated for 48 h with insulin/compounds/drug candidates. Two hours before the end of the treatment, 2.5 μCi [^{14}C] palmitate in ethanol is added to each well (24-well dish, 0.5 ml culture medium). The labeled cells are put on ice, washed in 3 × 0.5 ml ice-cold PBS, scraped off the dish in 1 ml cold PBS, and spun (3,000 × g, 10 min). The supernatant is discarded, and lipids in the cell pellet are extracted overnight in 1 ml chloroform/methanol (2/1, by vol.). The extractions are washed in 250 μl H2O, followed by an additional wash in 125 μl H_2O, and the resulting lipid-containing, organic phase is dried under a stream of nitrogen. The lipids are redissolved in 50 μl chloroform/methanol (2/1, by vol.), and a small fraction is used to determine the total amount of counts in the extracted lipids. The rest of the sample is spotted onto silica plates. The lipids are separated by TLCD-14C(U) in petroleum ether/diethyl ether/methanol/acetic acid (180/14/4/1, by vol.). Spots comigrating with TAG and cholesterol ester standards are individually scraped and counted by liquid scintillation spectrometry. Protein is measured in parallel using a bicinchoninic acid protein assay.

Incorporation of [^{14}C] Acetate into Lipids

This protocol has been adapted from methods previously described (Foretz et al. 1998; Garcia-Villafranca et al. 2003; Cool et al. 2006). Primary rat hepatocytes are isolated as described (see chapter "▶ Insulin Target Tissues and Cells") and plated at 5 × 104 cells per well on collagen-coated, black-walled 96-well plates in DMEM supplemented with 10 % FBS, 5 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES/KOH (pH 7.4), 0.1 mM nonessential amino acids, 5 μg/ml transferrin, 100 nM dexamethasone, 100 nM insulin, and 25 μg/ml gentamycin. After 4 h of incubation, medium is replaced with medium as described above but lacking FBS and containing 100 nM triiodothyronine. Following a 16-h incubation at 37 °C, the medium is removed and replaced with medium containing [^{14}C]acetate (2 μCi/ml) and, as a control, AICAR or compounds/drug candidates. Cells are incubated (4 h, 37 °C). Subsequently, the plates are rinsed with PBS three times. The final wash is replaced with scintillation cocktail (e.g., MicroScint-20, Perkin Elmer) and radioactivity incorporated into fatty acid monitored on a microplate β-reader.

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Lipolysis

General Considerations

TAG is accumulated in LD in the cytoplasm of skeletal muscle cells (Schick et al. 1993; Oscai et al. 1990). The energy content of this TAG store is higher than the energy content of the muscle glycogen pool. The muscle TAG concentration is increased by a high-fat diet (Van der Vusse et al. 1996) and in poorly controlled diabetes (Standl et al. 1980; Stearns et al. 1979), and is inversely related to whole-body insulin action (Pan et al. 1997). On the other hand, although existing studies are not unambiguous, the general view is that the intramuscular TAG stores can be mobilized by catecholamines (Froberg et al. 1975; Abumrad et al. 1980) and by exercise (Oscai et al. 1990; Van der Vusse et al. 1996). The exercise-induced decrease in muscle TAG concentration can be reduced by β-adrenergic blockade and must accordingly be due to some extent to sympathetic stimulation (Stankiewicz-Choroszucha and Gorski 1978). While it seems that the intramuscular TAG constitutes a dynamic energy store, the enzymatic regulation of TAG breakdown in the muscle is poorly understood (Van der Vusse et al. 1996; Martin 1996; Hagstrom-Toft et al. 1997; Kerckhoffs et al. 1998). The expression of HSL and its regulation by adrenaline has been investigated by Langfort and coworkers (1999).

It is thought that HSL is the major enzyme responsible for the hydrolysis of stored TAG in skeletal muscle similar to in adipose tissue. HSL protein or mRNA has been detected in rodent (Holm et al. 1987; Langfort et al. 1999) and in human (Roepstorff et al. 2004) skeletal muscle but with a considerable lower expression than in adipose tissue. The HSL protein expression also varies between fiber types, being higher in oxidative than glycolytic fibers (Langfort et al. 1999). Furthermore, it was recently shown (Hämmerle et al. 2002) that HSL-deficient mice accumulated DAG in adipose tissue and skeletal muscle, indicating that when HSL is missing, this leads to an incomplete hydrolysis of TAG with an interruption of the lipolytic cascade at the stage of diacylglycerol hydrolysis. This observation was supported by the findings of a marked reduction in the formation of fatty acids in skeletal muscle as in several other tissues (Hämmerle et al. 2002). Interestingly, when the specific TAG hydrolase activity (the enzymatic conversion of TAG to DAG) was calculated, the specific TAG hydrolase activity was reduced 50 % in adipose tissue, whereas no reduction was found in skeletal muscle when comparing HSL-deficient mice with wild type (Hammerle et al. 2002). These data suggest that HSL is rate limiting in the catabolism of DAG but not of TAG hydrolysis in skeletal muscle and furthermore points to the existence of one or more lipases with considerable activity, specifically to TAG hydrolysis in skeletal muscle. Further support for the existence of lipases other than HSL involved in basal TAG hydrolysis in skeletal muscle appears from recent studies, where neutral lipase activity in human skeletal muscle only decreased by – 25 % when antiserum against HSL was added to the assay medium (Roepstorff et al. 2004; Watt et al. 2004). When a similar approach was taken in basal, resting rat soleus muscle (Langfort et al. 1999, 2000), HSL activity was decreased by ~60 %, indicating that

HSL may be less dominating in human skeletal muscle than in rat skeletal muscle and that more lipases than HSL are involved in TAG hydrolysis in the resting state. It has been shown that electrically induced muscle contractions increase the neutral lipase activity of soleus muscle of rats (Langfort et al. 2000). It is surprising from the point of view that exercise is accompanied by increases in circulating catecholamine concentrations, and there is evidence for activation of HSL by epinephrine. Thus, in incubated rat soleus muscle, HSL was activated by epinephrine (Langfort et al. 1999). The impact of skeletal muscle lipid metabolism on exercise and insulin resistance has been reviewed by Kiens (2006). The following assays can be used for investigation of the effects of compounds/drug candidates on the regulation and activity of muscle lipases.

Lipolysis in Isolated Muscle Strips

Procedure

In studies of glycerol release, muscle strips are incubated for indicated periods of time (usually 90 min) in 2 ml of KRB buffer supplemented with 20 mg/ml BSA (pH 7.4), 1 mg/ml glucose, and 0.1 mg/ml ascorbic acid in a shaking water bath (37 °C), with air as gas phase according to the protocol of Enoksson and coworkers (2005). After incubation, an aliquot of the medium is removed for analysis of glycerol using an ultrasensitive bioluminescent method (Hellmer et al. 1989). Strips are incubated in duplicate or triplicate in the absence (basal) or presence of various concentrations of isoproterenol, which is a nonselective β-adrenoceptor agonist. The muscle strip is removed from the medium and frozen in liquid nitrogen. It is then removed from the clamps and weighed. Lipolysis is expressed as millimole of glycerol per total incubation medium per time unit per milligram of muscle strip. In studies of FFA release, muscle strips are incubated and analyzed as described for glycerol release, except that 20 mg/ml of fatty acid-free BSA is used as the protein component in the incubation buffer, the number of strips in each type of incubation should range between five and ten, and FFA is analyzed by an ultrasensitive chemiluminescence method as described for adipocytes (Näslund et al. 1993).

TAG Lipase Activity in Soleus Muscle Incubation and Electrical Stimulation

For electrical stimulation of the isolated soleus muscle after 1 h preincubation with compound/drug, the isolated soleus muscle is transferred to fresh incubation medium and fixed in the vertical position and at resting length by small clips attached to the tendons according to the procedure introduced by Langfort and coworkers (2000). The upper tendon is connected to a force transducer. If subsequent electrical stimulation is required, the muscles either remain resting or are stimulated electrically through electrodes in both ends to perform repeated (1 s–1) maximal tetanic contractions (200-ms trains of 100 Hz, impulse duration 0.2 ms, 25 V) for 1, 5, 10, or 60 min while tension is recorded.

At the end of the incubation, muscles are freeze clamped with aluminum tongs cooled in liquid nitrogen, and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to further ensure that findings reflected the biology of muscle cells, these are isolated before analysis from other tissue components by microdissection using a stereomicroscope after freeze-drying. Sometimes single fibers are isolated from fresh, nonfrozen muscle by microdissection after collagenase digestion (one muscle being incubated for 3 h at 37 °C in 5 ml of DMEM containing 2 % (w/v) collagenase).

Preparation of Homogenates

Generally, muscles are homogenized (e.g., Polytron PT 3100, maximum speed) on ice in 10 vol. of 0.25 M sucrose, 1 mM DTT, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 20 mM HEPES/KOH (pH 7.0), 0.31 μM okadaic acid, 20 μg/ml leupeptin, 10 μg/ml antipain, and 1 μ/ml pepstatin. The crude homogenate is centrifuged (15,800 × g, 1 min, 4 °C). The supernatant is recovered and stored at –80 °C until analysis within 1 week. In experiments evaluating the effect of phosphatase treatment on muscle homogenate, the homogenization buffer contained 20 mM PIPES (pH 7.0) instead of sodium pyrophosphate and glycerophosphate.

HSL Assay

HSL assays are based on measurement of the release of [^{3}H]oleic acid from 1(3)-mono[^{3}H] oleoyl-2-oleoylglycerol, a DAG analog not hydrolyzable at position 2 (referred to as MOME activity), and from tri[3H]olein (referred to as TOG activity). Upon phosphorylation by PKA, the TOG activity of adipose tissue HSL increases markedly (Fredrikson et al. 1981; Cook et al. 1982), whereas the MOME activity does not change significantly (Cook et al. 1982, 1983; Greenberg et al. 2001; for a review, see Yeaman 2004). Accordingly, MOME activity is a measure of the total enzyme concentration, whereas TOG activity is a measure of the activated form of HSL and represents the assay of choice for monitoring changes in the activation state of HSL. The TOG and MOME substrates are emulsified with phospholipids by sonication, and BSA is used as fatty acid acceptor. Samples of 14 µl (for TOG activity measurements) or 7 µl (for MOME activity measurement) of muscle supernatant (protein concentration ~3 mg/ml) or pellet (resuspended to initial volume in homogenization buffer; protein concentration ~4.4 mg/ml) are incubated for 30 min at 37 °C with 100 µl of 5 mM TOG (1.25 × 106 cpm) or MOME (0.4 × 106 cpm) substrate and enzyme dilution buffer (to a total volume of 200 µl). Hydrolysis is stopped by the addition of 3.25 ml of methanol/chloroform/heptane (10/9/7, by vol.), followed by 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid (pH 10.5). The mixture is vortexed vigorously for 10 s and centrifuged (1,100 × g, 20 min). A 1-ml portion of the upper phase containing the released fatty acids is mixed with 10 ml of scintillation liquid. Radioactivity is determined in a scintillation counter.

Magnetic Resonance Spectroscopy Study of Intramyocellular Lipid Content

Kuhlmann et al. (2003) performed a longitudinal in vivo ^{1}H-spectroscopic study of intramyocellular lipid content in Zucker diabetic fatty (ZDF) rats. Magnetic resonance spectroscopy (MRS) has been established as a dependable method for selective detection and quantification of intramyocellular lipid (IMCL) in humans. To validate the interrelation between insulin sensitivity and IMCL in an animal model of type 2 diabetes, volume-selective 1H-MRS at 7 Tesla to noninvasively assess IMCL in the rat was established. In male obese ZDF rats and their lean littermates, IMCL levels were determined repeatedly over 4 months, and insulin sensitivity was measured by the euglycemic–hyperinsulinemic clamp method at 6–7 and at 22–24 weeks of age.

In vivo MRS studies were performed using a 7-T Biospec system (Bruker BioSpin, Ettlingen, Germany), a resonator for excitation and an actively decoupled surface coil for signal detection. Rats were anesthetized with 2–3 vol.% isoflurane and 1:2 O_2:N_2O, and their temperature was kept at 37.5 °C. The animals were fixed in a nonmagnetic device allowing for accurate alignment of their hind leg on top of the surface coil. Voxels of ≈8 mm^3 size were located in M. soleus and in M. tibialis anterior, avoiding vascular structures and gross adipose tissue deposits. Volume-localized 1H-MR spectra [PRESS sequence, echo time (TE) = 17 ms, repetition time (TR) = 1 s, CHESS water suppression, 1,024 averages] were obtained with Bruker's ParaVision acquisition software. The integral of the IMCL signal (1.3 ppm) was related to that of total creatine (tCr; 3.05 ppm; program MRUI 97.2, cf. http://www.mrui.uab.es). The IMCL/tCr ratio corresponded to the total muscle IMCL value. In all cases, a clear distinction between EMCL and IMCL was possible. As the IMCL/tCr ratio did not change on using a relaxation delay TR of 2 s instead of 1 s (n = 4), the influence of relaxation time changes on observed IMCL values could be excluded. The tCr values for the M. soleus and M. tibialis anterior were determined in obese ZDF rats at 8 weeks and 15 months of age (n = 8 each). In both muscles, tCr concentrations proved to be constant (soleus 89 ± 1.1 and 81 ± 1.7 µmol/g dry wt.; tibialis 136 ± 2.2 and 132 ± 1.0 µmol/g dry wt., respectively) and therefore to be a good reference for quantification of IMCL.

For assessing hepatic fat content, the rats were placed prone with their upper abdomen on top of the detecting surface coil. Volume-localized,

respiration-triggered 1H-MR spectra were obtained without water suppression (TE = 28 ms, TR = 1 s, 8 mm^3). Fat content was expressed as the ratio of the fat-to-water signal (in percentage).

Determination of Other Metabolites in Muscle

Muscle non-lipidic metabolites are determined according to the procedure of Watt and coworkers (2004). For this, muscle samples are divided into aliquots under liquid nitrogen. One piece of muscle (~80 mg wet muscle) is freeze-dried, dissected free of non-muscle contaminants under magnification, and divided into four aliquots. Muscle for glycogen analysis (3 mg) is extracted in 2 M HCl and neutralized with 0.67 M NaOH, and glycogen content is determined as described above and adapted from Passoneau and Lauderdale (1974). A second aliquot of muscle (2 mg) is extracted according to Harris and coworkers (1974), and ATP, phosphocreatine, creatine, and lactate are determined by enzymatic fluorometric techniques according to well-established procedures (Passoneau and Lowry 1993). Intramyocellular TAG content is determined by extraction of the TAG from ~6 mg of tissue in chloroform/methanol (2/1), saponification of the TAG in an ethanol-KOH solution at 60 °C, and fluorometric determination of glycerol (Froberg and Mossfeldt 1971, 1975).

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Assays for Insulin and Insulin-Like Activity Based on Adipocytes

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Contents

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Assays for Insulin and Insulin-Like Metabolic Activity

Evaluation

Data from the metabolic assays (and signaling assays; see below) are calculated as stimulation factor above basal activity (absence of insulin/compound/drug candidate) for processes stimulated (e.g., lipogenesis, glucose transport, and GLUT4 translocation) or as difference between the basal and insulin/compound/drug candidate-induced values for processes downregulated (e.g., lipolysis). In each case, these data, which reflect the responsiveness of the metabolic effector system studied toward the respective stimulus (insulin/compound/drug candidate), are normalized to the basal (set at 0 %) and maximal insulin action (set at 100 %; elicited by maximally effective concentration of insulin). For characterization of the sensitivity of the metabolic effector system toward the respective stimulus, effective concentrations for the induction of 150 % (or higher) of the basal activity (set at 100 %) can be given. These so-called EC150-values facilitate the insulin-independent comparison of the relative potency of the insulin-like activity between compounds/drug candidates, in general, and in particular for those frequently observed stimuli, which do not elicit the same maximal response in % stimulation or inhibition and/or fail to approach the maximal insulin response.

Typically, at least three different cell/tissue preparations with two to four independent incubations with insulin/compound/drug candidate and two to four independent activity measurements (i.e., assays) should be performed. Concentration/response curves can be fitted to the equation using a Marquardt–Levenberg nonlinear least squares algorithm. When plotted on linear–log axes, this equation gives a sigmoidal curve where the parameters are associated with the following properties:

The term "insulin-like activity" is used in a wide range of definitions varying in the type and number of parameters evaluated by the respective assays which have to be fulfilled by a given compound/drug candidate. An appropriate criterium for a compound/drug candidate with insulin-like activity may represent that it exerts at least 20 % of the maximal insulin response in at least three major metabolic insulin actions (e.g. glucose transport, lipogenesis, anti-lipolysis) in at least one insulin target cell/tissue. However, the final judgement of a compound/drug candidate as being appropriate for further evaluation and development has to rely on the complete data set of its profiling in many metabolic and signaling assays rather than to stick to the fulfillment of fixed criteria for one or a few parameters.

Assays for Insulin and Insulin-Like Activity Based on Adipocytes

Differentiation

Purpose and Rationale

The increase in obesity and the identification of adipocyte-secreted proteins that regulate energy metabolism (Drevon 2005) have generated huge interest in adipocyte biology. Adipocytes are the primary storage site for energy in vertebrate animals. During fasting, adipocytes release energy-rich molecules that provide metabolic fuels to other tissues. Adipocytes also secrete hormones that orchestrate the storage, release, and oxidation of energy-rich molecules throughout the body and that control behavior, including feeding (Rajala and Scherer 2003). Insulin as anabolic hormone is of critical importance for the differentiation of adipocytes in humans. Consequently, assays that monitor the quantitative analysis of adipocyte differentiation may be helpful for studying the effects of insulin/compounds/drugs on pathways which are engaged both directly in the regulation of the differentiation and in the molecular mechanisms of metabolic insulin action.

Procedure

Understanding the molecular mechanism underlying adipocyte differentiation has been one of the major focuses of many researchers in the field of metabolic disorders. Methods in use for the

assessment of fat cell development and maturation after initiation of adipogenesis in cell culture include microscopic examination of cellular LD formation and cellular lipid staining by Oil Red O (von Goor et al. 1986). These methods, although excellent in detecting the presence of intracellular lipid, are ineffective in objectively quantifying the degree of fat accumulation if not used in conjunction with other extraction and analytic systems. Particularly, during the process of adipogenesis of preadipocytes, it is observable that cells are heterogeneous in their response to adipogenic agents in terms of speed of adipogenic conversion and degree of fat accumulation (Shigematsu et al. 2001). This heterogeneity of response may cause difficulties when evaluating and comparing the effects of several treatments that target only fat cells of certain status due to the masking influence of those nontargeted cells. Thus, an effective method to detect and classify cells with similar fat content will certainly increase analytical precision in monitoring fat cell development and the ability to quantify the effects of therapeutic agents. Currently, flow cytometry and fluorescence-activated cell sorters (FACSs) are extensively used in the analysis of adipocyte differentiation and lipid accumulation as well as their modulation by compounds/drugs as is exemplified best by the study from Lee and coworkers (2004).

Evaluation

A simple and sensitive method to detect and quantify lipid accumulation inside cells by flow cytometry has been introduced (Lee et al. 2004). Using this method, elevated levels of cytoplasmic granularity can be detected that correlate well with an increased level of lipid accumulated inside cells after adipogenic conversion. Furthermore, this method is appropriate to monitor and quantify adipose cell maturation within a complex population of cells and to identify and collect the adipose cells with similar lipid storage for further analysis. Flow cytometry offers distinct advantages over existing detection systems for cytoplasmic lipid staining and lipid extraction and can represent a powerful analytical tool to monitor the effect of chemicals and biological molecules, including compounds and putative anti-obesity drugs on adipose cell conversion and maturation. Moreover, in combination with a cell sorting facility, this method offers a simple and efficient means of collecting adipose cells of specific status for further analysis. In conclusion, flow cytometry enables the direct measurement of the lipid content of specific cells in a complex population of adipose cells. Not only is the system simple, sensitive, and quantitative, but it also offers an advantage over the existing methodology, with its capability of monitoring the degree of intracellular lipid accumulation in a precise, fast, and selective manner. These results provide a basis for developing a variety of applications in compound and drug screening procedures to monitor adipose cell development.

Lipogenesis

General Considerations

Lipogenesis encompasses the complete biosynthetic pathways for TAG stored in cytoplasmic LD and phospholipids building up the internal and plasma membranes (including their lipidic intermediary and degradation products). The type as well as the concentration of the labeled precursor (e.g., glucose, fatty acids) whose conversion or incorporation into TAG and phospholipids is followed determines which step(s) of the lipogenic pathway is monitored by the lipogenesis assay (e.g., glucose transport, acylation). Thus, simple variations of the assay conditions allow the selective analysis of individual rate-limiting steps of lipogenesis and their modulation by compounds/drug candidates.

Method Based on the Incorporation of Radiolabeled Glucose

Purpose and Rationale

This assay measures the complete pathway of lipid synthesis (lipogenesis) encompassing the transport of the radiolabeleled glucose across the plasma membrane of the adipocytes, its conversion into glycerol and/or fatty acids and their subsequent esterification into predominantly

neutral TAG and phospholipids, and finally the deposition of TAG in LD in the cytoplasm of the adipocytes. Since in adipocytes each of these steps is stimulated by insulin, albeit to varying degrees, the lipogenesis assay is perfectly suited for the analysis of effects of compounds/drugs on the complex insulin signaling cascade regulating lipogenesis (Humbel 1959, Lingsoe 1961). Dependent on the experimental conditions chosen (i.e., low or high glucose), the assay monitors predominantly the effect of insulin/compound/drug on glucose transport or on the subsequent esterification. At low glucose concentrations (up to 0.1 mM glucose), when according to TLC analysis of the total TAG after enzymatic digestion, the majority of the radiolabel is incorporated into the glycerol backbone, the glucose transport step is rate limiting and monitored. In contrast, at higher glucose concentrations (above 2 mM), when two thirds of the radiolabel are recovered with the fatty acid moieties of TAG, the rate-limiting and monitored step is the esterification rather than glucose transport, which is driven by mass action. Since in adipocytes the insulin stimulation of glucose transport is significantly more pronounced than that of esterification, the lipogenesis assay performed at low glucose concentration (<0.1 mM glucose) exhibits the highest insulin responsiveness, whereas at intermediary glucose concentrations (0.1–2 mM), it represents a compromise for the measurement of both transport and esterification with intermediary insulin responsiveness, and at high glucose concentrations (>2 mM glucose), it predominantly monitors the esterification with lowest insulin responsiveness.

Procedure

For measurement of lipogenesis monitoring effects on both glucose transport and esterification, isolated rat adipocytes are incubated with D-[3H]glucose (0.55 mM final concentration, 0.1–1 μCi). The cells are lysed and the total lipids separated from water-soluble products and the incubation medium including the unincorporated [3H]glucose by addition of toluene-based scintillation cocktail. After phase separation, radioactivity incorporated into total lipids/phospholipids is determined by liquid scintillation counting directly without removal of the lipid phase based on determination of the radiolabel of the lipidic products partitioned into the toluene phase containing the scintillator rather than of the [3H]glucose left in the aqueous phase lacking scintillator (Moody et al. 1974).

The reaction is started by the transfer of 0.2 ml of adipocyte suspension (3.5 × 105 cells/ml) in KRHB to scintillation vials containing 0.1 ml of [3-3H]glucose (2 μCi/ml, 4.4 mM), 0.4 ml of twofold KRHB, and 0.3 ml of insulin or compound/drug with insulin-mimetic activity dissolved in vehicle (e.g., DMSO) and diluted with KRHB to the appropriate concentration of compound and vehicle (e.g., 3 % DMSO). The scintillation vials are placed under a stream of carbogen for 10 s and then closed and placed in a very slowly shaking water bath (37 °C). After incubation for 90 min, the reaction is terminated by the addition of 10 ml of toluene-based scintillation cocktail. The vials are mixed rigorously using a vortexer and subsequently left standing for 2–4 h to allow phase separation. The 3H-radioactivity is determined with a liquid scintillation counter.

Evaluation

Blank values obtained from a typical reaction mixture containing buffer and [3-3H]glucose but lacking either adipocytes or compound/drug/insulin have to be included in each experiment. Since the quality of the cells decreases with time (resulting in increased lipogenesis in the basal state), it is recommended to set up 2 basal incubations for every 20–25 test mixtures. In addition, for direct comparisons (e.g., of insulin analogs), incubations with identical (insulin) concentrations should be performed immediately for one agent (insulin) after the other rather than with the complete concentration series for one agent (insulin) after the other. This procedure allows the resolution of potency differences of less than 10 % between two insulin analogs as reflected in corresponding shifts of the apparent EC50-values with identical maximal responses. The blank values lacking adipocytes (usually 500–600 dpm) are subtracted from the values measured

for the corresponding set of test mixtures containing adipocytes to correct for 3H-radiation originating from the aqueous phase (i.e., [3H]glucose left in the incubation medium). Fold stimulations reflecting the responsiveness of the glucose transport and/or esterification systems of the adipocytes toward insulin/compound/drug are calculated as ratio between the corrected test values (presence of insulin/compound/drug) and basal values (absence of insulin/compound/drug). Typically, insulin induces 15- to 20-fold, 8- to 12-fold, and 2.5- to 4-fold stimulations in lipogenesis at 50 μM, 0.55 mM, and 2 mM glucose, respectively. Due to the very limited number of pipetting steps (with the most critical one being the transfer of an equal number of cells to each assay mixture), the standard deviations are rather low. Usually, two to four measurements per data point are sufficient.

An insulin-concentration/response curve should be performed for each experiment to test the insulin sensitivity and responsiveness (and thus the quality) of the adipocytes prepared. Appropriate insulin concentrations are 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0, 12, 0.15, 0.2, 0.5, 1, and 5 nM (final concentration in the assay). Typically, the EC50 for human insulin is 0.06–0.10 nM. The insulin-like activity of compounds/drugs can be expressed as % of the maximal insulin stimulation (i.e., as the ratio between the fold stimulation provoked by the compounds/drugs with insulin-like activity at the maximally effective concentration and that induced by 5 nM insulin) to correct for the varying quality (i.e., insulin responsiveness) of the cells from different preparations.

Method Based on the Incorporation of a Fluorescent Fatty Acid Analog

Purpose and Rationale

Usually lipogenesis is studied by the incorporation of radiolabeled glucose into toluene-extractable acylglycerols in the presence of glucose at concentrations below 2 mM (see above). Under these conditions, glucose transport and, in consequence, supply of glycerol-3-phosphate are rate limiting. For uncoupling of glucose transport/glycerol-3-phosphate synthesis from (re)esterification, lipogenesis has to be performed in the presence of high glucose. To circumvent analysis of lipogenesis at high glucose (>2 mM) by (radio) labeling the glycerol moiety of TAG, which necessitates the application of glucose of high specific radioactivity, a fluorescent fatty acid analog can be used for its insulin-sensitive incorporation into TAG in isolated rat adipocytes.

Several recent reports suggest that the fluorescent fatty acid derivative, NBD-stearate, behaves like naturally occurring fatty acids, since it has been shown to enter rat hepatocytes by the same uptake mechanism as that described for unmodified fatty acids (Elsing et al. 1995; Storch et al. 1995). Furthermore, measurement of the uptake of a fluorescent long-chain fatty acid derivative into COS7 cells transfected with a 3T3-L1 adipocyte cDNA library led to the identification of fatty acyl-CoA synthetase and a putative fatty acid transport protein (Schaffer and Lodish 1994). Recently the efficient incorporation of NBD-FA, 12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) dodecanoic acid, into mono- and diacylglycerol (-3-phosphate) in isolated rat adipocytes and its significant stimulation by insulin have been reported by Müller and coworkers (1997).

Procedure

Incubation of the Adipocytes

Two hundred microliters of adipocyte suspension is supplemented with 190 μl of KRHB containing 0.1/2 mM glucose (=KRHB low/KRHB high) and 10 μl of an insulin solution (routinely 200 nM) or compound/drug candidate (routinely 200 μM in 20 % DMSO) and incubated for 15 min at 37 °C in a slowly shaking water bath. The assay is started by the addition of 50 μl of 0.9 mM NBD-FA (prepared daily from a 100 mM stock solution in ethanol by dilution with KRHBlow/KRHBhigh under mild heating). After incubation at 37 °C under mild shaking (stage 11, thermomixer, Eppendorf) for 90 min, lipogenesis is terminated by filtration of the total mixtures over GF/C filters under vacuum. The filters are rapidly washed three times with 1 ml of KRHB low/KRHB high each, placed in 20 ml plastic

scintillation vials, and extracted with 400 µl THF for 15 min under rigorous shaking. Three hundred microliters of the extract is transferred into new tubes and centrifuged (15,000 × g, 5 min). The supernatant is dried (SpeedVac) and suspended in 50 µl THF. Five microliters of samples are analyzed by TLC on silica gel Si-60 plates using 78 ml diethyl ether, 22 ml petrol ether, and 1 ml acetic acid as solvent system. Fluorescent lipid products on the dried plates are visualized under UV (312 nM) and photographed (Polaroid CU5). The amount of acylglycerols (AG) is determined by fluorescence scanning with excitation at 342 nm and emission at 505 nm or fluorescence imaging with excitation at 460 nm and emission at 540–560 nm. The relative peak area of each lipid product minus a background value (derived from an equal-sized region of the TLC plate which does not contain any lipid product) is calculated as arb. units.

Characterization of the Fluorescent Lipids

For elucidation of the type of lipid(s), the synthesis of which is stimulated by insulin/compound/drug, the fluorescently labeled lipids are analyzed. This enables the characterization of the signaling and enzymatic steps preferentially activated during stimulus-induced lipogenesis. For this, adipocytes are labeled with 0.5 mM NBD-FA for 4 h at 37 °C. Total lipids are extracted from the washed cells with chloroform/methanol (3/1 by vol.), dried, and suspended in THF. NBD-fatty acylglycerol-3-phosphate, NBD-fatty acyl-palmitoylglycerol-3-phosphate, and NBD-fatty acyl-palmitoylglycerol and NBD-fatty acyl-dipalmitoylglycerol are separated by TLC (Silica Gel Si-60 F254) using toluene/ethyl acetate (9/1 by vol.) as solvent system and subsequently eluted from the plate using the line elution method with ethyl acetate as eluent. Larger amounts are separated by flash column chromatography (Still et al. 1978) on YMC-spherical silica (60 A, 40 µM, YMC Europe GmbH, Schermbeck).

Evaluation

The nitrobenzyldiazolyl (NBD) moiety of NBD-FA is a strong fluorophore and is located far away from the carboxyl terminus. Furthermore, the apparent overall length of NBD-FA (carbon backbone including NBD moiety) is comparable to palmitic acid. It has been shown that insulin stimulation of the synthesis of the three lipid species harboring one NBD-fatty acyl residue each, in isolated rat adipocytes, closely resembles that of radioactive labeling of total lipids by [3-3H]glucose with regard to both insulin responsiveness and sensitivity. The data available suggest that incorporation of one residue of NBD-FA into glycerol-3-phosphate blocks further acylation, resulting in the accumulation of mono-NBD-fatty acylglycerol-3-phosphate, NBD-fatty acyl-palmitoylglycerol-3-phosphate, and NBD-fatty acyl-palmitoylglycerol rather than of NBD-fatty acyl-dipalmitoylglycerol. One possibility is a stringent substrate specificity of diacylglycerol acyltransferase (DGAT), for the acceptor, diacylglycerol, and donor acyl-CoA.

Stimulation of the rate-limiting enzyme of the esterification steps, GPAT, by insulin has been well established (Sooranna and Saggerson 1976; Vila and Farese 1991; Farese et al. 1994). It is generally accepted that in rat adipocytes, the glucose transport step is rate limiting for insulin-stimulated lipogenesis in the presence of 0.1 mM glucose in the incubation medium (see above). Thus, under these conditions, the stimulation of synthesis of these AG by insulin may mainly reflect the effect of insulin on glucose transport. At 2 mM glucose, however, processes subsequent to glucose transport and glycerol-3-phosphate production will become rate limiting. Both transport and esterification may be studied more conveniently using incorporation of NBD-FA rather than of [3-3H]glucose into lipids (see "Method Based on the Incorporation of Radiolabeled Glucose"), in particular under conditions of screening for compounds/drug candidates. Thus, assaying lipogenesis with NBD-FA can be used as a sensitive and reliable (nonradioactive) method for analyzing insulin signaling and lipid synthesis in rat adipocytes, in particular, if there is interest on the impact of high glucose concentrations in the incubation medium (and thus of conditions of insulin resistance; see "► Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes") on these processes.

Glucose Transport

General Considerations

Glucose transport is generally assumed to represent the rate-limiting step for lipogenesis in adipose tissue in vivo and in adipocytes in vitro, at least under conditions of low to moderate concentrations of glucose in the plasma and incubation medium, respectively (see above). Its stimulation by insulin is of exquisite sensitivity and responsiveness. Insulin resistance is defined as the reduced ability of cells or tissues to respond to physiological levels of insulin and is a characteristic of non-insulin-dependent diabetes mellitus (type II diabetes). Skeletal muscle is the primary tissue responsible for the postprandial uptake of glucose from the blood in humans. The two major transporters expressed in adipose tissue/skeletal muscle are the muscle-/fat-specific glucose transporter GLUT4 and the ubiquitous transporter GLUT1. The insulin-stimulated acute activation of glucose transport mainly occurs by one of two mechanisms: translocation of GLUT4 and GLUT1 from intracellular vesicles to the plasma membrane and augmentation of the intrinsic catalytic activities of the transporters. The molecular mechanisms underlying the glucose transport and its regulation are similar for adipose and muscle cells. Consequently, adipocytes are more widely used for transport studies than myocytes due to their more convenient accessibility and more pronounced insulin responsiveness. However, studies for clarification of the mechanism by which compounds/drugs with insulin-like and/or insulin-sensitizing activity stimulate glucose uptake in skeletal muscle for the treatment of type II diabetes have to be performed with assay systems based on muscle cells or tissues. When differentiated into myotubes, cultured L6 muscle cells possess many of the properties of mature skeletal muscle tissue. They express both GLUT4 and GLUT1 and are capable of increasing glucose transport via insulin stimulation.

Purpose and Rationale

Uptake of glucose in cultured cells is commonly determined by using non-metabolizable radioactive hexoses, such as 3-O-methylglucose (3MG) or 2-deoxyglucose (2DG), labeled with a high specific activity of tritium. [3H]3MG necessitates a very short incubation time due to fast equilibration of the analog across plasma membranes and requires either rapid separation of the cells from the aqueous incubation medium of the reaction mixture, usually by centrifugation through a suitable oil cushion with a buoyant density of less than 1, or the prevention of 3MG efflux by washing with a mercuric chloride solution. Uptake assays based on [3H]2DG are more convenient because 2DG is phosphorylated to a stable and membrane-impermeable derivative, 2-deoxyglucose-6-phosphate (2DG6P), by hexokinase or glucokinase, which may accumulate at a specific intracellular compartment, ensuring less rapid equilibration, slower kinetics, and more convenient measurement of 2DG transport compared to 3MG (Frost and Lane 1985). However, routine use of these radiolabeled analogs is costly and requires a specialized institution where isotopes can be handled. Consequently, considerable efforts have been spent during the last decade in the development of assays using nonradioactive tracers, which are compatible with the analysis of glucose transport with sufficient insulin responsiveness.

Method Based on Radiolabeled 2-Deoxyglucose

Isolated Tissues

[2-3H]2DG (12 mCi/mouse) is injected i.p. into mice fasted for 6 h. After 40 min, mice are sacrificed and tissues are excised and rinsed in ice-cold PBS, 1 mM EDTA. Tissue samples are homogenized in 0.5 % perchloric acid and centrifuged. The protein pellet is solubilized in 0.3 N NaOH, 0.1 % SDS for protein determination. The supernatant is neutralized with KOH and an aliquot is counted to yield total tissue counts (2DG and 2DGP). A second aliquot is treated with Ba(OH)2 and ZnSO4 to remove 2DGP and counted to yield [2-3H]2DG. Specific [2-3H]2DGP accumulation in tissues is calculated as difference between total tissue counts and [2-3H]2DG counts.

Primary and Cultured Adipocytes

Fifty microliters of portions of adipocyte cell suspension (1 × 106 cells/ml) are pipetted into MiniSorp tubes (Nunc, Denmark) and equilibrated at 25 °C for 30 min. Insulin (80 nM stock in KRH/5 % BSA) and compounds/drugs (lyophilized, 2 mg/ml or 2 % DMSO, 30 μM) are added in 50 μl KRHB and incubated for 30 min at 25 °C. Thereafter, 50 μl of 2-deoxy-D-[1-3H]glucose (2 μCi per ml of KRHB containing 10 μl/ml of 3H stock, 0.2 mCi/ml specific activity 20–30 Ci/mmol, 0.3 mM) is added (total volume of the final incubation mixture =150 μl). After further incubation for 20 min at 25 °C in a shaking water bath, the assay is terminated by transfer of 100 μl samples on top of 250 μl dinonyl phthalate (density <0.93–0.96 g/ml, Merck, Darmstadt, FRG) in 500 μl plastic tubes (Beckman) and centrifugation in a microfuge (10,000 × g, 1 min, room temperature). The adipocytes remain on top of the oil layer, while the buffer is below the oil layer. The tube is cut with a special knife at the position of the oil layer below the adipocyte layer, then transferred into scintillation vials containing 5 ml aqueous scintillation cocktail (Zinsser Nr. 312 or Beckman Ready Safe), and counted for radioactivity. To correct for 2DG unspecifically associated with the adipocyte plasma membrane or entrapped in extracellular spaces or penetrated into the adipocytes by diffusion, 2 μl of cytochalasin B (20 μM final conc.) from a 1 mM stock solution (in 10 % ethanol, diluted with H_2O from a 10 mM stock in ethanol at the day of use) and, as a control, 2 μl of 10 % ethanol are added prior to addition of radiolabeled 2DG and incubated under identical conditions (Gliemann et al. 1972; Foley and Gliemann 1981; Müller and Wied 1993).

Evaluation

Specific transport is calculated as the difference between total cell-associated radioactivity (absence of cytochalasin B) and unspecifically associated/entrapped radioactivity (presence of cytochalasin B). Under the conditions used, 2DG transport is linear with time up to 20 min. The fold stimulation for glucose transport is calculated as ratio between stimulated specific transport (presence of insulin/compound/drug) and basal-specific transport (absence of insulin/compound/drug). For insulin (2 nM), this stimulation factor usually lies in the range between 15 and 25. To compensate for the varying insulin responsiveness of different adipocyte preparations, the insulin-like activity of compounds/drugs can be calculated as percentage of the maximal effect as described above for lipogenesis.

This assay measures the total glucose uptake encompassing the transport of the non-metabolizable glucose analog, 2DG, via the specific glucose transporters, GLUT1 and GLUT4, and the molecular mechanism of their movement from intracellular vesicles to the plasma membrane (GLUT translocation) including the underlying signaling cascade through which insulin stimulates glucose transport in adipocytes. Since 2DG taken up by the cell is immediately phosphorylated by hexokinase, the assay actually monitors the accumulation of 2DG6P (presumably in specialized intracellular compartments). However, the rate-limiting step of glucose uptake at low glucose concentrations is the transport via GLUT1/4 rather than the phosphorylation. This results in apparently longer linear periods of glucose uptake in comparison to the use of non-phosphorylatable glucose analogs, such as 3MG (see "Method Based on 3-O-Methylglucose"), and thereby considerably facilitates the experimental procedure and kinetic analysis. Subsequent conversion of the 2DG6P into lipids and glycogen or its oxidation does not occur. Thus, the use of 2DG is a reliable measure for glucose transport into adipocytes.

Method Based on Unlabeled 2-Deoxyglucose

Purpose and Rationale

A non-radioisotope, enzymatic assay based on the methods of Manchester and coworkers (1990) and Sasson and coworkers (1993) for measuring of 2-deoxyglucose-6-phosphate (2DG6P) in tissues and cultured cells has been developed by Yamamoto and coworkers (2006). These methods enable the detection of 2DG6P accumulating in cells by measurement of the fluorescence of NADPH produced from NADP+, which is

coupled to the oxidation of 2DG to 2DG6P by glucose-6-phosphate dehydrogenase (G6PDH). This approach, however, requires the cultivation of many cells on large plates and the preparation of cell extracts because the fluorescence of NADPH is rather weak. Consequently and more recently, a diaphorase–resazurin system that produces a potent fluorescent substance in the presence of NADPH has been linked. Uptake of 2DG into the cells can be measured by the addition of a single assay solution to the cell culture, followed by a simple incubation.

Procedure

The differentiated 3T3-L1 adipocytes or L6 myotubes are incubated with 170 µl/well of α-MEM with 2 % FBS in the presence of compounds/drugs/insulin for 10–30 min. After incubation, the cells are washed twice with Krebs–Ringer–phosphate–Hepes (KRPH) buffer (pH 7.4,20 mM HEPES, 5 mM KH2PO4, 1 mM MgSO4, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl) containing 0.1 % BSA. The washed cells are then incubated with KRPH buffer containing 1 mM 2DG and 0.1 % BSA for 1–2 h (termed the 2DG-uptake period) at 37 °C in 95 % O_2/5 % CO_2. After incubation, the cells are washed twice with KRPH buffer containing 0.1 % BSA, and then 25 µl of 0.1 N NaOH was added. To degrade NAD(P)H, NAD(P)+ and any enzymes in the cells, the culture plate is subjected to one freeze–thaw cycle and incubated at 85 °C for 40 min on a temperature-controlled bath. The components in the wells are then neutralized by the addition of 25 µl of 0.1 N HCl, and then 25 µl of 150 mM triethanolamine (pH 8.1) is added.

To determine the low concentrations of 2DG and/or 2DG6P present in a 96-well microplate, a diaphorase–NADPH amplifying system is combined with previous methods (Sasson et al. 1993; Manchester et al. 1990) for measuring 2DG6P in tissues and cultured cells. Fifty microliters of 2DG solution at various concentrations is dispensed into each well of a 96-well plate and incubated for 90 min at 37 °C after the addition of 150 µl of an assay cocktail of 50 mM triethanolamine (pH 8.1), 50 mM KCl, 0.5 mM $MgCl_2$, 0.02 % BSA, 670 µM ATP, 0.12 µM NADP+, 25 µM resazurin sodium salt, 5.5 units/ml hexokinase, 16 units/ml G6PDH, and 1 unit/ml diaphorase. The assay cocktail is prepared before each assay from stock solutions of enzyme, coenzyme, and substrate that are maintained in the freezer or refrigerator. At the end of the incubation, fluorescence at 590 nm with excitation at 530 nm is measured by a microplate reader to detect the resorufin derived from reduced resazurin. A standard curve is generated by placing 2DG standard solutions in wells of the culture plate that had been prepared without cells.

Evaluation

Glucose uptake in cultured cells is routinely determined by using non-metabolizable radioactive hexoses, such as 3MG or 2DG, labeled with a high specific activity of tritium. Assaying the uptake of [3H]2DG is more convenient than assaying the uptake of [3H]3MG because 2DG is converted to a stable and impermeable derivative 2DG6P through phosphorylation by hexokinase or glucokinase (see above). Because both methods rely on the use of substantial amounts of radioactive material, the concentration of the unlabeled analog is usually kept low (50–500 µM) in the uptake mixture to maintain a high specific activity. Measuring the uptake of these analogs at concentrations comparable to normal or pathological blood glucose levels (5–10 mM) is not practical because of the excessive amount of radioactive analog that would be required to maintain a specific activity high enough to obtain reliable data. Furthermore, the high concentration of analog needed is very expensive, and a specialized institution registered for using radioactive isotopes is required. Although new fluorescently labeled glucose analogs for use as tracers of glucose uptake have been synthesized, none of these molecules has been found to show the same biological behavior as 2DG (Perret et al. 2004). Therefore, 2DG is often considered to be the gold standard in reference tracers of glucose transport and phosphorylation.

The resazurin–diaphorase-amplifying system facilitates the measurement of NADPH in the 2DG uptake assay, because the resorufin fluorophore produced is water soluble and has high fluorescence intensity. In addition, there

will be little background interference from other biochemical entities in the cell or from chemical compounds/drugs added during the assay because the emission wavelength of resorufin is longer and its fluorescence wavelength is much longer than those of NADPH. Furthermore, analysis of resorufin can be measured on standard, clear tissue-culture plates because the material comprising the culture plate, such as polystyrene, does not interfere with the fluorescence signal of resorufin.

Method Based on 3-*O*-Methylglucose

The rate of 3MG transport is determined according to Whitesell and Gliemann (1979), Karnieli and coworkers (1981), and Basi and coworkers (1992) by a modification of the L-arabinose uptake method described by Foley and coworkers (1978).

Procedure

Five milliliter polyethylene miniscintillation vials are prepared with 50 µl of incubation medium containing L-[1-3H]glucose and 3-O-[methyl-14C]glucose such that their concentration is 0.5 mM (58.2 and 11.6 µCi/µmol, respectively). L-Glucose is included as a marker for the extracellular space. Sugar uptake is initiated by the rapid addition of 200 µl of adipose cell suspension (2×108 cell/ml) and then rapidly stopped by the addition of 10 µl of a 10 mM solution of cytochalasin B in 25 % ethyl alcohol. The duration of this "pulse" incubation is adjusted from 3 to 25 s so that the uptake achieved will be approximately one third of the equilibrium level. The equilibrium level of uptake is determined by incubating cells for 5–6 min in the presence of 3MG only and then for one additional min following the addition of L-glucose. Experimental blanks are obtained by adding cytochalasin B to the labeled sugar solution prior to the addition of cells. Following incubation, two 100 µl portions of each incubation mixture are separated from the incubation medium by centrifugation through dinonyl phthalate, and the separated cells are counted for radioactivity (see above for 2DG transport). The net uptake of 3MG is then counted, and the initial uptake velocity or rate of transport is determined.

Evaluation

3MG is a non-phosphorylatable glucose analog which can be used for the accurate measurement of the true initial glucose transport rate without interference with the subsequent glucose-metabolizing steps (e.g., phosphorylation to glucose-6-phosphate). Since the volume of the adipocyte cytosol is rather small, the time period required for equilibration of the extracellular and intracellular glucose concentration is rather short. This necessitates sophisticated experimental procedures enabling start and termination of the transport reaction within a few seconds. Therefore, for routine purposes, 2DG rather than 3MG is commonly preferred.

Method Based on a Fluorescent Glucose Analog

Zou and coworkers (2005) described a sensitive and nonradioactive assay for direct and rapid measuring glucose transport in single living cells. The assay is based on direct incubation of mammalian cells with a fluorescent D-glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), followed by flow cytometric detection of total fluorescence produced by the cells due to the accumulation of 2-NBDG-6-phosphate with time. However, despite a series of experiments has been conducted to define the optimal conditions for this assay, Zou and coworkers (2005) failed to demonstrate a clear-cut stimulation of 2-NBDG transport into these cells by insulin.

Method Using CHO/L6 GLUT4myc-HIR Cells

Purpose and Rationale

Sometimes it is of advantage for studying insulin-induced glucose transport to use cells ectopically expressing GLUT4 and/or the (human) insulin receptor (HIR). This is of particular importance if the relevant cells express low amounts of GLUT4 and insulin signaling components, only, such as CHO or L6 myocytes (in contrast to L6 myotubes). Furthermore, the detection of GLUT4 by immunoblotting is considerably facilitated using cells overexpressing myc epitope-tagged

GLUT4, since anti-myc antibodies of high quality are available. In addition, due to the extracellular topology of the myc epitope, the cell-surface expression of GLUT4 (in response to insulin or insulin-mimetic signaling) can be monitored using intact cells by "On-Cell Western" or imaging, in contrast to wild-type endogenously expressed GLUT4, which is recognized by anti-GLUT4 antibodies typically raised against intracellular epitopes, exclusively.

Procedure

CHO/L6 GLUT4myc(-HIR) are prepared by plating 3.0–5.0 × 104 cells/well into CytoStar 96-well plates (scintillating bottom) in standard medium for up to 48 h. The medium is exchanged to uptake medium (alpha MEM +2 % newborn calf serum (NCS) + penicillium/streptomycin) together with compounds for 2–24 h, depending on the experimental design. For the uptake assay, the following reagents have to be prepared freshly: KRB buffer (per 96-well plate) containing 50 ml of 308 mM NaCl (18.00 g/l), 10.5 ml of 317 mM NaHCO3 (13.30 g/0.5 l), 2 ml of 309 mM KCl (4.60 g/0.2 l), 0.5 ml of 308 mM MgSO4*7H_2O (7.58 g/0.1 l), 0.5 ml of 310 mM KH_2PO_4 (4.22 g/0.1 l), 63.5 ml H_2O (bidest.), and 1.53 ml of 144 mM $CaCl_2$*H_2O (2.12 g/0.1 l) under 20 min carbogen gas incubation at pH 7.4 and 37 °C; 10 mM cytochalasin B stock (10 mg/2,085 µl DMF, stored at −20 °C); 40 µM cytochalasin B stop solution (24 µl of 10 mM stock/6 ml KRB, per plate); 135 µM insulin stock by dissolving 1 mg powder in 0.33 ml of 50 mM HCl and subsequent addition of 0.67 ml in H_2O bidest. and mixing (stored at −20 °C) and sequential 1:10 dilutions for 0.1 mg/ml in H_2O bidest. from 1 mg/ml stock, for 0.01 mg/ml in H_2O bidest. from 0.1 mg/ml stock, and for 0.001 in H_2Obidest from 0.01 mg/ml stock; 250 µM 14C-2-deoxyglucose solution (150 µl stock of 200 µCi/ml/6 ml KRB per plate, giving a total of 0.3 µCi per well); 10 or 30 mM compound stock solutions in 100 % DMSO; 100 or 300 µM compound working solutions diluted with uptake medium (depending on the solubility) resulting in final assay concentrations of 10 and 30 µM, respectively; and 0.1 % and 0.3 % DMSO (the final concentration of DMSO should not exceed 0.5 %) upon direct 1:10 dilution into the wells.

Uptake Assay

Compound working solutions or DMSO control is mixed with uptake medium (150 µl total volume per well) to yield the desired final compound concentrations and max. 0.5 % DMSO (v/v). The culture medium is aspirated from the cells, and uptake medium with compound, insulin, or DMSO control is added. The cells are grown and incubated for 20 min (short-term treatment) or for 12 h to overnight (long-term treatment). The desired insulin working dilutions are prepared in KRB (500 µl for 1 row/8 wells). The 20 µM cytochalasin B negative controls are prepared in KRB (500 µl for 1 row/8 wells supplemented with 1 µl of 10 mM cytochalasin B). The culture medium is aspirated and the cells are washed twice with 200 µl of KRB using an 8-channel pipette. Fifty microliters of KRB are added per well using an 8-channel pipette. Fifty microliters of KRB insulin working solutions or cytochalasin B controls is added per well using an 8-channel pipette. Aspiration and addition of solutions have to occur on the same side of the well. The integrity of the cell layer should be checked using a microscope. After incubation for 25 min at 37 °C and 5 % CO_2 in an incubator, 50 µl of 14C-2-deoxyglucose/KRB is added and the incubation continued for 25 min. Thereafter, 50 µl of cytochalasin B/KRB stop solution is added using an 8-channel pipette. The plates are sealed with self-adhesive sheet and stored for up to 1.5 h. The amount of 14C-2-deoxyglucose taken up by the cells is determined by scintillation counting in a Wallac MicroBeta reader (1 min measurement period; see 14C-protocol for CytoStar plates). Subsequently, the plates are removed from the reader and discarded as radioactive waste. The primary data are analyzed by subtracting mean cytochalasin B-insensitive nonspecific background uptake from each value of total uptake resulting in the specific glucose uptake component.

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Glucose Transporter Translocation

Purpose and Rationale

The transport of glucose across the plasma membrane of mammalian cells represents one of the most important nutrient transport events, since glucose plays a central role in cellular energy homeostasis and intermediary metabolism. Apparently, rather than mediated by a single transporter molecule, glucose transport is mediated by a family of highly related transporters characterized by 12 transmembrane regions and amino-/carboxy-termini facing the cytoplasm (Assimacopoulos-Jeannet et al. 1991; Gould and Holman 1993; Mueckler 1994); the most important ones with regard to carbohydrate metabolism and regulation by insulin are:

GLUT1: the erythrocyte-type glucose transporter
GLUT2: the liver-sstype glucose transporter
GLUT3: the brain-type glucose transporter
GLUT4: the insulin-responsive glucose transporter
GLUT5: the small intestine sugar/fructose transporter
GLUT8: a small intestine/hypothalamic glucose sensor and ion channel lacking a transport function

Among these isoforms, the insulin-responsive GLUT4 is the only one which is expressed in mammalian muscle and adipose tissue, exclusively, and localized in cytoplasmic vesicles (GLUT4 vesicles) derived from the tubulovesicular structures of the trans-Golgi network in the unstimulated state. In animal and human adipose and muscle tissues, insulin induces severalfold increases in glucose transport by causing the movement and fusion of GLUT4 vesicles to and with the plasma membrane, respectively. This so-called GLUT4 translocation leads to increase in the number of GLUT4 molecules exposed at the plasma membrane which results in elevated glucose transport velocity (i.e., increase in Vmax). Numerous cellular, animal, and human studies have revealed significant impairment of GLUT4 translocation in the insulin-resistant and diabetic state. The molecular basis remains unclear so far but may include alterations in the complex machinery regulating the targeting, docking, and fusion of the GLUT4 vesicles as well as in the phosphorylation of protein (SNARE) components involved (see below). This is suggested by the findings that GLUT4 is inactivated by phosphorylation in the insulin-resistant state and activated by dephosphorylation (Reusch et al. 1993) and that the phosphorylation state of GLUT4 is increased in primary rat adipocytes made insulin-resistant in vitro by incubation with high glucose and insulin (Müller and Wied 1993; see ▶ Insulin Target Tissues and Cells).

Methods Based on the Determination of GLUT Molecules in Isolated Plasma Membranes

General Considerations

The insulin-stimulated GLUT4 translocation in adipocytes can be assayed by a number of different procedures either dependent on or independent of subcellular fractionation, i.e., the preparation of plasma membranes of high purity. This admittedly tedious and time-consuming procedure is prerequisite for the majority of techniques appropriate for the analysis of cell-surface expression of GLUT4 (and thus reflecting GLUT4 translocation) in primary rat adipocytes,

which had been challenged with insulin/compounds/drugs. These techniques rely on the determination of the amount of immunoreactive GLUT4 by immunoblotting with the recently introduced specific anti-GLUT4 antibodies in isolated and sufficiently pure plasma-membrane vesicles. Alternatively, radioactively or fluorescently labeled glucose analogs which are recognized but not transported by GLUT4 can be used for the determination of the number of GLUT4 at the plasma membrane of intact rat adipocytes upon covalent cross-linking (see "Exofacial Labeling"). In contrast, in cultured murine or human adipocytes, GLUT4 translocation can be followed upon transfection with a recombinant GLUT4 which is tagged with an extracellular epitope recognized by a specific antibody, thereby circumventing the necessity for subcellular fractionation or covalent cross-linking (see "Determination of GLUT Molecules in Plasma Membrane "Sheets" and "Methods Based on the Determination of GLUT Molecules at the Plasma Membranes of Intact Cells").

Preparation of Rat Adipocyte Plasma-Membrane Vesicles

Adipocytes are isolated from epididymal fat pads of 160–180 g male Wistar rats by collagenase digestion under sterile conditions and incubated with insulin or compounds/drugs as described (see "Primary Rat Adipocytes" in chapter "▶ Insulin Target Tissues and Cells"). The subcellular fractionation is performed according to McKeel and Jarett (1970) and Simpson and coworkers (1983) with the following modifications: The postnuclear supernatant is centrifuged (12,000 × g, 15 min). The washed pellet is suspended in 35 ml of buffer and recentrifuged (1,000 × g, 10 min). The washed pellet (12,000 × g, 20 min) is suspended in 5 ml of buffer; layered onto a 20 ml cushion of 38 % (w/v) sucrose, 20 mM Tris/HCl (pH 7.4), and 1 mM EDTA; and centrifuged (110,000 × g, 60 min, 4 °C). The membranes at the interface between the two layers (1 ml) are removed by suction, diluted with three volumes of buffer, and layered on top of an 8 ml cushion of 24 % Percoll, 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), and 1 mM EDTA. After centrifugation (45,000 × g, 30 min), the plasma membranes are withdrawn with a Pasteur pipette from the lower fourth of the gradient (1 ml), diluted with ten volumes of buffer, and recentrifuged (150,000 × g, 90 min, 4 °C). The pellet is suspended, recentrifuged, and finally dissolved in buffer at 2–5 mg protein/ml and stored at −80 °C. The majority of plasma membranes are recovered as outside-out vesicles as revealed by the low cryptic activity of exofacial 5′-nucleotidase which is detected after detergent solubilization of the vesicles.

Determination by Immunoblotting

The methods described above lost their importance with the introduction of antibodies specific for GLUT4 representing the major (insulin-) regulated GLUT isoform which accounts for more than 90 % of the maximal insulin-stimulated glucose transport activity in primary rat adipocytes and is localized in the basal state almost exclusively (95 %) at intracellular vesicles and in the maximally stimulated state with 50 % at the plasma membrane. Consequently, the amount of GLUT4 in plasma-membrane vesicles derived from basal and stimulated (by insulin/compound/drug) rat adipocytes is now usually determined by immunoblotting for GLUT4. In case of weak expression of GLUT4 (e.g., cultured human adipocytes) or limited availability of starting materials (e.g., human adipose tissue), the sensitivity of the immunological detection can be considerably improved by immunoprecipitation of GLUT4 from the solubilized plasma-membrane vesicles prior to immunoblotting.

Polyclonal affinity-purified rabbit antibodies have been successfully raised against a synthetic peptide corresponding to the COOH-terminal domain of rat GLUT4 (residues 495–509) (James et al. 1989). The plasma-membrane vesicles are subjected to SDS-PAGE (25 µg protein/lane) and electrophoretically transferred onto nitrocellulose filters (Towbin et al. 1979). Incubations of the filters with primary and secondary antibodies (125I-labeled or peroxidase-labeled anti-rabbit IgG from goat) and quantitative

evaluation of the immunoreactive material by autoradiography and densitometry or enhanced chemiluminescence and luminescence imaging, respectively, are carried out as described previously (Müller and Wied 1993) for the demonstration of the sulfonylurea-induced GLUT4 translocation in isolated rat adipocytes, which may be based on phosphorylation of GLUT4. Evidence has been presented suggesting that insulin promotes serine phosphorylation of GLUT4 at the carboxyl terminus (Lawrence et al. 1990a, b) arguing for a causal relationship between GLUT4 translocation and phosphorylation in response to insulin and insulin-like acting sulfonylureas, such as glimepiride.

Determination of GLUT Molecules in Plasma-Membrane "Sheets"

Purpose and Rationale

In nonrecombinant adherent murine and human adipocytes as well as nonadherent primary adipocytes which express endogenous GLUT4, only, its translocation can be monitored by the plasma-membrane "sheet" assay. This is based on placing glass coverslips onto the adherent cells (6- or 12-well culture plates) following incubation with insulin/compounds/drugs. Upon removal of the coverslips, the adherent cells are sheared and their plasma membranes attached to the coverslip become disrupted, leaving the cytosolic components and the intracellular particulate materials (including the intracellular GLUT4 vesicles) with the medium of the plate. Subsequent immunofluorescent detection of GLUT4 remaining attached to the coverslips enables the semiquantitative determination of GLUT4 located at the plasma membrane and apparently deprived from intracellular GLUT4.

Procedure

The protocol for the preparation of plasma-membrane sheets is derived from that of Moore and coworkers (1987) with modifications adapted from Robinson and coworkers (1992) and Fingar and coworkers (1993). 3T3-L1 adipocytes or human cultured adipocytes are plated on poly-D-lysine-coated coverslips the day before an experiment. Adipocytes are incubated for 1.5 h at 37 °C in Leibovitz's L-15 medium containing 0.2 % BSA and treated in the absence or presence of insulin/compounds/drug candidates. The cells are washed twice in ice-cold buffer A (100 mM NaCl, 50 mM HEPES/KOH, pH 7.3) and once in buffer B (100 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 µM leupeptin, 2 µg/ml trypsin inhibitor, 0.5 mM benzamidine, 20 mM HEPES/KOH, pH 7.2) and then sonified in buffer B for 3 s using a Branson Sonifier 250 with a microtip placed 1 cm above the coverslip (output control = 4, duty cycle = 30). Adherent plasma-membrane sheets are washed twice in ice-cold buffer B, fixed in 3 % paraformaldehyde for 10 min, and processed for indirect immunofluorescence using affinity-purified anti-GLUT4 antisera and rhodamine-conjugated secondary antibodies as described (Garcia de Herreros and Birnbaum 1989).

Methods Based on the Determination of GLUT Molecules at the Plasma Membranes of Intact Cells

General Considerations

For recombinant cultured murine and human adipocytes, the need for subcellular fractionation (i.e., preparation of plasma-membrane vesicles of sufficient purity) can be circumvented by heterologous expression of GLUT4 tagged at the extracellular loop with an epitope for which specific antibodies are available (e.g., c-myc) and subsequent immunological detection of GLUT4 in the intact and appropriately fixed cells. Alternatively, GLUT exposed at the plasma membrane of intact adipocytes can be photoaffinity labeled with membrane-impermeable specific and radioactive probes which enable the subsequent detection upon immunoprecipitation of GLUT4/1 from total membranes. Moreover, the methods of "in-cell" immunoblotting or colorimetry enable accurate and straightforward detection of plasma-membrane GLUT4 of cultured and, subsequent

to the treatment, fixed adipocytes without the need for cell fractionation or plasma-membrane sheet generation.

In-Cell Immunoblotting

Purpose and Rationale

The "in-cell immunoblot" is an immunocytochemical assay performed in microtiter plate formate. Target-specific primary antibodies and infrared-labeled secondary antibodies are used to detect target proteins in fixed cells, and fluorescent signal from each well is quantified. Two targets at 700 and 800 nm using two spectrally distinct dyes are monitored. Separate lasers and fluorescence detectors are used for each dye and offer a wide linear detection range. With two detection channels, two separate targets can be probed, or the quantification accuracy is increased by using the second channel for normalization against a second target or DNA stain. Quantification accuracy is maximized by normalization because adjustments can be made for differences in cell number from well to well. Two color normalization also helps to prevent false negatives and provides more accurate evaluation of the treatment of cells with compounds/drug candidates. The use of near-infrared probes yields high sensitivity for measuring small changes in the protein amount. The direct detection of proteins (e.g., GLUT4) in their cellular context eliminates variabilities and artifacts caused by cell lysis, gel loading, electrophoresis, and membrane transfer.

This method enables the analysis of cell-surface expression of proteins (e.g., GLUT4) in adherent cells without the need for cumbersome and labor-intensive cell fractionation procedures. Upon incubation of the adherent adipocytes with insulin/compounds/drug candidates, GLUT4 expression at the plasma membrane is detected by "in-cell" immunoblotting using conditions for fixation and permeabilization of the adipocytes, which enable access of the anti-epitope antibodies to plasma-membrane GLUT4, only, rather than to intracellular GLUT4 vesicles.

Procedure

3T3-L1 fibroblasts or human adipocytes are seeded in 96-well clear round bottom plates (BD Bioscience cat# 353077) at 200,000 cells/well, grown to confluency and then differentiated into mature adipocytes as described above. After serum deprivement overnight by replacement of the differentiation medium with serum-free DMEM, the adipocytes are incubated with insulin/compound/drug in 200 µl of the same medium for 15 min at 37 °C. For fixation, 25 µl of 36 % formaldehyde is added gently to each well using side of the wells (to avoid detaching of the cells from the well bottom). After incubation for 20 min at room temperature with very gentle rotation, the fixation solution is removed. For permeabilization, the cells are washed three times with 100 µl of PBS containing 0.1 % TX-100 for 5 min each by carefully pipetting the permeabilization solution down the sides of the wells to avoid detaching the cells from the well bottom. Thereafter, the permeabilization solution is removed and the washing step is repeated two more times. After addition of 100 µl of blocking buffer (Odyssey, LI-COR Biosciences) to each well and incubation for 1 h at room temperature with very gentle shaking on a rotator, 50 µl of anti-GLUT4 antibody (1:200–1:500, Calbiochem) is added and the incubation continued overnight at 4 °C. Thereafter, the primary antibody solution is removed, and the plates are washed five times with 200 µl of PBS containing 0.1 % Tween-20 for 5 min each. Fifty microliters of fluorescently labeled secondary antibody solution (goat anti-rabbit IRDye 800CW, Rockland Immunochemicals, 1:800 diluted in Odyssey blocking buffer with 0.2 % Tween-20) is added to each well. After incubation for 1 h at room temperature on a rotator under protection from light, the secondary antibody solution is removed, and the plates are washed five times with 200 µl of PBS containing 0.1 % Tween-20 at room temperature for 5 min each. After the final wash, the washing solution is completely removed from the wells. The plates are immediately scanned by placing on the Odyssey scan surface with detection in the 800 nm channel and at 169 µm resolution at

3.0–3.5 mm focus offset and an intensity setting of 5 or less.

In-Cell Colorimetry

Purpose and Rationale

Understanding the molecular mechanisms of insulin action has been of major interest since the discovery, several decades ago, that insulin stimulates glucose transport in vivo. Subcellular fractionation (see section "Methods Based on the Determination of GLUT Molecules in Isolated Plasma Membranes" and "▶ Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes"), cell photolabeling coupled to immunoprecipitation, and immunofluorescence (see below) or immunoelectron microscopy have been used to detect translocation of GLUT4 to the cell surface (Wilson and Cushman 1994; Kozka et al. 1991; Smith et al. 1991). All of these methods are laborious and suffer from methodological inaccuracies. Subcellular fractionation is cumbersome and produces membranes that are rarely pure. Moreover, quantitative recovery of all membrane compartments is difficult or impossible. Affinity labeling of surface glucose transporters followed by immunoprecipitation depends on the ability to obtain quantitative immunoprecipitation and recovery upon SDS-PAGE. This technique results in the incorporation of about 1/10.000 of the label added; thus, the signal to noise ratio is low. Moreover, the reactivity of the photolabel can depend on the level of activity of the transporter (Vannucci et al. 1992) in addition to the amount of transporter exposed at the cell surface. Immunofluorescence detection does not distinguish the native GLUT4 molecules incorporated into the cell membrane from molecules in subplasmalemmal vesicles and is not a quantitative technique. Immunogold electron microscopy detects antigens at the plasma membrane accurately, but has not been successfully used in a quantitative fashion. None of the above techniques is suitable for large numbers of experiments, as would be required for screening of antidiabetic drugs stimulating insulin signaling or GLUT4 translocation. Therefore, Wang and coworkers (1998) intended to develop a fast and quantitative approach to measure GLUT4 translocation in intact cells. The method described uses cells in culture and does not require subsequent immunoprecipitation, SDS-PAGE, or use of radioactivity, as required by the photolabeling technique. It does not require large amounts of cells nor laborious subcellular fractionation and is rapid, sensitive, and quantitative.

Procedure

Construction of L6 Cells Expressing c-myc Epitope-Tagged GLUT4 (GLUT4myc)

GLUT4myc cDNA is constructed by inserting the human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 as described by Kanai and coworkers (1993). The epitope does not affect GLUT4 activity. GLUT4myc cDNA is subcloned into the mammalian expression vector pCXN (pCXN-GLUT4myc) as described by Niwa and coworkers (1991). L6 myoblasts are transfected with pCXN-GLUT4myc and pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride.

Cell Culture

L6-GLUT4myc myoblasts in cell monolayers are maintained in α-MEM supplemented with 10 % FBS in a humidified atmosphere containing 10 % CO_2 and 90 % air at 37 °C. Cells are grown in 12- or 24-well plates and, prior to experiments, incubated with serum-free α-MEM supplemented with 25 mM glucose for 5 h.

Colorimetric Assay of Surface GLUT4myc

Quiescent L6-GLUT4myc cells are incubated with compounds/drug candidates, then washed once with PBS, and fixed in 3 % paraformaldehyde in PBS for 3 min at room temperature. The fixative is immediately neutralized by incubation with 1 % glycine in PBS at 4 °C for 10 min. The cells are blocked with 10 % goat serum and 3 % BSA in PBS at 4 °C for at least 30 min. Primary monoclonal antibody (anti-c-myc, 9E10) is then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4 °C. The cells are

extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1,000). After 30 min at 4 °C, the cells are extensively washed and 1 ml o-phenylenediamine dihydrochloride reagent is added to each well. The colorimetric reaction is stopped by addition of 0.25 ml of 3 N HCl for 10 min at room temperature. The supernatant is collected and the optical absorbance is measured at 492 nm.

Evaluation

Standard curves can be generated using either peroxidase-conjugated anti-mouse IgG alone or myc-tag peptide. For this, myc-tag peptide at various concentrations is coated onto 24-well plates by incubation at 4 °C for 24 h and then allowed to dry. The plates are rinsed with PBS to remove excess salt, and the uncoated spaces are blocked with 10 % goat serum and 3 % BSA.

Exofacial Labeling

Purpose and Rationale

Alternatively, in nonrecombinant primary and cultured adipocytes, GLUT translocation can be analyzed by exofacial labeling of the GLUT molecules. For this, the membrane-impermeable photoreactive substrate analogs, radiolabeled bismannose propylamine (ATB-BMPA; Ryder et al. 2000), and biotinylated and spacer-equipped glucose compound (GP15; Hashimoto et al. 2001) which are all recognized but not transported by GLUT1 and GLUT4 are used. In addition, a series of photoaffinity probes based on a dihexose structure, in which the hexose moieties (D-mannose or D-glucose) are linked through their 4-OH positions to a 2-aminopropyl spacer, to probe the cell-surface exposure of GLUT4 has been generated. Comparisons of aryl azide, benzophenone, and aryl diazirine trifluoroethane photoaffinity labeling derivatives of hexoses (Holman et al. 1986, 1988, 1990) have suggested that the latter are the most suitable as they are highly efficient cross-linkers with good specificity. A (diazirine trifluoroethyl)benzoyl moiety has been attached through an amide link to the central amine group. More recently, a biotinylated version of this compound has been developed in which a linker to biotin is attached through the hydroxy group of the 2-hydroxy-4-(1-diazirine-2,2,2-trifluoroethyl)benzoyl moiety (Koumanov et al. 1998). This compound has been used to assess the cell-surface exposure of GLUT4 in the human muscle of control and type II diabetic patients, and marked impairments in the insulin-stimulated translocation of GLUT4 have been identified using this technique (Ryder et al. 2000). All these substrate analogs can be covalently cross-linked to the GLUT isoforms by photoaffinity labeling via the attached diazirine trifluorobenzoyl moiety upon UV irradiation following incubation of the cells with insulin/compounds/drugs. After removal of free substrate analog by washing of the adipocytes, total membranes are prepared from the lysed cells and counted for radioactivity (ATB-BMPA) or detected by immunoblotting for interaction with streptavidin (GP15). Both measures reflect the amount of total GLUT1/4 located at the plasma membranes at the time point of cell lysis, exclusively. For specific analysis of GLUT4 or GLUT1 translocation, the isoforms have to be immunoprecipitated with specific antibodies since the substrate analogs do not discriminate between GLUT4 and 1 (Holman et al. 1990).

Procedure

For the use of photoaffinity labeling with GP15 (Hashimoto et al. 2001), primary rat adipocytes, which have been incubated with insulin/compounds/drugs, are maintained at 18° C in the presence of 300–500 μM photoreactive probe for 1 min either in the presence or absence of 0.5 M glucose. The samples are then irradiated for 1 min in a Rayonet photochemical reactor using 300 nm bulbs. Excess reagent is removed by extensive washing in physiological buffers, and then 0.3 μM avidin is added to block the cell-surface-tagged transporters. Excess avidin is then removed by washing the cells. For detection of biotinylated GLUT4 by blotting, adipocyte samples are homogenized in HEPES/EDTA/sucrose

(HES) buffer (255 mm sucrose, 1 mM EDTA, 20 mM HEPES (pH 7.2), 1 μg/ml antipain, aprotinin, pepstatin, and leupeptin, each, and 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). Homogenates are washed once with HES buffer and subjected to centrifugation (554,000 × g, 30 min, 4° C) to obtain a total membrane fraction. This pellet is solubilized in PBS, pH 7.2, with 2 % of Thesit (C12E9) and 1 μg/ml each of antipain, aprotinin, pepstatin, and leupeptin and 100 μM AEBSF. The samples are solubilized for 50 min at 4° C with rotation and then subjected to centrifugation (20,000 × g, 20 min, 4° C). Biotinylated proteins in the supernatants are either immunoprecipitated using a GLUT4 antiserum as described above or precipitated with streptavidin beads (Pierce, Rockford). Following GLUT4 immunoprecipitation, complexes are released into electophoresis-sample buffer (62.5 mM Tris/HCl, pH 6.8, 2 % SDS, 10 % glycerol) at room temperature. The streptavidin precipitates are washed four times with PBS buffer containing 1 % Thesit with protease inhibitors, four times with PBS containing 0.1 % Thesit plus protease inhibitors, and once in PBS. Electrophoresis-sample buffer is added to each pellet. The sample is then heated to 95° C for 30 min and subjected to centrifugation (2,300 × g, 1 min). After removal of the supernatants, the pellets are washed with additional electrophoresis-sample buffer, heated to 95° C for 30 min, and resubjected to centrifugation. Mercaptoethanol is added (10 % final concentration) to the above samples in electrophoresis-sample buffer, and these samples are then subjected to SDS-PAGE (10 % gel). Proteins are transferred to nitrocellulose membranes. Membranes are blocked with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween (TBS-T) and washed six times with TBS-T. Membranes are incubated either with streptavidin-HRP (Amersham) or with affinity-purified anti-GLUT4 C-terminal antibody in TBS-T containing 1 % BSA (2 h, room temperature), followed by washing (six times in TBS-T) and detection using secondary antibody linked to horseradish peroxidase. GLUT4 protein is visualized with enhanced chemiluminescence (ECL).

Method Based on the Reconstitution of GLUT4 Translocation

Purpose and Rationale

Insulin action on peripheral tissues including fat, heart, and skeletal muscle leads to increased glucose transport. The basis for this action lies in the increased translocation of GLUT4-containing vesicles from an intracellular reservoir compartment and ultimately exposure of the GLUT4 at the cell surface where it facilitates transport (see "Glucose Transport" and "Glucose Transporter Translocation"). Kinetic studies have identified that GLUT4 traffics through multiple intracellular compartments involving endosomes, the trans-Golgi network (TGN), and a specialized compartment in which GLUT4 is enriched, and it is the exocytosis process that is stimulated by insulin (Satoh et al. 1993; Yang and Holman 1993). However, the site of insulin action along the exocytic pathway is unknown and potentially could involve increased release or budding of GLUT4 vesicles from tubular-vesicular structures, increased movements of vesicles along microtubules, increased transit through cortical actin near the cell surface, increased docking and then fusion of GLUT4 vesicles at the plasma membrane, and then possibly stimulation of catalytic activity of plasma-membrane-inserted GLUT transporters. For further resolution of the key-regulated step, an in vitro fusion reaction has been developed in which GLUT4 vesicles are immunoisolated and fused with reconstituted plasma membrane in the presence of cell cytoplasm. The separate analysis of this partial reaction in the exocytic limb of the translocation sequence allows direct analysis of the extent to which fusion is stimulated by insulin or insulin-like compounds/drug candidates. Remarkably, the magnitude and time course for insulin's action on glucose transport are fully recapitulated in the vesicle fusion reaction.

Cell-free reconstitution of vesicle fusion reactions has many advantages for studying cell biology processes, as the separate components of the fusion reaction can be separately manipulated and combined. The cell-free approach is particularly powerful in studying insulin-regulated fusion as separate mixing of vesicles containing

insulin-activated and nonactivated components is feasible. With this approach, it can be studied whether the action of insulin/compounds/drugs leads to activation of GLUT4 vesicles, activation of components of the cytoplasm fraction, or activation of components of the plasma-membrane fraction. For insulin, recent data reveal that it is the activation of that plasma-membrane fraction that is the key-regulated step in the stimulation of fusion (Koumanov et al. 2005).

However, the exact nature of insulin signals that regulate GLUT4 translocation and how they affect the donor membrane, plasma membranes, or some other components in the translocation machinery remain unclear. In attempts to further characterize the translocation machinery and to study its modulation by insulin/compounds/drugs, two distinct in vitro reconstitution assays for GLUT4 translocation have been developed (Inoue et al. 1999; Koumanov et al. 2005).

Analysis by Immunoblotting

Preparation of Plasma Membranes and Donor Membranes

In an attempt to define the mechanism of insulin-regulated GLUT4 translocation, Inoue and coworkers (1999) developed an in vitro reconstitution assay based on immunoblotting detection of the membrane fusion reaction. For this, differentiated 3T3-L1 adipocytes (stably transfected with mycGLUT4 cDNA by using a retroviral expression plasmid for the preparation of donor membranes) in 10 cm dishes are serum starved for 18 h and then incubated in the presence or absence of insulin/compounds/drugs. After washing twice with ice-cold PBS, the cells are homogenized immediately by using 20 strokes of a 1 ml Teflon-in-glass homogenizer in a buffer containing 10 mM HEPES/KOH (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1 mM Na3VO4, 50 nM okadaic acid, 1 mM PMSF, and 0.1 mg/ml aprotinin. For preparing the donor membranes containing my-cGLUT4, the homogenization buffer is supplemented with 0.5 mM $ZnCl_2$. The homogenate is centrifuged (500 × g, 1 min). For collection of plasma membranes, the supernatant is centrifuged (1,500 × g, 5 min). The supernatant is centrifuged (12,000 × g, 15 min). For collection of donor membranes (consisting of both low- and high-density microsomes), the supernatant is centrifuged (100,000 × g, 1 h). The resulting pellets of plasma membranes and donor membranes are suspended in the homogenization buffer without $ZnCl_2$, adjusted to a protein concentration of 2–4 mg/ml using the Bradford method and immediately used for the in vitro association assay.

In Vitro Association Assay

Donor membranes and plasma membranes (15–30 μg each) are mixed at 4 °C in 75 μl of assay buffer (20 mM HEPES/KOH, pH 7.0, 250 mM sucrose, 0.5 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 50 μg/ml BSA, 50 mM KCl), supplemented with an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 25 units/ml creatine phosphokinase). The amount of protein for plasma membranes and donor membranes in the assay is equal. The reactions are initiated by incubation at 37 °C. After 2–10 min, the reactions are returned to 4 °C and subjected to one of the two procedures to separate the plasma membranes or plasma-membrane-associated donor membrane vesicle structures from the donor membranes. Two different methods for the demonstration of the fusion of donor membranes and plasma membranes have been used successfully by Inoue and coworkers (1999).

Method I

The mixture is applied to the top of a discontinuous 28–44 % sucrose gradient with 2 % steps and subjected to centrifugation (100,000 × g, 18 h). Each 4 ml step layer is diluted with ice-cold water and centrifuged (200,000 × g, 1 h). The final pellets are dissolved in SDS-sample buffer.

Method II

The mixture is centrifuged (15,000 × g, 15 min) after dilution with 1 ml of the ice-cold homogenization buffer supplemented with 0.5 M KCl. The final pellet is dissolved in SDS-sample buffer. For detection of mycGLUT4 translocated from the donor membranes into the re-isolated plasma membranes during the in vitro association

reaction, plasma-membrane proteins are tested for the myc epitope by immunoblotting with appropriate primary antibodies and secondary anti-IgG antibodies coupled to peroxidase. The chemiluminescent signals for mycGLUT4 on the blot, which are quantitated by densitometric scanning, have to be proportional to the load over the range used.

Analysis by Fluorescent Resonance Energy Transfer (FRET)

Isolation of Tagged GLUT4 Vesicles

For demonstration of GLUT4 translocation in the reconstitution system introduced by Koumanov and coworkers (2005), rat adipocytes at 40 % cytocrit are stimulated with 5 nM insulin for 20 min at 37 °C. In a typical experiment, 10 ml of cells are used. After cooling the cells to 18 °C, the surface GLUT4 is labeled with 500 μM biotinylated photolabel GP15 (see "Exofacial Labeling") and incubated with 10 μg/ml of cells DyLight 647 streptavidin for a further 15 min. Insulin/compound/drug is removed and GLUT4 is internalized. The cells are then homogenized and processed as described by Simpson and coworkers (1983) to obtain a post-high-density-microsome supernatant, containing the fluorescently tagged GLUT4 vesicles. The GLUT4 vesicles are purified by immunoisolation using an anti-GLUT4 antibody (Satoh et al. 1993) prebound to the MBP-pa construct attached to a 0.7 ml amylose resin column. After 2 h incubation, the column is washed with intracellular buffer (IC buffer, 20 mM HEPES/KOH, pH 7.4, 140 mM potassium glutamate, 5 mM NaCl, 1 mM EGTA) and the GLUT4 vesicles are eluted in 500 μl fractions with 20 mM maltose in IC buffer. The presence of GLUT4 in each fraction is monitored by measuring the DyLight 647 fluorescence. Usually, fractions 1–3 are pooled together and used as a source of purified GLUT4 vesicles. GLUT4 in the fractions is analyzed using a sheep anti-GLUT4 antibody.

Preparation of Cytosol and Plasma Membrane: Liposomes

Concentrated cytosol is prepared from rat adipocytes in the basal state or following treatment with insulin/compounds/drugs for 20 min. Cells are washed once with buffer without BSA and once with IC buffer. As much buffer as possible is removed and the cells are vortexed and then spun (265,000 × g, 70 min). The cytosol is recovered between the pellet of crude membranes at the bottom and the fat layer at the top and kept frozen at −70 °C until further use. Basal and insulin-/compound-/drug-treated plasma membranes are prepared (Simpson et al. 1983) and reconstituted in phospholipid liposomes using an adaptation of previously described methods (Kono 1983; Schurmann et al. 1989). Purified plasma membrane is solubilized in 1.25 % octyl glucoside and applied to Sephadex G50 column. Two hundred microliters of aliquots of solubilized eluted protein (300 μg/ml) are gently vortex mixed with 40 μl of 150 mg/ml soya bean phosphatidylcholine (Type IV-S, Sigma) in 20 mM HEPES (pH 7.4). A biotin-europium (TMT) ligand is added at 55 nM final concentration, and the mixture is sonicated for 15 s and frozen to −70 °C for at least 2 h. The samples are then thawed, resonicated, and purified on flotation Histodenz gradients. The liposomes are mixed 1:1 with 80 % Histodenz in reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 1 mM DTT) containing 10 % glycerol. This is overlayed with 30 % Histodenz in reconstitution buffer containing glycerol, and then with buffer only. After centrifugation (35,000 rpm, 12 h, SW41Ti rotor), liposomes are collected at the 0/30 % Histodenz interface. The amount of encapsulated biotin-Eu(TMT) is monitored by measuring the fluorescence against a standard curve. Typically 1–2 nM of biotin-Eu(TMT) is encapsulated in 1 ml of isolated liposomes.

Fusion Assays

Fusion assays are performed in duplicate. Fifty microliters of GLUT4 vesicles, liposomes, and cytosol are mixed with 25 μl of IC buffer containing 1 μM biocytin on ice. Fusion is initiated by adding 50 μl of ATP-regenerating system (final concentrations: 1 mM ATP, 5 mM $MgCl_2$, 8 mM phosphocreatine, and 31 U/ml creatine phosphokinase type 1) at 37 °C. In some cases, GLUT4 vesicles and plasma-membrane

liposomes are mixed with the ATP-regenerating system on ice, and the reaction is initiated by adding cytosol. Fusion is stopped by returning the samples on ice and addition of 1 % TX-100 containing excess biocytin.

Evaluation

The extent to which fusion had occurred prior to this endpoint is detected by TR-FRET between europium (TMT) and DyLight 647 in a FARCyte microplate reader. The TR-FRET signal is calculated from the ratio of the emission at 670 nm/612 nm with excitation at 340 nm. The zero time point is used as a measure of the background and is subtracted from each value. Generally the extent of fusion at 5 min is compared with the maximal fusion obtained by incubation at 37 °C for 30 min.

The in vitro reconstitution approach for GLUT4 translocation provides an unequivocal evaluation of the role of compounds/drug candidates in the fusion step as it represents a partial reaction in the GLUT4 vesicle translocation process. Although several recent microscopy studies on GLUT4 movement in cells have suggested that a regulated fusion step is likely (van Dam et al. 2005; Kanda et al. 2005; Lizunov et al. 2005), it is difficult to interpret such studies as many factors before and after the fusion step can influence the appearance of tagged GLUT4 at the cell surface. Although the use of TIRF microscopy (Lizunov et al. 2005) has narrowed down the number of partial reactions involved in the insulin stimulation of exocytosis, the interpretation of these microscopy images is equivocal because the net incorporation of GLUT4 into the plasma membrane can be interpreted as indicating an involvement of the cytoskeleton, docking and fusion, and endocytosis. In addition, fusion and endocytosis may be tightly coupled, and the location of the membrane site at which these partial reactions occur may not be distinct. The in vitro analysis of the fusion reaction allows the fusion step to be studied in isolation from any involvement of the cytoskeleton or the endocytosis steps.

Applications

Li and McNeill (1997) reviewed various quantitation methods for the insulin-regulatable glucose transporter GLUT4 including reconstituted glucose transport, cytochalasin B binding assays, immunocytochemistry, immunoblots, ELISA, and exofacial labels. The effect of streptozotocin-induced diabetes on GLUT4 phosphorylation in rat adipocytes has been studied by Begum and Draznin (1992). The effect on glucose transporters by sulfonylureas was studied by Jacobs and Jung (1985) and Jacobs and coworkers (1989) and that of metformin by Matthei and coworkers (1991). Cusin and coworkers (1990) found that hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles. Hofmann and coworkers (1991) determined GLUT4 content by Western immunoblot protein analysis and by Northern blot analysis of GLUT4 mRNA in epididymal fat or soleus muscle tissue of streptozotocin-treated rats and of obese male KKAY mice after treatment with pioglitazone. The effect of in vivo thyroid hormone status on insulin signaling and GLUT1 and GLUT4 glucose transport systems in rat adipocytes was studied by Matthei and coworkers (1995). Galante and coworkers (1994) studied insulin-induced translocation of GLUT4 in skeletal muscle of insulin-resistant Zucker rats. Recycling of GLUT4 was studied by Laurie and coworkers (1993) and Rampal and coworkers (1995). Bähr and coworkers (1995) studied the stimulation of myocardial glucose transport and glucose transporter-1 (GLUT1) and GLUT4 protein expression in cultured cardiomyocytes from rats. Teresaki and coworkers (1998) studied the influence of an insulin sensitizer on GLUT4 translocation in adipocytes of rats fed a high fat diet. Ren and coworkers (1995) evaluated the effect of increased GLUT4 protein expression in muscle and fat on the whole body glucose metabolism by the euglycemic hyperinsulinemic clamp technique in conscious mice. Uphues and coworkers (1995) used cardiac ventricular tissue of lean and genetically obese (fa/fa) Zucker rats to study the expression,

subcellular distribution, and insulin-induced recruitment of the glucose transporter GLUT4. Zeller and coworkers (1995) studied the GLUT1 distribution in adult rat brains using a newly developed immuno-autoradiographic method. Abe and coworkers (1997) reported molecular cloning of bovine GLUT4. Dalen and coworkers (2003) reported that the expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent in liver X receptor α.

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Method Based on the Confocal Image Analysis of GLUT4 in Recombinant Rat Myoblasts

Purpose and Rationale

The major function of insulin stimulation of major insulin-sensitive tissues such as muscle and fat is to increase the uptake of glucose. This uptake results mainly from an increase in the plasma-membrane concentration of the insulin-sensitive glucose transporter, GLUT4 (glucose transporter 4)/SLC2A4 (solute carrier family 2 member 4), from intracellular compartments to the plasma membrane (Watson and Pessin 2006). This translocation is stimulated by the insulin receptor's activation of the phosphatidylinositol-3-kinase (PI3K) and Akt (protein kinase B [PKB]) signaling cascade. The translocation is mediated in part by AS160 (Akt substrate of 160 kDa) (Kane et al. 2002). Phosphorylated AS160, also known as TBC1D4 (tre-2/USP6, BUB2, cdc16 domain family member 4), was first identified as an important regulator of insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (Sano et al. 2003). Other studies demonstrated that AS160 is functional in mouse, rat, and human skeletal muscle (Bruss et al. 2005; Desmukh et al. 2006; Treebak et al. 2006). Several stimuli are described as increasing phosphorylation of AS160 in vitro and in vivo, including insulin, AICAR (5-aminoimidazole-4-carboxamide 1 ß-D-ribonucleoside), AMPK (AMP-activated protein kinase), and contraction. AS160 is phosphorylated at two main sites (Ser588 and Thr642) by Akt (Kane et al. 2002). Although AS160 phosphorylation is reduced in patients with T2D, overexpression of AS160 in adipocytes did not alter basal or insulin-stimulated surface-to-total distribution of GLUT4 (Sano et al. 2003; Karlsson et al. 2005). Recently, the identification of a novel splice variant of AS160 lacking exons 11 and 12, namely, AS160v2, has been described (Baus et al. 2008). Overexpression of this isoform significantly improved glucose uptake rates in a rat myoblast cell model. This increase in glucose consumption is accompanied by increased surface-to-total GLUT4 translocation after stimulation with insulin or insulin-like growth factor-1 (IGF-1) (Baus et al. 2008). There is mounting evidence that small molecule activators of GLUT4 translocation should act as insulin mimetic for treatment of T2D. The discovery of such novel molecules requires reliable cellular assays. Traditionally, such assays are performed in differentiated myotubes or adipocytes (Wang et al. 1998), making the assays costly, time intensive, and unsuitable for high-throughput screening (HTS). Besides extended timelines, poor statistical values, a lack of sensitivity, and high variability between cell batches, as well as variations from plate to plate, limited the utility of these assays. Consequently, a novel cell-based assay has been developed using a rat myoblast cell line that coexpresses myc-tagged GLUT4 and AS160v2. This assay should be able to identify novel GLUT4 translocation activators. Compared with traditional assays, this system does not require costly and time-consuming differentiation steps and provides a more robust signal. The assay detects the translocation of GLUT4 from cytoplasm to cell surface using an automated confocal imager. This assay allows to measure GLUT4 translocation, cell viability, and cell morphology in the same experimental setting. EC50 values are compared with those from radioactive glucose uptake in response to human insulin and insulin analogs (e.g., insulin glargine, insulin detemir, and [AspB10]insulin). In addition, small molecules, such as resveratrol (Breen et al. 2008), dinitrophenol (Yamaguchi et al. 2005), compound A (Zhang et al. 1999; Webster et al. 2003), and TLK19780 (Laborde and Manchem 2002), known to interfere with glucose uptake and/or GLUT4 translocation may be assessed.

Procedure

Cell Line Creation

To create a stable myocyte cell line coexpressing AS160_v2 and GLUT4-myc rat skeletal muscle cells, L6 is chosen. This cell line constitutively expresses GLUT4-myc (Wang et al. 1998). The transcript variant 2 of AS160 (AS160v2) is amplified from human testis complementary DNA (cDNA, cat. no. 7117-1, Clontech) using forward primer 50-GGAGGAGGATGCCCATTTAAC-30 and reverse primer 50-TCTAAGGAG-CACTTTCTGCTGAG-30. The insert is cloned into pDONR221 (Invitrogen) and subsequently into the expression vector pCDNA5-TO (Invitrogen) by standard methods under control of a tetracycline-inducible promoter. Sequence analysis reveals that in comparison with full-length AS160 (NM014832), exons 11 and 12 are missing in AS160v2. Two mismatches have been identified at positions nucleotide (nt) 202 and nt 1275. The tetracycline repressor (TR) is isolated from pCDNA3.1 (+)/TR (Invitrogen) and cloned into the Nhe I and Not I sites of pIRESpuro2 (Clontech) to obtain pIRESpuro2/TR, which is transfected into L6 cells stably expressing GLUT4-myc (L6-GLUT4-myc) as described (Wang et al. 1998). For L6-GLUT4-myc-tetR-AS160v2 cells, pCDNA5 vector (Invitrogen) containing the AS160v2 gene is also introduced into the cells. Selection of stable clones is performed with 200 μg/ml hygromycin (Invitrogen).

Cell Culture Conditions and Compounds

L6-AS160v2-GLUT4 cells are grown in MEMα supplemented with 10 % fetal calf serum (FCS) (PAA, tet free), 2 μg/ml blasticidin (Calbiochem), 0.5 μg/ml puromycin (InvivoGen), and 200 μg/ml hygromycin (Invitrogen). Expression of AS160v2 protein is induced with 1 μg/ml doxycycline (Sigma). L6-wild-type cells are obtained from American Type Culture Collection (CRL-1458) and grown in MEMα harboring GlutaMax (Gibco) supplemented with 10 % FCS (PAA, tet free) and 1 % penicillin/streptomycin (PAA). L6-GLUT4-myc cells are grown in MEMα harboring GlutaMax (Gibco) supplemented with 10 % FCS (PAA, tet free), 1 % penicillin/streptomycin (PAA), and 2 μg/ml blasticidin (Calbiochem). All cells are grown at 37 °C and 5 % CO_2. L6-AS160v2-GLUT4-myc cells are incubated in starvation medium (MEMα) 3–4 h prior to each experiment. For differentiation, the cells are grown in MEMα harboring GlutaMax (Gibco) supplemented with 1 % horse serum (Lonza) for 7 days.

Laser-Scanning Fluorescence Microplate Cytometer

Cells are plated in 96-well plates (Becton Dickinson Biocoat collagen-coated plates), serum starved (if necessary), and treated with insulin and/or compounds. After treatment, cells are fixed in 3.75 % paraformaldehyde (Sigma) for 20 min, quenched with 100 nM NH4Cl, and blocked with 2 % bovine serum albumin (BSA), 2 % FCS, and 0.2 % gelatin in phosphate-buffered saline (PBS) for a minimum of 1 h. Primary antibody (monoclonal anti-myc 9E10, sc-40, Santa Cruz Biotechnology) is incubated for 1 h. The secondary goat anti-mouse immunoglobulin G (IgG) (Alexa Fluor 488, Invitrogen) is incubated in the presence of SY-TOX Orange (Invitrogen) for 1 h. Signals are detected and quantified with a microplate cytometer (Acumen).

Confocal Image Analysis of GLUT4 Translocation

Cells are plated in black glass bottom 384-well plates (Matri-Cal) in growth medium with 1 μg/ml doxycycline. After 24 h of incubation, cells are washed with 1 μg/ml doxycycline in starvation medium on a BioTek 384 HTS cell washer, and thereafter, thw cells are serum starved for an additional 16 h. Using a WellMate (Matrix Technologies), compounds are diluted and transferred onto the cells with a Biomek FX workstation (Beckman Coulter). Plates are then incubated at 37 °C and 5 % CO_2 for 60 min. Insulin is added to a final concentration of 2 nM and incubated for 20 min at 37 °C and 5 % CO_2. To stop the response to insulin and fix the cells, formaldehyde

is added to each well to a final concentration of 4 % for 60 min at room temperature. After fixing, the cells are washed (BioTek 384 HTS cell washer) and blocked with Odyssey blocking solution (LI-COR) for 1 h at room temperature. The monoclonal anti-c-myc tag antibody 9E10 solution (sc-40, Santa Cruz Biotechnology) in SuperBlock buffer T-20 (Pierce) is prepared and added to each well. Plates are incubated at room temperature for 2 h. After the incubation time, the plates are washed four times with PBS + 0.02 % Tween 20. The secondary antibody solution, Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) and DRAQ5 (Biostatus) in SuperBlock buffer T-20 (Pierce), is added, and plates are incubated for 2 h with light protection at room temperature. After four washes with PBS containing 0.02 % Tween 20, the plates are sealed with black tape plate seal and measured within 72 h.

Image Acquisition and Processing

Plates are warmed to room temperature and imaged on the IN-Cell Analyzer 3,000 (GE Healthcare) with a 488 nm green laser and a 535 to 545 nm emission filter to detect GLUT4-myc staining and a 647 nm red laser and a 695 to 755 nm emission filter to identify DRAQ5 nuclear staining. Two 0.75 mm fields are acquired with 22 binning per well. Images are analyzed using the Object Intensity Analysis Module from GE Healthcare. The Object Intensity Analysis Module measures the average green fluorescence intensity (Ipos) that represents GLUT4-myc staining and the average cell number per well (Npass) as the primary parameters. Images are reanalyzed with MatLab. For data analysis of the confocal image-based assay, GLUT4-myc translocation signal is measured by average cellular fluorescence intensity (Ipos). Compound activities are compared with high and low controls of 400 and 2 nM human insulin, respectively.

Evaluation

Muscle and fat cells translocate GLUT4 to the plasma membrane when stimulated by insulin. Usually, this event is measured in differentiated adipocytes, myotubes, or cell lines overexpressing tagged GLUT4 by immunostaining. However, measurement of the translocation in differentiated adipocytes or myotubes or GLUT4 overexpressing cell lines is difficult because of high assay variability caused by either the differentiation protocol or low assay sensitivity. The identification of a novel splice variant of AS160 (substrate of 160 kDa), namely, AS160v2, has been described that its coexpression with GLUT4 in L6 myoblasts increased the insulin-stimulated glucose uptake rate due to an increased amount of GLUT4 on the cell surface. L6 cells, which coexpress myc-tagged GLUT4 and AS160v2, can be efficiently used to generate an assay useful for identifying compounds that affect cellular responses to insulin. The EC50 values are comparable for radioactive glucose uptake and GLUT4 translocation for different insulin analogs and several small insulin-mimetic molecules, thereby validating the assay. The use of L6 cells overexpressing AS160v2 in combination with an image-based assay can be considered as a novel tool for the characterization of molecules modulating insulin signaling and GLUT4 translocation. So far, assays targeting GLUT4 translocation or glucose uptake have been performed only in differentiated adipocytes and myotubes or in artificial cellular models (Hill et al. 1999; Liu et al. 2009). However, these cell systems not only are time and cost intensive but also suffer from methodological inaccuracies. Overexpression of AS160v2 significantly improves glucose uptake rates in rat myoblasts and increases GLUT4 expression at the plasma membrane (Baus et al. 2008), thereby providing a larger assay window for measuring the effect of insulin or insulin mimetics. Moreover, the system provides high reproducibility and reliable statistical values.

The differentiated parental cell line L6-GLUT4-myc is widely used for quantitative assessment of GLUT4 translocation (Wang et al. 1998). However, in comparison with the differentiated parental cells, the novel cell system eliminates time-consuming differentiation of cells and also improves assay variability. Differences in the fold induction of glucose uptake rates of undifferentiated and differentiated L6-GLUT4-AS160v2 cells are not observed. Stimulation of myoblasts with insulin results in a well-defined phenotype of GLUT4 translocation. For assay

validation, the orders of potencies have been compared for insulin, different insulin analogs, and IGF-1 using either the classical radioactive glucose uptake measurement or the GLUT4 translocation assay. For both assays, it is possible to demonstrate differences between the activities of the different molecules as reported previously (Kurtzhals et al. 2000; Wada et al. 2008). In general, the EC50 values determined with the translocation assay are higher when compared with the radioactive assay format. However, the relative rankings are comparable. Phenotypes and morphologies after stimulation with insulins or IGF-1 are similar. Distinct staining of GLUT4 in ruffle-like structures at the plasma membrane indicates an intact cell membrane and a reorganized cytoskeleton (Brozinick et al. 2004).

Assay validation for the GLUT4 translocation assay using L6-AS160v2-GLUT4 cells is based on the reproducibility of the assay as demonstrated in the day-to-day correlation. GLUT4 translocation (activity in %) after stimulation with a first replicate of a small random compound library (activity [%] replicate 1) is correlated with the activity obtained after a second validation replicate (activity [%] replicate 2) as well as the distribution of positives in a small random library. Setting a criterion of 20 % activation, the hit rate was approximately 2.05 %.

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Fatty Acid Transport

Purpose and Rationale

To gain knowledge of the biochemical pathways of fat metabolism in human physiology is central to understanding the derangements and pathology in the adipocyte handling of fatty acids associated with obesity and type II diabetes. The adipocyte stores large amounts of TAG, which continually turn over during lipolytic hydrolysis and reacylation of exogenous fatty acids. Human adipocytes do not have the capacity to synthesize significant amounts of fatty acids de novo. The large part of TAG synthesis comes from fatty acids provided by lipoprotein lipase hydrolysis of chylomicron or very low-density lipoprotein TAG in the capillary blood vessels provided by the intestinum and the liver, respectively. Fatty acids act as detergents that rapidly dissolve the plasma membrane, causing cell lysis if allowed to accumulate. More conflicting, the abnormal storage of TAG in the LD of cells from nonadipose tissues (muscle, liver, pancreatic β-cells) may interfere with the insulin regulation of lipid and carbohydrate metabolism as well as with glucose-induced insulin secretion via ill-defined molecular mechanisms which are collectively termed the lipotoxicity hypothesis for the pathogenesis of type II diabetes. The flow of fatty acids into the adipocyte can be high, and these fatty acids have to be efficiently and rapidly converted to TAG and stored away in the cytoplasmic LD. The synthesis of lipids, in particular of TAG, including the biogenesis of LD by adipocytes depends on the uptake of free fatty acids (FFA) from the blood stream. FFA transport across the adipocyte plasma membrane involves both passive diffusion (at high FFA concentrations) and facilitated diffusion via specific transport proteins (at low concentrations). Current evidence indicates that several classes of fatty acid transport proteins, long-chain fatty acyl-CoA synthetases (ACS), and cytosolic fatty-binding proteins participate in fatty acid transport and activation. In eukaryotic systems, fatty acid transport proteins (FATPs), fatty acid-binding proteins (FABPs), ACS, and fatty acyl-CoA-binding proteins are suggested to function in concert to facilitate long-chain fatty acid uptake. There is considerable evidence that insulin stimulates fatty acid transport up to threefold in adipocytes by inducing the translocation of specific FATPs (CD36) from intracellular vesicles to the plasma membranes, a molecular mechanism resembling GLUT4 translocation (see above). Potential drugs targeting these proteinaceous

components of the transport pathway are promising. They may specifically block fatty acid uptake, cytoplasmic trafficking, and/or activation in adipocytes for the pharmacotherapy of obesity, diabetes, and the metabolic syndrome in humans. Conversely, stimulation of fatty acid uptake into adipocytes by potential drugs with insulin-like activity may be beneficial for the antidiabetic therapy on the basis of storage of excess of FFA in adipose tissue, the site specifically designed for the deposition of carbons taken in by daily food. The removal of FFA from the circulation would thereby circumvent the putative lipotoxic effects of FFA in other nonadipose tissues. It is hypothesized that high circulating levels of FFA occur when uptake and activation of fatty acids exceed the adipocyte's capacity for storage or β-oxidation. This leads to excess fatty acid internalization and resultant lipotoxicity of nonadipose cells and tissues (muscle, liver, pancreas). However, so far no drugs are available, which either increase or decrease the storage of TAG (by stimulation/inhibition of fatty acid transport and esterification or inhibition/stimulation of lipolysis in adipose tissue or nonadipose tissues, respectively). Their identification requires the development of assay systems for monitoring fatty acid transport, esterification, and lipolysis appropriate for throughput screening.

Method Based on Radiolabeled Fatty Acids

Adipocytes

The [3H]oleic acid uptake assays are performed with confluent 3T3-L1 adipocyte cell monolayers according to the procedure developed by Stremmel and coworkers (1986) and Pohl and coworkers (2005). [3H]Oleic acid (173 μM, 0.05–0.20 μCi) is dissolved in a defatted BSA solution (173 μM) at a ratio of 1:1. 2 ml of the oleate/BSA solution is incubated with the cell monolayer of each well of a 6-well culture dish at 37 °C. The uptake is terminated after 5 min by the removal of the solution followed by addition of 5 ml of an ice-cold stop solution (PBS containing 0.5 % BSA and 200 μM phloretin). The stop solution is discharged after 2 min, and the culture dishes are washed by dipping them six times in ice-cold oleate/BSA solution (173 μM). NaOH (2 M) is added to lyse the cells. After neutralization, aliquots of the lysates are used for protein and radioactivity determination (in a liquid scintillation counter after addition of 10 ml of scintillation cocktail Ultima-Gold).

Hepatocytes

For measurement of fatty acid uptake into hepatocytes according to the procedure described by Chang and coworkers (2006), 200 μl of a suspension of freshly isolated hepatocytes (5×105 cells in PBS with >95 % viability according to trypan blue exclusion) is added to 200 μl of oleate-BSA buffer solution containing 40 μM of oleic acid, 10 μM BSA, and 5 μCi/ml [3H]oleate in PBS. At increasing periods of time (0–120 s) afterwards, 5 ml of buffer containing 0.1 % BSA and 500 μM phloretin in BSA are added to the mixture to stop the uptake. The entire mixture is immediately run through a glass fiber filter under low vacuum (<10 mmHg) to collect the cells. The glass fiber is placed in a vial with scintillation fluid to count radioactivity. A blank vial and a vial with an aliquot of oleate-BSA buffer solution is used as negative and positive controls, respectively. The assays done at the time point zero are used as the background uptake activity and subtracted from the activity measured at each time point. The final values are corrected for cell viability, determined after isolation of the hepatocytes by trypan blue exclusion or MTT.

Viability of Cultured Cells: MTT Assay

To evaluate the cytotoxic effect of the drugs, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is performed. Cells are seeded in 12-well plates and incubated with compounds/drug candidates. Subsequently, 200 μl of 0.25 % (w/v) MTT is added to each well, and cells are further incubated for 2 h. The resulting formazan crystals are then solubilized by adding 1 ml of MTT lysis solution (10 % SDS and 1 mM acetic acid in DMSO). Absorbance at 570 nm is measured. The results are expressed as the percentage of absorbance related to control cells.

Viability of Cultured Cells: Trypan Blue Assay

After incubation of the cells in the absence (control group) or presence of compound/drug candidate, the cells are exposed to the membrane-impermeable dye, trypan blue (0.1 %, w/v) for 15 min at 37 °C. The presence of dye is determined by light microscopy, and the numbers of unstained and stained cells in the field are counted to obtain an estimate of the percentage of the cells taking up the dye.

Viability of Cultured Cells: Lactate Dehydrogenase (LDH) Release

After incubation of the cells with compound/drug candidate, LDH release into the medium is measured by determining LDH activity (cytotoxicity detection kit-LDH, Roche Applied Science). The amount of color formed in the assay is proportional to the number of lysed cells. The LDH activity in the total of dead islet cells (high control) is measured after solubilization of the cells with 5 % (by vol.) Triton X-100.

Evaluation

To determine the percentage cytotoxicity, the absorbance at 490 nm is measured in multiple samples with subtraction of values obtained in control incubation (low control with cells but without compound) using the following equation:

Method Based on Fluorescent Fatty Acids

General Considerations

Fatty acid transport can be more easily studied in yeast compared to mammalian cells, which makes a yeast-based assay system attractive for throughput screening in drug discovery. Fatty acid uptake has been demonstrated to be essential for growth of yeast under conditions of blocked de novo synthesis. This occurs in the natural state when cells are grown under hypoxic conditions due to the requirement of the acyl-CoA desaturase for oxygen. During hypoxia, cells specifically require an exogenous source of long-chain unsaturated fatty acids. Fatty acid uptake is dependent upon two proteins, Fat1p, the orthologue of the mammalian FATPs, and an ASCL, either Faa1p or Faa4p. Yeast with deletions in the structural gene encoding Fat1p (FAT1) or Faa1p and Faa4p (FAA1 and FAA4) cannot grow anaerobically whether or not unsaturated fatty acids are added to the growth media due to a defect in fatty acid activation (Faergeman et al. 1997, 2001; Dirusso et al. 2000). These deficiencies in transport and growth can be alleviated by expression of a selected group of FATPs (mmFATP1, mmFATP2, and mmFATP4; Kazantzis and Stahl 2012). The yeast system was recently successfully exploited as a method for screening fatty acid uptake inhibitors, which function by specific interaction with a mammalian FATP conditionally expressed in yeast (Li et al. 2005).

Purpose and Rationale

This method (Li et al. 2005) is based on the use of the fluorescent fatty acid analog, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodeca noic acid (C1-BODIPY-C12). The length of C1-BODIPY-C12 is approximately equivalent to that of an 18-carbon fatty acid. The BODIPY fluorophore is 11 carbons away from the ca rboxylate group. Both nonhomogeneous and homogeneous assay designs have been developed. Cellular uptake of C1-BODIPY-C12 ca n be monitored using fluorescence microscopy. For this, cells are first incubated with C1-BODIPY-C12 to allow uptake, and then unincorporated ligand is removed using repeated washes with the fatty acid-binding protein BSA. For detection of fatty acid uptake, the cells are atta ched to microscope slides by coating with polylysine and then visualized using confocal microscopy. For high-throughput screening, this is not practical, however, due to the requirement of several washing cycles and centrifugations or of microscopy as detection method. The homogeneous assay avoids multiple washing steps and relies on a standard microtiter plate fluorescence reader. In this method, extracellular fluorescence is quenched by the addition of trypan blue which does not penetrate into intact 3T3-L1 a dipocytes. The assay has been validated with the nonfluorescent natural fatty acid, oleic acid, in addition to sodium azide and Triacsin C, compounds that interfere with fatty acid uptake by blockade of the fatty acid activation step.

Procedure

Yeast cells with the genes for Fat1p and Faa1p deleted and transformed with the expression vector encoding murine FATP2 (mmFATP2) under the control of the GAL10 promoter are grown to 0.1 A600 (mid-log phase) in yeast-supplemented minimal media containing 2 % galactose and 2 % raffinose (without leucine and histidine) at 30 °C to induce expression of the mmFATP2 (Johnson 1994). Cells are harvested by centrifugation (5,000 × g, 5 min) and then resuspended in PBS at a cell density of 6 × 107/ml prior to use. In a standard uptake assay, 50 μl of yeast cells (3 × 106) is dispensed into each well and incubated with 5 μM C1-BODIPY-C12 in the dark for 3 min in 100 μl total volume. C1-BODIPY-C12 is prepared in PBS containing BSA (7.5 μM final concentration in the assay). Then, 50 μl of trypan blue (0.33 mM final concentration) is added to the reaction to quench the extracellular fluorescence of C1-BODIPY-C12. Fluorescence intensity is measured on a fluorescence microtiter plate reader (e.g., molecular devices) with filter set at 485 nm excitation and 528 nm emission. Fluorescence intensity is recorded after adding trypan blue at room temperature for various times (0, 30, 60 min). The ratio of measured fluorescence intensity of yeast cells expressing and lacking mmFATP2 is calculated and used to assess the C1-BODIPY-C12 uptake activity of the corresponding mammalian FATP. Cells are incubated with compounds/drugs on an orbital shaker (250 rpm, 30 °C) for 2 h prior to assaying C1-BODIPY-C12 uptake.

Evaluation

This yeast-based assay system introduced by Li and coworkers (2005) for the identification of compounds/drug candidates that target the mammalian FATPs is critically dependent on the bypass of the time-consuming repeated washing steps with BSA required to limit the fluorescent signal from unincorporated ligand. For this, a quenching reagent was chosen, which decreases the signal of non-cell-associated C1-BODIPY-C12, which obscures the cell-associated fluorescence. Consequently, the quenching dye must have an absorbance spectrum that significantly overlaps the fluorescence emission of the BODIPY fluorophore, such as trypan blue, with a peak absorbance at 607 nm. This dye has been shown to quench the extracellular fluorescent probes used in phagocytosis assays but does not quench internal fluorescence since it is effectively excluded from living cells due to impermeability of the cell membrane to this compound. BSA is added to the assay mixture to solubilize the hydrophobic fatty acid analog. It has been found that the apparent fluorescence measured in the presence of BSA is much more consistent compared to that measured in the absence of BSA. The signal difference between the positive and the negative controls (ratio of cells expressing and lacking the mammalian FATP) is usually in the range of four to fivefold. Therefore, utilizing trypan blue to quench the non-cell-associated C1-BODIPY-C12, it is possible to omit the washing cycles for monitoring fatty acid uptake.

One advantage of the yeast cell-based assay to target the mammalian FATPs is that it relies on a phenotype (i.e., fluorescence signal) specific to the target protein. Thus, it mimics the biological context of the protein more closely than the cell-free system (Fernandes 1998). A drawback of the cell-based assay, however, is the lack of knowledge about the mechanism of interaction with and specificity toward the target protein. Therefore, secondary screens must be performed, which are required to eliminate toxic compounds. Compounds which kill the cells allow the nonspecific entry of trypan blue leading to quench of the internalized ligand. Additionally, some selected compounds might work by non-FATP-mediated mechanisms, including those that increase the permeability of the cell membrane and facilitate trypan blue entry into the cells. In conclusion, the data presented so far demonstrate that the yeast cell-based fatty acid uptake assay employing a *S. cerevisiae* strain deficient in fatty acid uptake that expresses mmFATP2 and tests the mmFATP2-dependent C1-Bodipy-C12 transport activity can be used for high-throughput screening of inhibitors or activators of the fatty acid transport and may be successfully applied to other members of the FATP and ACS families.

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Cellular Esterification

Purpose and Rationale

The following assay monitors the complete pathway of esterification of glycerol-3-phosphate synthesized via glycolysis from glucose, which is transported into the adipocytes upon insulin-dependent GLUT4 translocation with fatty acids taken up by the adipocytes via the fatty acid transport proteins (in insulin-dependent fashion) (see "Lipogenesis" and "Fatty Acid Transport").

Procedure

For this assay described by Sooranna and Saggerson (1976), adipocytes are treated with low concentrations of saponin to permeabilize the plasma membrane (without disrupting internal membranes) and subsequently incubated with L-[U-14C]glycerol-3-phosphate for 10 min at 25 °C. Toluene-based scintillation cocktail is added and the lipid and aqueous phases are separated by centrifugation. The lipid phase is removed and counted for radioactivity.

Evaluation

This assay measures the complete esterification pathway of glycerol-3-phosphate into lipid-soluble products (TAG, phospholipids, lipidic intermediates such as [lyso]phosphatidic acid) including the regulatory cascade which is stimulated by insulin. One or the other of the activation or acylation steps may be affected by compounds/drug candidates. Glucose transport does not interfere due to the use of the permeabilized adipocytes as is revealed by lack of inhibition of basal esterification by cytochalasin B.

Lipid-Synthesizing Enzymes

Purpose and Rationale

The effects of insulin/compounds/drugs on the three acylating enzymes involved in the

esterification pathway, glycerol-3-phosphate-acyltransferase (GPAT), monoacylglycerol-3-phosphate-acyltransferase (AGPAT), and 1,2-diacylglycerol acyltransferase (DGAT) (for a review, see Coleman and Lee 2004), can be assayed individually using microsomes (GPAT, AGPAT, DGAT) and mitochondria (GPAT, AGPAT) prepared from adipocytes which have been pretreated correspondingly. Care must be taken to preserve the activation state of the acylating enzymes during preparation of the microsomes after challenge of the adipocytes with insulin/compounds/drugs by the inclusion of mixtures of phosphatase and protease inhibitors. For the analysis of putative direct effects of compounds/drugs on the acylation enzymes, they are added to the cell-free incubation mixtures containing microsomes/mitochondria from untreated adipocytes.

Preparation of Microsomes/Mitochondria

Isolated rat adipocytes, which have been incubated with insulin/compounds/drugs, are homogenized by 10 strokes in a motor-driven Teflon-in-glass homogenizer in TES buffer (20 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA) supplemented with 10 μM okadaic acid, 10 mM sodium pyrophosphate, 100 mM NaF, 10 mM glycerol-3-phosphate, 1 mM sodium orthovanadate, 0.1 mM PMSF, and 10 μg/ml each of antipain, leupeptin, pepstatin, and E-64. After differential centrifugation steps for the removal of cell debris (500 × g, 5 min), plasma membranes (2,000 × g, 15 min), and mitochondria (10,000 × g, 20 min), the supernatant is centrifuged (100,000 × g, 60 min, 4 °C). The pellet is resuspended in TES buffer and centrifuged (16,000 × g, 10 min). The microsomes are recovered from the supernatant by centrifugation (100,000 × g, 60 min, 4 °C). Microsomes and mitochondria are resuspended in TES buffer (at 4 mg protein/ml) and finally stored at −80 °C and used only once after thawing. According to the distribution of the marker enzymes (NADPH-cytochrome-c reductase, GRP-78, and BiP for microsomes, succinate dehydrogenase and porin for mitochondria, adenylate cyclase, and Na+/K+ −ATPase for plasma membranes), the microsomes are contaminated to varying degree with mitochondria and are significantly deprived of plasma membranes.

Glycerol-3-Phosphate-Acyltransferase (GPAT)

Procedure

Radioactive Assay

The incubation mixture contains 50 mM Tris/HCl (pH 7.4), 200 mM KCl, 1 mM DTT, 150 μM palmitoyl-CoA, 4 mM $MgCl_2$, 10 mM NaF, 2 mg/ml BSA, 0.2 mM [3H]glycerol-3-phosphate (2 μCi), and microsomes from primary rat adipocytes (25–100 μg) in a final volume of 0.5 ml. The reaction is started by addition of the microsomes in the absence or presence of 2 mM *N*-ethylmaleimide (NEM). After incubation (3 min, 37 °C), the reaction is stopped with 2 ml of water-saturated butanol, followed by 1.5 ml of butanol saturated water. The butanol phase is separated and washed twice. An aliquot is counted for radioactivity. Under these conditions, the rate of product formation is linear for up to 15 min.

Fluorescent Assay

Up to 100 μl of microsomes/mitochondria are incubated with 100 μl of reaction mixture containing 40 mM Tris/HCl (pH 7.4), 8 mM $MgCl_2$, 4 mg/ml BSA, 10 mM NaF, 1 mM NDC, and 0.3 mM glycerol-3-phosphate in a total volume of 200 μl. After incubation (60–180 min, 25–30 °C) in the absence or presence of 2 mM NEM, the reaction is terminated by the addition of 300 μl of chloroform/methanol (2/1, by vol.) containing 0.1 M HCl and thoroughly mixing. After phase separation (5,000 × g, 10 min), 180 μl of the lower organic phase is removed and dried. The resin is solubilized in 50 μl of dichloromethane by thorough mixing. Portions (5 μl) are analyzed by thin layer chromatography (Merck, silica gel Si-60) using acetic acid methylester/toluol (2/1 or 1/1, by vol.) with synthetic NBD-labeled mono-, di-, and tridodecanoylglycerol run in parallel as markers.

Evaluation

In case of using microsomes which are equipped with the complete lipid-synthesizing machinery, the predominant radiolabeled/fluorescent product formed is TAG with accumulation of only minor amounts of its precursors, phosphatidic acid (3–8 %), and lysophosphatidic acid (1–4 %) as determined by thin layer chromatography and fluorography. However, under the conditions used, GPAT activity is rate limiting for the formation of radiolabeled or fluorescent TAG, and consequently, the rate of TAG formation is a direct reflection of the activity of GPAT. In case of using mitochondria, which are typically contaminated by microsomes, their total GPAT activity is determined in the absence of NEM. mtGPAT1 activity, which is resistant to inhibition of NEM (Bell and Coleman 1980), is determined in the presence of 2 mM NEM and subtracted from the total activity to yield the NEM-sensitive GPAT activity, representing the activity from the microsomal GPAT and mtGPAT2 (Bell and Coleman 1980; Lewin et al. 2004). Typically, 2 mM NEM is sufficient to inhibit all of the microsomal GPAT and mtGPAT2 activity.

Time and concentration dependence of sulfonylurea-stimulated GPAT was tested by Müller and coworkers (1994). Insulin has been reported to stimulate microsomal GPAT by two- to threefold in primary rat adipocytes. The molecular mechanism of this regulation remains to be elucidated. Phosphorylation (by casein kinase II) and allosteric activators (polar head groups derived from glycosyl-phosphatidylinositol lipids) have been discussed (Vila et al. 1990; see "► Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes").

Acylglycerol-3-Phosphate-Acyltransferase (AGPAT)

Procedure

Radioactive Assay

One microliter of incubation mixtures are prepared by sonication of monoacylglycerol-3-phosphate (MAGP) (up to 100 μM), TX-100 (0.5 %), phosphatidylcholine (0.2 mg/ml), [14C] palmitoyl-CoA (40 μM), NaF (50 mM), and $MgCl_2$ (1.5 mM) in 150 mM Tris/HCl (pH 8.0). The reaction is started by addition of up to 20 μg of microsomes. After incubation (10 min, 37 °C, constant shaking), the reaction is terminated by the addition of 4.5 ml of chloroform/methanol (1/2, by vol.), 1.5 ml of chloroform, and 1.5 ml of 2 M KCl/0.2 M H3PO4. Diacylglycerol-3-phosphate contained in the chloroform layer is dried, redissolved in chloroform/methanol (2/1, by vol.), and then analyzed by TLC using chloroform/methanol/acetic acid/water(25/10/3/1, by vol.). Dried TLC plates are subjected to fluorography (ENHANCE spray, NEN/DuPont; Kodak X-Omat AR films).

Spectrophotometric Assay

AGPAT can be assayed by measuring the reaction of released CoA-SH with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB = Ellman's reagent) spectrophotometrically. The reaction mixture consists of 100 mM Tris/HCl (pH 7.4), 20 μM oleoyl-CoA, 50 μM 1-acylglycerol-3-phosphate, 1 mM DTNB (10 mM stock in 1 M Tris/HCl, pH 8.0), and up to 50 μl of sample (~200 μg microsomal protein) in the presence of compounds/drug candidates in the wells of a 96-well microtiter plate (200 μl total vol.). The reaction is initiated by the addition of oleoyl-CoA after preincubation of the microsomes with all other components for 2–4 min. The increase in absorbance at 405 nm is followed at 37 °C with a microtiter plate spectrophotometer. Control incubations are performed in the absence of 1-acylglycerol-3-phosphate, and the observed rates are subtracted from the rates measured in the presence of acyl acceptor.

Diacylglycerol Acyltransferase (DGAT)

Procedure

The incubation mixture (250 μl) contains 150 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 1 mg/ml BSA, and 50 μM [14C]palmitoyl-CoA (3 μCi, 25,000 cpm/nmol). The reaction is started by the addition of up to 50 μl of a mixture containing 1.5 mM 1,2-diacylglycerol and phosphatidylcholine (0.2 mg/ml) in absolute ethanol and up to 100 μg of microsomal protein. After incubation (15 min,

37 °C), the reaction is terminated by the addition of 1 ml of 2-propanol/heptane/water (80/20/2, by vol.). TAG contained in the heptane layer is further purified. The dried samples are redissolved in chloroform/methanol (2/1, by vol.) and analyzed by TLC using petroleum ether/diethyl ether/acetic acid (80/20/1, by vol.) or hexan/diethyl ether/acetic acid (70/291/1, by vol.) and fluorography or radioactivity scanning.

Formation of Lipid Droplets (LD)

General Considerations

In vertebrate animals, the most abundant energy reserve is stored as TAG in the LD of adipocytes. These LD can be as large as 100 pm and are composed of a core of TAG surrounded by a phospholipid and cholesterol monolayer into which numerous proteins are embedded (DeLany et al. 2005; Brasaemle et al. 2004; Wolins et al. 2005; Robenek et al. 2004, 2005a, b). Most other types of cells contain tiny LD that store primarily cholesterol esters and serve as a reservoir of cholesterol for the synthesis and maintenance of membranes. Steroidogenic cells of the adrenal cortex, testes, and ovaries use stored cholesterol additionally as a source of substrate for steroid hormone synthesis. Little is known about the mechanisms that control the flux of neutral lipids into and out of LD in any type of cell, but it is clear that the processes that control lipid traffic in adipocytes are central to the regulation of whole body energy metabolism. Moreover, the biogenesis of these structures, in general, and adipocytes, in particular, remains to be elucidated. Several proteins have been identified on their surface, including adipocyte differentiation-related protein (ADRP or adipophilin), perilipins, and caveolin, but their roles in LD assembly are unknown.

ADRP and perilipins are abundant on LD. ADRP is found mostly on smaller LD. Caveolin is a 21 kDa membrane protein with a hairpin structure whose N- and C-termini face the cytosol. This is in agreement with the model proposed for the assembly of LD. According to this model, the TAG will during the biosynthesis "oil-out" between the leaflets of the bilayers, forming lens-shaped structures in the membrane. The LD, covered by a monolayer, will be formed by the budding of such a lens from the membrane. The structure will be stabilized by proteins bound to both the luminal and cytosolic surface of the membrane.

Purpose and Rationale

Cell-free systems are extremely useful for studies of sorting processes, such as the budding of transport vesicles, and are the only method for determining the mechanism by which such complex structures are formed. A microsome-based, cell-free system has been developed by Marchesan and coworkers (2003) to study the transport of TAG from microsomes to the cytosol. This system assembles TAG-containing LD similar to the small LD isolated from 3T3-L1 cells. Caveolin, ADRP, vimentin, and the GRP-78 are present on these LD, and the release of the LD from microsomes is dependent on PLD and the formation of PA. This cell-free system may be helpful for the screening and characterization of compounds/drug candidates activating or inhibiting LD biogenesis by interference downstream of TAG synthesis (e.g., DGAT activity, assembly of LD-associated proteins at the ER, LD release from the ER) as well as for the elucidation of the mode of action of compounds/drug candidates identified in cell-based assays for lipogenesis (see "Lipogenesis").

Preparation of Microsomes

3T3-L1 cells are cultured and differentiated for 2 days (unless stated otherwise) as described above. The cells (2 × 106 cells/incubation) are homogenized (30 strokes in a dounce glass homogenizer in 10 mM Tris/HCl) (pH 7.5) containing 250 mM sucrose, 1 mM EDTA, 100 KIU/ml Trasylol, 0.1 mM leupeptin, 0.1 μM pepstatin, and 1 mM PMSF, and the microsomes are isolated by two distinct gradient ultracentrifugation methods as described (see "Cell-Free System"). To remove loosely bound (nonintegral) proteins from the microsomes, the pellet is resuspended in 500 mM Tris/HCl (pH 7.5) containing 1.2 M KCl (high salt wash) and

incubated (30 min, 4 °C) with head-over-tail mixing. The microsomes are then recovered by ultracentrifugation (160,000 × g, 70 min, 12 °C). The washed microsomes are resuspended in 10 mM Tris/HCl (pH 7.5) containing 250 mM sucrose.

Cell-Free System

Incubation

The in vitro system used to investigate the formation of LD and introduced by Marchesan and coworkers (2003) is based on microsomes that had been subjected to a high salt wash, an activator from the 160,000 × g-supernatant from homogenized rat adipocytes, and a substrate for DGAT. To prepare the DGAT substrate, 0.25 mg of 1,2-diacylglycerol is dissolved in 40 µl of 10 mM Tris/HCl (pH 7.5) with 1.0 mM palmitoyl-CoA by gentle vortexing: 1.25 µl of [14C]palmitoyl-CoA (55 mCi/mmol; total 0.06 µCi) and 360 µl of 10 mM Tris/HCl (pH 7.5) are added, and the mixture is vortexed again. To start the reaction, 600 µl of the partially purified cytosolic activator in 10 mM Tris/HCl (pH 7.5) containing 250 mM sucrose, 150 µl of 1.4 M $MgCl_2$ (in the same buffer), and 50 µl of the microsome solution (0.5–1 mg of microsomes) is added to the DGAT substrate. Incubation is carried out (37 °C, 60 min) (the production of LD plateaued between 30 and 60 min). After the incubation, the total production of radioactive TAG is measured, and the incubation mixture is subjected to gradient ultracentrifugation as described below. Before centrifugation, the mixture is supplemented with (final concentrations) Trasylol (100 KIU/ml), leupeptin (0.1 mM), PMSF (1 mM), pepstatin A (1 µM), *N*-acetyl-Leu-Leu-norleucinal (5 µM), and EDTA (0.5 mM).

Gradient Ultracentrifugation

For gradient I, the samples are adjusted to 25 % sucrose, and 1 ml is layered under 2 ml of 50 mM Tris/HCl (pH 7.5) with 10 % sucrose and 10 mM EDTA, which in turn is overlaid with 2 ml of 50 mM Tris/HCl (pH 7.5) and 10 mM EDTA, and centrifuged (160,000 × g, 17 h, 4 °C, Beckman SW55 Ti rotor). Homogenates of 3T3-L1 cells (differentiated for 10 days) are used to establish the gradient. The gradient is divided into four fractions with mean densities of <1.018 (2 ml), 1.034 (1 ml), 1.055 (1 ml), and 1.098 (1 ml) g/ml, respectively. The major amount of TAG (~60 %) is recovered in the d <1.018 g/ml fraction. The pellet (microsomes and cell debris) contains only ~3 % of the total amount of TAG recovered from the gradient. Caveolin is present in all fractions and in the pellet.

For gradient II, the samples are adjusted to 40 % sucrose, and 1.2 ml is layered under 3 ml of 50 mM Tris/HCl (pH 7.5) with 25 % sucrose and 10 mM EDTA, which is in turn overlaid with 1 ml of 50 mM Tris/HCl (pH 7.5) and 10 mM EDTA. After centrifugation (160,000 × g, 17 h, 4 °C Beckman SW55 Ti rotor), the gradient is arbitrarily divided into 5 fractions with mean densities of 1.055 (1.2 ml), 1.099 (1 ml), 1.112 (1 ml), 1.141 (1 ml), and 1.161 (1 ml) g/ml, respectively. Most (75 %) of the TAG is present in the top fraction (d <1.055 g/ml). Caveolin is present in the top fraction and in the two bottom fractions.

Characterization of Lipid Droplets

SDS-PAGE

LD fractions in microcentrifuge tubes are delipidated with 1.5 ml of cold acetone overnight at −20 °C followed by centrifugation (14,000 × g, 30 min, 4 °C) and removal of solvent from the protein pellet. The pellet is further extracted with acetone (20 °C) followed by acetone/ether (1/1, by vol.) and ether. Residual solvents are evaporated under nitrogen, and proteins are solubilized in 2 × Laemmli sample buffer by incubation in a bath sonicator at 65 °C for 4–5 h with frequent mixing using a vortex mixer. 2-Mercaptoethanol is added to samples before loading onto SDS-PAGE gels. Typically, LD proteins from 28 dishes of adipocytes are loaded onto 30 cm long SDS-PAGE gels for staining and further identification. Typically, proteins from two dishes of cells are loaded onto gels for transfer to nitrocellulose membranes and immunoblotting. Gels containing greater protein loads are stained for 2 h in 0.25 % Coomassie Blue G250 in 10 % acetic acid and 50 % methanol and then destained

in 7 % acetic acid and 5 % methanol for 4–6 h. To confirm the identification of known LD-associated proteins, nitrocellulose membranes containing LD proteins are probed with antisera raised against the amino terminus of rat perilipin A, mouse adipophilin (Research Diagnostics Inc., NJ), mouse TIP47 (Research Diagnostics), mouse S3–12, and caveolin-1 (Signal Transduction Inc.).

In-Gel Tryptic Digestion of LD-Associated Proteins

Coomassie-stained protein bands are excised from the gels and destained with 45 % acetonitrile in 100 mM ammonium bicarbonate. The resulting gel slices are incubated with 10 mM Tris (2-carboxyethyl)phosphine hydrochloride, alkylated by the addition of 50 mM iodoacetamide, and then digested in situ with trypsin (100 ng per band in 50 mM ammonium bicarbonate). The tryptic peptides are extracted using POROS 20 R2 beads (Applied Biosystems) in 0.2 % trifluoroacetic acid in 5 % formic acid. The extracted peptides are concentrated using C18 zip-tips and eluted with 0.1 % trifluoroacetic acid in 30 % acetonitrile followed by 0.1 % trifluoroacetic acid in 75 % acetonitrile. The eluates are dried under vacuum using a SpeedVac concentrator.

Mass Spectrometry

The resulting peptides are dissolved in 2–25 µl of HPLC sample solvents containing water/methanol/acetic acid/trifluoroacetic acid (70/30/0.5/0.01, by vol.). The volume used is proportional to the staining intensity of the given band. Micro-HPLC-MS/MS analysis is conducted on an LCQ electrospray ionization ion trap mass spectrometer coupled with an online MicroPro-HPLC system. Two microliters of tryptic peptide solution is injected into a Magic C18 column (0.2 × 50 mm, 5 µm, 200 Å, Michrom BioResources) which had been equilibrated with 70 % solvent A (0.5 % acetic acid and 0.01 % trifluoroacetic acid in water/methanol (95/5, by vol.) and 30 % solvent B (0.5 % acetic acid and 0.01 % trifluoroacetic acid in methanol/water (95/5, by vol.). Peptides are separated and eluted from the HPLC column with a linear gradient from 30 % to 95 % solvent B in 15 min at a flow rate of 2.0 µl/min. The eluted peptides are sprayed directly into the LCQ mass spectrometer (2.8 kV). The LCQ mass spectrometer is operated in a data-dependent mode for measuring the molecular masses of peptides (parent peptides) and collecting MS/MS peptide fragmentation spectra.

Database Search and Protein Identification

The measured molecular masses of parent peptides and their MS/MS data are used to search for information of nonredundant DNA/protein sequence database using the program KNEXUS (Genomic Solutions). The mass error tolerance used in the database search is usually ± 3 Da for the parent ions and ± 0.5 Da for the fragment ions, respectively. Protein identifications are made based on expectation values $<1 \times 10–2$ or the quality of MS/MS spectra of peptides identified. BLAST searches are performed for hypothetical and unknown proteins.

Reesterification

Purpose and Rationale

In adipose tissue under conditions of enhanced lipolysis, i.e., during fasting or β-adrenergic stimulation, synthesis of TAG from endogenous fatty acids released from the LD during lipolysis is usually not completely repressed. This apparently vicious cycle of the so-called reesterification is regulated in complex fashion and critically depends on the concentration of the exogenous fatty acids, which in combination with the lipolytically released fatty acids form a common pool for reesterification. Thus, the supply from/removal by the circulation of fatty acids ultimately determines the rate of reesterification which thereby ensures rapid and hormone-independent delivery of fatty acids into the circulation in case of immediate demand for nutrients. Previous studies of the regulation of FFA reesterification in vitro have used the balance technique (Vaughan 1962), in which reesterification is calculated from the difference between glycerol and FFA release (Gilbert et al. 1974; Hammond and Johnston

1987). This method does not distinguish between FFA reesterification and TAG synthesis. Other studies that measured both processes simultaneously did not provide exogenous FFA in the incubation medium (Brooks et al. 1982) and so do not accurately mimic the situation in vivo, in which FFA substrate may be derived from the plasma, either from hydrolysis of circulating lipoproteins or from albumin-bound FFA.

Whether FFA reesterification may be regulated independently from TAG synthesis can be addressed by the recently developed dual-isotopic technique for measuring FFA reesterification in adipose tissue (Leibel and Hirsch 1985). This method allows TAG synthesis and reesterification to be measured independently and simultaneously. Studies using this technique have suggested that the rate of FFA reesterification is not regulated at the cellular level, but rather by changes in the extracellular environment (Leibel et al. 1989; Edens et al. 1990).

Procedure

Preincubation medium consists of Krebs–Henseleit bicarbonate buffer containing 5 g/100 ml BSA and 4.17 mM glucose. Dual isotope incubation medium is a composition identical to preincubation medium, except that it also contains 0.5 mM palmitic acid and both [U-14C] glucose and [9,10-3H]palmitic acid. Palmitic acid is complexed to BSA as described by Leibel and coworkers (1984) with minor modifications. Briefly, a film of mixed unlabeled and [3H] palmitic acid is dissolved with ethanol and a small (12 %) molar excess of NaOH. This solution is heated at 60 °C for 30 min with gentle shaking. It is then dried under a stream of N2. Pre-warmed (60 °C) 10 % BSA in distilled, deionized water is added. The 10 % BSA is heated and gently shaken until clear. The 10 % BSA (now containing 1 mM palmitic acid) is combined with an equal volume of double-strength KHB bicarbonate buffer with glucose to make the incubation medium. The final medium-specific activity of [14C]glucose ranges from 0.4 to 0.9 μCi/μmol, and that of [3H]palmitate is 2.0 μCi/μmol. The final concentration of glucose in the incubation medium is 5 mM. The gas phase above both preincubation and incubation media is 5 % CO_2/95 % O_2. All incubations are carried out 2 h at a shaking speed of 80 cycles/min and temperature of 37 °C. Zero time samples are run and all other values are corrected accordingly. The isolated rat adipocytes are suspended at a 50 % concentration and aliquoted (200 μl) into 2 ml of incubation medium. Incubations are done in 20 ml polypropylene and screw-top vials at 37 °C, with shaking at 80 cycles/min. Incubations in medium containing either no added FFA or 0.5 mM palmitic acid are done in the basal state (no additions) and either with adenosine deaminase to prevent inhibition of lipolysis or with isoproterenol (1 μM) to stimulate lipolysis. At the end of the incubations (2 h), the adipocytes and medium are poured into iced Falcon tubes containing PE90 catheters. The tubes are spun gently for 1–2 min at 0 °C to float the adipocytes. Incubation medium is withdrawn from beneath the adipocytes through the catheter and frozen for subsequent glycerol assay by the method of Laurell and Tibbling (1966). The cells remaining in the tube are extracted by the method of Dole and Meinertz (1960). The extracted lipids are subjected to TLC to isolate TAG. The TAG is recovered and counted simultaneously for both 3H and 14C. The incorporation of [14C]glucose into TAG calculates the rate of reesterification as described (Leibel and Hirsch 1985).

Evaluation

Under these experimental conditions, 14C is not incorporated into the fatty acid moiety of AG and 3H is not incorporated into the glycerol moiety. FFA reesterification can be measured because both unlabeled FFA arising from lipolysis and medium-derived [3H]palmitate are esterified in newly synthesized [14C]AG. The degree of dilution of [3H]palmitate by unlabeled, endogenous FFA in [14C]TAG is an index of the rate of reesterification. Reesterification is calculated as the difference between the total theoretical moles of FFA in TAG (μmol[14C]TAG × 3) minus the actual total μmoles of [3H]palmitate esterified into TAG. Therefore, FA reesterification or the esterification of unlabeled FFA in TAG equals [(3 × μmol[14C]TAG)-μmol[3H]TAG], where μmoles

of [3H]TAG represents the amount of [3H]palmitate esterified into [14C]TAG. The data are expressed as μmol FFA reesterified per μmol newly synthesized TAG (3 × μmol[14C]TAG/μmol[3H]TAG/μmol[14C]TAG), a value that theoretically can vary between zero (no reesterification) and three (all newly esterified fatty acids derived from lipolysis). Adipocyte size is measured in aliquots of isolated adipocytes by microscopy. The lipid content of aliquots of isolated adipocytes is determined gravimetrically in Dole extracts. Adipocyte concentration in the incubation mixture is calculated by dividing lipid content by cell size (assumed to be 100 % lipid).

Cellular Lipolysis

General Considerations

Deregulation of lipid metabolism has long been recognized as essential in the development of obesity and the metabolic syndrome. White adipose tissue (WAT) lipolysis plays a pivotal role in controlling the quantity of TAG stored in fat depots and in determining plasma-free fatty acid levels (Frayn 2002). Hence, targeting critical steps of this catabolic process constitutes one of the strategies to combat obesity and the metabolic syndrome (Nisoli and Carruba 2004).

Activators of lipolysis represent a pharmacological interest only if the same molecule or another compound stimulates oxidation of fatty acid and energy expenditure. The lipolytic and thermogenic β3-adrenergic agonists highly efficient in rodents to decrease fat mass and insulin resistance have so far not been effective in humans. There are several potential explanations. Unlike rodents, adult humans have very little brown adipose tissue, the tissue specialized in thermogenesis in rodents, which possesses a lot of β3-adrenoceptors. The receptors are expressed at low levels in human white adipocytes, again a major difference with rodents. Finally, the first generation of β3-adrenergic agonists may have low efficacies and potencies for the human β3-adrenergic receptor. Nevertheless, the approach is interesting conceptually. Ideally, a strategy could be to stimulate lipolysis and use of the fatty acids by newly formed brown-fat cells (Tiraby and Langin 2003; Langin 2006). Concomitant stimulation of fat oxidation in skeletal muscle is an alternative strategy. The recently characterized lipolytic receptors (e.g., natriuretic peptide and pituitary adenylate cyclase-activating polypeptide receptors) and pathways (e.g., residual lipolytic effect of catecholamines in mice with no β-adrenoceptors) may constitute novel drug targets.

Since the launching of nicotinic acid (niacin) as a lipid-lowering drug a long time ago, suppression of lipolysis to decrease free fatty acid level has attracted much interest (Karpe and Frayn 2004). However, most known antilipolytic receptors are expressed in several organs raising the risk for side effects. The recent cloning of the receptor for nicotinic acid which is mainly expressed in WAT has undoubtedly led to important screening efforts for the identification of agonists with less side effects than niacin and its long-lasting form, acipimox (Tunaru et al. 2003; Wise et al. 2003). Inhibition of HSL is also attractive as the enzyme has little homology with other mammalian lipases and shows a rather limited tissue distribution. Indeed, several series of agonists have been synthesized (Slee et al. 2003; Ebdrup et al. 2004) with an apparent high specificity for some of them (Langin et al. 2005). The effect of chronic treatment in rodent models of obesity and dyslipidemia is now awaited.

Purpose and Rationale

The inhibition of lipolysis induced by starvation or β-adrenergic stimulation in response to insulin is of exquisite sensitivity (IC50 $<$ 0.1 nM) and responsiveness (lipolysis rate less than 5 % of that in the basal state) toward the hormone. This reflects the enormous physiological importance of a tight regulation of fatty acid release from adipose tissue to ensure both adequate nutrient supply and prevention from the detrimental so-called lipotoxic effects of FFA in the circulation. The complex molecular mechanism of the signal transduction in the adipocytes from the insulin receptor downstream to the lipolytic end effector systems, i.e., the TAG-degrading lipases

(see "Triacylglycerol (TAG) Lipases (HSL, ATGL) Activity"), has been characterized in detail (for a review, see Müller and Petry 2005). It encompasses the translocation of lipases (HSL) and LD surface proteins (perilipins) from the cytosol to the LD and vice versa, respectively, which is stimulated in the fastened/β-adrenergic state and blocked in response to insulin. These translocation processes are regulated by (PKA-dependent) phosphorylation of both the lipases and LD surface proteins and presumably additional regulatory proteins. These phosphorylations are downregulated by insulin through stimulation of cAMP degradation by phosphodiesterase 3B which is linked directly to distal elements of the insulin signaling cascade in adipocytes. Basal and stimulated lipolysis as well as its inhibition is monitored in vitro with cultured and primary rodent and human adipocytes and can be used to study insulin-like effects on the negative regulation (i.e., activation of the insulin signaling) or direct inhibition of lipolysis (i.e., of the lipases involved) by compounds/drug candidates. Several procedures are available based on the determination of glycerol and fatty acids released from unlabeled, fluorescently labeled, or radiolabeled TAG.

Method Based on Isolated Fat Pads

Gonadal fat pads are removed from male rats and washed several times with PBS. Tissue pieces (~20 mg) are incubated in DMEM containing 2 % fatty acid-free BSA with or without 10 μM isoproterenol at 37 °C. Portions are collected after 30, 60, 120, 180, and 240 min and analyzed for the FFA and glycerol content by using commercial kits (Wako Chemicals).

Method Based on the Release of Fluorescent Fatty Acids from Isolated Adipocytes

Procedure

This method which avoids the use of radioactivity but nevertheless enables the visualization and quantitative evaluation of the released fatty acids has been introduced by Müller and coworkers (2003). For fluorescent labeling of adipocyte TAG, 250 μl portions of adipocyte suspension in 1.5 ml plastic cups are incubated (15 min, 37 °C) with 200 μl of KRH containing 1 % BSA in the presence of 0.55 nM insulin prior to addition of 50 μl of NBD-FA (500 μM, prepared daily by dissolving 39.8 mg NBD-FA in 1 ml ethanol under moderate heating and subsequent 1:200 dilution with KRH containing 1 % BSA) and further incubation (1 h, 37 °C) under mild shaking (thermomixer 5436, setting 10, Eppendorf, Hamburg, Germany). For the removal of free NBD-FA left in the incubation medium, the adipocyte suspensions are combined in 50 ml plastic vials and centrifuged (500 × g, 1 min, swing-out rotor). The infranatant below the cell-surface layer is aspirated. The adipocytes are suspended in 30 ml of medium L (medium 199 containing 25 mM HEPES, 4 mM L-glutamine, 5.5 mM glucose, 3 % BSA). After two further cycles of centrifugation and resuspension, the washed adipocytes are finally adjusted to 2.5 × 106 cells/ml in medium L. For treatment with compounds/drug candidates directly inhibiting relevant lipases which may bind to proteins, the adipocytes are washed with medium L lacking BSA and then incubated (15 min, 37 °C) with the inhibitors in the same medium. For the initiation of lipolysis, 400 μl portions of adipocyte suspension are supplemented with 500 μl of medium L containing 2 or 4 % BSA (in case of preincubation with inhibitors) and 5.5 mM glucose and incubated (37 °C) for 30–180 min in the presence or absence of combinations of isoproterenol and ADA in plastic scintillation vials (total vol. 1 ml) under mild shaking in a water bath. One hundred fifty microliters of portions of the suspension are transferred into precooled Eppendorf cups containing 750 μl chloroform/heptane/methanol (3/3/2 by vol.), mixed vigorously, and centrifuged (12,000 × g, 1 min). The lower organic phase is removed, dried (SpeedVac), and then suspended in 20 μl of THF. 5 to 10 μl portions are analyzed by TLC using Si-60 F254 plates (Merck, Darmstadt, Germany) and diethyl ether/petroleum ether/acetic acid (78/22/1, by vol.) as solvent system.

Evaluation

Quantitative evaluation of the amount of NBD-FA is performed by fluorescence imaging (Storm 860, Molecular Dynamics, Germany) using imaging software (Molecular Dynamics, ImageQuant). Alternatively, 20 µl aliquots of the lower phase are supplemented with 325 µl of methanol/chloroform/heptane (10/9/7, by vol.) and 1.05 ml of 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5), vortexed, and then centrifuged (12,000 × g, 1 min). One hundred fifty microliters of portions of the upper phase are removed and measured for fluorescence (automatic fluorometric analyzer, Cobas Bio, Roche Diagnostics, Germany).

Method Based on the Release of Glycerol from Isolated Adipocytes

Procedure

The determination of glycerol released from unlabeled adipocytes is the most common and convenient method and can be performed according to published procedures (Petry et al. 2005) with the following modifications. Three hundred fifty microliters of aliquots of the incubation mixtures are rapidly transferred into 1.5 ml Eppendorf cups and precooled to 4 °C containing 300 µl of 10 % (w/v) HClO4. After incubation (1 h, 4 °C), the precipitates are removed by centrifugation (12,000 × g, 2 min). The infranatants recovered are neutralized with 20 % KOH, followed by addition of 60 µl of 1 M Tris/HCl (pH 7.4). One hundred fifty microliters of samples are removed for glycerol determination, frozen in liquid N2, and stored at −20 °C. Glycerol is measured fluorometrically by a modified version of the enzymatic method (Wieland 1974). The reaction mixture contains 0.1 M Tris/HCl (pH 9.3), 0.9 mM ATP, 1.25 mM NAD, 0.1 M $MgCl_2$, 0.25 % (v/v) hydrazine hydrate, 5–50 µl sample or glycerol standard, and a mixture of glycerol kinase (0.2 U/ml final conc.) and glycerol 3-phosphate dehydrogenase (0.7 U/ml final conc.) in a total volume of 240 µl. Fluorescence is determined using an automatic fluorometric analyzer.

Method Based on the Release of [3H] Oleic Acid from Isolated Adipocytes

Procedure

Adipocytes are washed with KRPH buffer (128 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 25 mM sodium phosphate, 1 mM sodium pyruvate, 5.5 mM glucose, 50 mM HEPES/KOH, pH 7.4) and then incubated (2 h, 37 °C) in fresh KRPH containing [3H]oleic acid (50 µCi per 2 × 106 cells and ml) and 1 % BSA in the presence of 0.55 nM human insulin. The adipocytes are washed with KRPH (10 °C) containing 5.5 mM glucose and 2 % BSA or lacking BSA (for subsequent treatment with direct lipase inhibitors for 15 min at 37 °C) three times by centrifugation and then incubated (2 h, 37 °C) under mild shaking in KRPH containing 5.5 mM glucose, 2 % BSA, and 400 µM phloretin (for reduction of fatty acid uptake by 85 %) at 1 × 106 cells/ml in the presence of lipolytic stimuli. Thereafter, 150 µl portions of the adipocyte suspension are transferred into narrow centrifugation tubes (Beckman) and then overlaid with 100 µl of dinonyl phthalate (Merck, Darmstadt, Germany). After centrifugation (2,000 × g, 30 s, 25 °C), the tubes are cut through the oil layer and the lower parts of the tubes containing the incubation medium transferred into 5 ml scintillation vials and mixed with 3 ml of chloroform/heptane (1/1, by vol.) containing 2 % methanol. After centrifugation (2,000 × g, 5 min), the upper aqueous phase is removed by suction. Following addition of 5 ml of liquid scintillation cocktail to the lower organic phase, radioactivity is measured.

Evaluation

Control values of the 0 min incubation with the corresponding lipolytic stimulus are subtracted in each case to correct for [3H]oleic acid left from the labeling period, accounting for 2–7 % of the total radioactivity recovered with the medium. TLC and phosphorimaging analysis revealed that under both basal and lipolytic conditions, [3H] oleic acid accounts for more than 70 % of the total radioactivity measured, radiolabeled

degradation products (recovered mainly with the aqueous phase) for 15–20 %, and TAG for up to 6 %.

Method Based on the Release of Unlabeled Fatty Acids from Isolated Adipocytes

Purpose and Rationale

Studying lipolysis with human adipocytes and adipose tissues is hampered by methodological problems. The amount of human adipose tissue that is available is usually limited, and the rate of lipid mobilization from human adipocytes is low compared to that of rat adipocytes. Both chemical and enzymatic methods for the determination of FFA in human blood and serum have been developed (e.g., Matsubara et al. 1983; Shimizu et al. 1980; Duncombe and Rising 1973; Miles et al. 1983; Okabe et al. 1980). However, incomplete recovery of FFA from plasma (Duncombe and Rising 1973) and interference of BSA with the FFA assays (Matsubara et al. 1983; Shimizu et al. 1980; Okabe et al. 1980) have been reported, which results in an underestimation of FFA values in serum and plasma samples. To overcome this, the extraction method has been modified (Duncombe and Rising 1973), BSA has been included in the standard curve (Miles et al. 1983), or p-toluenesulfonic acid has been used (Matsubara et al. 1983).

These methods are unsuited for FFA determination for human in vitro studies since the detection limit is too high using small amounts of human adipose cells with usually low lipolytic activity. Näslund and coworkers (1989) have established a chemiluminometric method for the determination of low levels of FFA based on H_2O_2 determination in a peroxidase luminescence reaction. Since albumin (which must be added to the incubation medium as FFA carrier) infers with this assay resulting in nonlinear FFA standard curves, Näslund and coworkers (1993) modified the chemiluminometric assay for compatibility with biological samples containing albumin.

Procedure

A semiautomatic luminometric method for determination of small amounts of FFA released from human adipocytes in vitro has been introduced by Näslund and coworkers (1993). BSA is used as acceptor of FFA in the incubation medium of isolated fat cells. The assay involves pretreatment with the detergent SDS to liberate the FFA from the BSA before activation by acyl-CoA synthetase (ACS). This is followed by oxidation of the resulting thioesters by acyl-CoA oxidase (ACO). The H_2O_2 formed is subsequently measured in a horseradish peroxidase (HRP)-catalyzed luminol reaction. The assay is linear in the interval of 0.01–1 nmol in the cuvette corresponding to 2–200 μmoles in the sample, and 25 samples can be automatically assayed in the luminometer within 75 min. FFA release can easily be studied in a small incubation volume (200 μl) of very diluted (104 cells/ml) human adipocyte suspensions. Samples (25 μl) containing 0.25 % BSA from incubates of adipose tissue cells do not interfere with the standard curve. This method (Näslund et al. 1993) is a sensitive, simple, and inexpensive luminometric assay for FFA release. It is 100-fold more sensitive than standard spectrophotometric methods and can be used for serial studies of lipid metabolism (lipolysis, FFA reesterification) in small amounts of human adipose tissue or for investigations of FFA release from very diluted human adipose cell samples.

Cell-Free Lipolysis

Purpose and Rationale

For analysis of effects of compounds/drug candidates on the translocation to the LD and direct activation of HSL including the increase in accessibility of the TAG of LD for degradation by HSL (or other/additional lipases), these individual steps of lipolysis can be reconstituted in a cell-free system consisting of purified LD and crude HSL (and other lipases) prepared from primary rat adipocytes.

Procedure

Preparation of LD from Adipocytes

LD are prepared according to Egan and coworkers (1992) and adaptations from Wolins and coworkers (2005) with the following modifications: After the incubation, the adipocytes are washed two times with KHH by flotation and removal of the infranatant by suction and then homogenized in 25 mM Tris/HCl (pH 7.4), 250 mM sucrose, 5 mM NaF, 10 mM NaPPi, 1 mM Na3VO4, 1 mM EDTA, 20 μg/ml leupeptin, 1 mM benzamidine, and 0.5 mM PMSF at 15 °C. After centrifugation (1,000 × g, 5 min, 15 °C), 1 ml of the supernatant is combined with 1.5 ml of 65 % sucrose (w/v) and poured into a 5 ml centrifuge tube. 1.5 ml of 10 % sucrose (w/v) is then layered on top of the sucrose cushion. The tube is filled to capacity with buffer A. The gradient is centrifuged (172,000 × g, 60 min, 15 °C) and then allowed to coast to rest. The floating fraction of LD is visualized as the upper white layer of the gradient and is isolated by suction with a syringe (0.8 ml). After one washing cycle with buffer A, the LD are suspended in an equal volume of fourfold sample buffer containing 10 % SDS by incubation (10 min, 65 °C) and treatment in a bath sonicator (10 min, 25 °C, max. power) for extraction of proteins. After centrifugation (10,000 × g, 5 min, 25 °C), proteins contained in the infranatant are separated by SDS-PAGE. Alternatively, the gradient is fractionated from bottom to top by suction with a syringe (0.8 ml each). Proteins of fractions 1–5 are precipitated with 5 % TCA (15 min, 2 °C, 15,000 × g, 10 min), washed three times with ice-cold acetone, dried, and finally suspended in sample buffer. Fraction 6 is extracted for protein (see above). All samples are analyzed by SDS-PAGE and immunoblotting.

Cell-Free System

Adipocytes labeled with NBD-FA (see "Method Based on the Incorporation of a Fluorescent Fatty Acid Analog") are washed with KRH containing 0.5 % BSA, suspended in the same medium at 3.5 × 105 cells/ml, and incubated (5 min, 37 °C) in plastic scintillation vials under mild shaking in a water bath in the presence or absence of isoproterenol, insulin, or compound/drug candidate. Adipocytes (3.5 × 106 cells) are washed twice with BSA-free KRH by flotation (500 × g, 1 min) and then suspended in 2.5 ml of homogenization medium (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 2 mM DTT, 50 mM glycerol-3-phosphate, 10 mM sodium pyrophosphate, 25 mM NaF, 1 μM microcystin, 2 μM cantharidin, 1 mM benzamidine, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 2 μg/ml antipain, 25 μg/ml soy bean trypsin inhibitor, 5 μg/ml PKA inhibitor). The mixture is vortexed, homogenized in a Teflon-in-glass homogenizer (10 strokes using a rotating Teflon pestle) at 15 °C, transferred into plastic vials (under rinsing of the homogenization vessel with homogenization medium), and then centrifuged (12,000 × g, 30 min, 4 °C, swing-out rotor). The infranatant (cytosol) is separated from the (fluorescent) TAG-containing LD by aspiration with a syringe. To eliminate entrapped unbroken cells, the LD are resuspended in 8 ml of homogenization medium (15 °C) containing 120 mM sucrose and 140 mM KCl, vortexed vigorously, and subsequently subjected to centrifugation (200,000 × g, 1 h). The infranatant (washing medium) is removed by suction. For initiation of NBD-FA release, 1 ml of ice-cold homogenization medium containing 2 % BSA is added to the plastic vial, while the LD adhering to the vessel wall are removed by repeated pipetting. A Teflon-coated magnetic stirring disk is placed into the vial. The mixture is warmed up to 30 °C and incubated under stirring (250 rpm) for 1–4 h. The digestion is terminated by addition of 2 ml of chloroform/methanol (3/1; by vol.). The extracts are dried (SpeedVac), suspended in 50 μl of THF, and then analyzed by TLC as described below.

Cell-free lipolysis is performed in glass tubes as described by Okuda and coworkers (1994) and Morimoto and coworkers (2000, 2001) with the following modifications: 40 μl of packed NBD-FA-labeled LD is incubated (90 min, 30 °C, constant shaking) with 80 μl of a solution enriched for HSL (100–200 μg protein) and 120 μl of buffer C

containing 2 % BSA and 2 mM DTT with or without 4.2 mg gum arabic. For incubation of the cell-free lipolysis system, compounds are added directly from the 10 mM stock solutions in DMSO to the reaction mixture containing the LD without prior dilution. The final DMSO concentration during all incubations is kept constant at 0.1 %. Portions of the lipolysis reaction are terminated by extraction of the total incubation mixture with 720 µl of chloroform/methanol/0.1 N HCl (3/1/1, by vol.). After vortexing and centrifugation (2,000 × g, 2 min) of the mixture, the organic phase is collected, dried under a stream of N2, and resuspended in 40 µl of tetrahydrofuran. Ten microliters of portions are analyzed by TLC (Silica Gel G plates, Merck Darmstadt, Germany; diethyl ether/petroleum ether/acetic acid, 78/22/1 by vol.). NBD-FA released from the total NBD-FA-labeled AG is quantitatively evaluated by phosphorimaging (e.g., Storm 860, Molecular Dynamics, Germany) using imaging software (ImageQuant, Molecular Dynamics), as described previously (Müller et al. 2003).

Evaluation

A blank value of a control reaction terminated immediately after addition of HSL-solution to the LD is subtracted in each case. Other portions of the lipolysis reaction are supplemented with 760 µl of buffer C containing protease inhibitors and centrifuged through sucrose cushions for the recovery of LD which are then analyzed for protein composition, in particular for the amount of HSL (HSL translocation; see "Translocation of Hormone-Sensitive Lipase (HSL)").

Translocation of Hormone-Sensitive Lipase (HSL)

Purpose and Rationale

The molecular mechanism of the regulation of lipolysis in adipocytes by starvation, β-adrenergic stimuli, and insulin as well as compounds/drugs (e.g., nicotinic acid) relies on the movement of HSL from the cytosol, its predominant location in the basal state, to the LD (Egan et al. 1992; Brasaemle et al. 2000; Clifford et al. 2000, for reviews see Holm and Osterlund 1999; Holm 2003; Londos et al. 2005; Müller et al. 2005). Candidate components involved in this HSL translocation initiated by β-adrenergic stimuli and inhibited by insulin and lipid-lowering drugs are elements of the cytoskeleton and LD-associated proteins, such as perilipin. Interestingly, perilipin has been observed to undergo reverse translocation from the LD to the cytosol upon challenge of adipocytes with insulin. The relative contribution of HSL and perilipin translocation to lipolysis stimulation seems, however, to depend on size, age, and origin of the adipocytes. HSL translocation blocked by insulin or compounds can be easily monitored by immunoblotting of isolated LD for HSL

Procedure

For the identification of HSL associated with LD in the induced compared to the basal state of the adipocytes, LD are prepared, purified, suspended in 20 % SDS, vortexed vigorously, then incubated (10 min, 95 °C), and finally centrifuged (12,000 × g, 5 min). Solubilized LD-associated proteins recovered from the infranatant by suction are analyzed by SDS-PAGE (7.5 % separating gel). The proteins are then transferred onto polyvinylidene difluoride (PVDF) membranes for 4 h at 200 mA using 20 mM 3-(cyclohexylamino)-1-propanesulphonic acid (pH 11.0) in 10 % methanol. Transfer efficiency is evaluated by Amido black staining in control lanes. The membrane is washed with PBS containing 0.1 % Tween-20, then incubated (1 h, 25 °C) with PBS containing 0.5 M NaCl and 0.75 % BSA, and subsequently incubated (18 h, 4 °C) in the same buffer with anti-HSL antibody (1:1,000 dilution, 0.5–2 µg/ml). This antibody was raised in white New Zealand rabbits against recombinant full-length His-tagged human HSL. This 84 kD polypeptide was encoded by a pET-derived expression vector under the control of the T7 promoter, expressed in *E. coli*, then partially purified from inclusion bodies by SDS-PAGE and subsequent elution from the gel band, and finally homogenized in PBS prior to emulsification in Ribi Adjuvant

System (RIBI Immunochem Research Inc., USA) and subsequent intraperitoneal injection into rabbits. The antibody was purified by chromatography on protein A-Sepharose. Subsequently, the blotting membrane is washed two times with PBS containing 0.1 % SDS, 0.2 % Nonidet P-40, and 0.25 % sodium deoxycholate and then once with PBS. Thereafter, the filters are incubated (2 h, 25 °C) with horseradish peroxidase-conjugated anti-rabbit-IgG (1:5,000 dilution) in PBS containing 0.75 % BSA.

Evaluation

Following three washing cycles (see "► Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes"), the amount of HSL is determined by ECL Western blotting detection reagents (Amersham-Pharmacia, Germany) according to the manufacturer's instructions. Quantitative evaluation is performed by luminometric scanning (Roche Diagnostics, Germany) using ImageQuant software.

Protein Composition of LD

Purpose and Rationale

Stimulation as well as inhibition of lipolysis in adipocytes by hormones, nutritional signals, or compounds/drug candidates exerts marked effects on the protein composition of LD favoring TAG/LD degradation or TAG/LD synthesis, respectively. These alterations can be monitored by modern proteomic analysis as has already been performed by Brasaemle and coworkers (2004) for 3T3-L1 adipocytes, Liu and coworkers (2004) for CHO fibroblasts, Fujimoto and coworkers (2004) for cultured human HuH7 hepatoma cells, Umlauf and coworkers (2004) for cultured human A431 epithelial cells, and Wu and coworkers (2000) for mouse mammary glands. The unexpected identification of caveolin as LD-associated protein in adipocytes (for a review, see Liu et al. 2002) demonstrates the power of modern proteomic analysis. These data may be useful to characterize and differentiate the molecular mechanisms of compounds which interfere with the lipid metabolism of physiologically relevant cells, such as adipocytes, at unknown site(s).

Procedure

For proteomic analysis of adipocyte LD, 6 days after the initiation of differentiation, 40 dishes of 3T3-L1 adipocytes are incubated either under lipolytically stimulated conditions (e.g., 10 μM isoproterenol and 0.5 mM IBMX for 15 min at 37 °C) or without the additions for basal conditions and subsequently in the absence or presence of antilipolytic stimuli (e.g., insulin or compound/drug candidates for 2 h) before harvest. Culture medium is removed, and cells are rinsed twice with ice-cold PBS before scraping off the cells into PBS using typical cell scrapers. Cells from sets of ten dishes of cells are pooled into 15 ml conical screw-capped tubes (Falcon) and centrifuged (500 × g, 5 min). Cell pellets are resuspended in a hypotonic medium containing 10 mM Tris (pH 7.4), 1 mM EDTA, 10 mM sodium fluoride, 20 μg/ml leupeptin, 1 mM benzamidine, and 100 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride by pipetting and incubated on ice for 10 min before homogenization by ten strokes in a Teflon-in-glass homogenizer. Cell lysates are centrifuged (26,000 × g, 30 min, 4 °C, SW41 Ti-rotor Beckman), and the rotor is allowed to coast to a stop. The floating LD layers are harvested using a tube slicer, and the harvested fractions are adjusted to 25 % sucrose and 100 mM sodium carbonate (pH 11.5) using a 60 % sucrose stock solution and a 1 M sodium carbonate stock solution with protease inhibitors followed by gentle mixing by pipetting. The density-adjusted fractions (~4 ml) are layered into centrifuge tubes containing 1 ml cushions of 60 % sucrose and then overlaid with ~5 ml of 100 mM sodium carbonate (pH 11.5) with protease inhibitors followed by ~3.5 ml of the hypotonic lysis medium with protease inhibitors. Tubes are centrifuged (26,000 × g, 30 min, 4 °C, SW41 Ti-rotor Beckman) and the rotor is allowed to coast to a stop. Floating LD are harvested using a tube slicer into 1.5 ml microcentrifuge tubes. Residual carbonate solution is removed by centrifuging tubes (14,000 × g, 20 min, 4 °C, Eppendorf microcentrifuge). The infranatant is removed with an 18-gauge needle from below the floating LD fraction, and the LD fraction is

rinsed once with hypotonic lysis solution containing protease inhibitors.

Evaluation

Typically, LD are isolated from 8 separate sets of 40 dishes of adipocytes grown and differentiated at different times. Four sets of dishes are used for basal conditions, and four sets of dishes are used for lipolytically stimulated conditions. Proteins from LD preparations from 3 sets of 28 dishes of basal adipocytes are separated in 3 lanes of a single SDS-PAGE gel. Coomassie-stained bands are compared and found to be equivalent for all lanes. Bands from one lane are excised for analysis by mass spectrometry. Proteins from LD preparations from 3 sets of 28 dishes of lipolytically stimulated adipocytes are separated in 3 lanes of a single SDS-PAGE gel and compared with a single lane containing proteins from 28 dishes of basal adipocytes on the same gel. Coomassie-stained bands are compared and found to vary slightly between the three preparations from the lipolytically stimulated adipocytes and significantly between basal and stimulated preparations. Bands are excised for all stained proteins from two lanes of stimulated preparations and analyzed by mass spectrometry.

The LD of adipocytes are unique among LD of many types of cells with respect to size, the perilipin-enriched protein composition, and the dynamic rearrangements in structure that occur in response to stimulation of the β-adrenergic signaling pathway. Although perilipin A is the most abundant protein associated with the large LD of basal adipocytes, when lipolysis is stimulated, these LD fragment into myriad micro-LD. Interestingly, the total mass of perilipin does not increase in proportion to the greatly increased surface area of the micro-LD. Some of the excess surface becomes coated with other PAT family members, adipophilin, TIP47, and S3–12 (Wolins et al. 2005). Additionally, the association of numerous unrelated proteins with the LD increases.

Adipophilin was found by proteomic analysis of proteins on LD isolated from lipolytically stimulated 3T3-L1 adipocytes but not basal adipocytes. This finding was confirmed by immunofluorescence microscopy experiments. The appearance of adipophilin on the LD of lipolytically stimulated adipocytes may be a consequence of increased availability of binding sites on micro-LD to stabilize nascent adipophilin. The results strongly suggest that adipophilin plays a role in lipolysis. The identification of caveolin1 on LD isolated from lipolytically stimulated but not basal adipocytes is consistent with previous observations (Cohen et al. 2004). Caveolin-1, perilipin, and the catalytic subunit of PKA can be coimmunoprecipitated from lysates of adipocytes preincubated with β-adrenergic receptor agonists but not from basal adipocytes (Cohen et al. 2004). Thus, the three proteins form a complex in stimulated cells with caveolin-1 thereby bridging between perilipin and the catalytic subunit of PKA to facilitate the phosphorylation of perilipin. The identifications of lipid metabolic enzymes on the LD of adipocytes and several other types of cells shows that these structures are not passive repositories for neutral TAG but, instead, comprise a dynamic pool of lipids.

The identifications of several proteins in the adipocyte LD preparations carry implications regarding the immediate subcellular neighborhood of LD. The intermediate filament protein, vimentin, is identified in the basal and stimulated state. Previous studies show that fibrous vimentin intermediate filaments collapse into a cage structure around developing LD in differentiating adipocytes (Franke et al. 1987). The disruption of vimentin cage formation halts LD formation (Lieber and Evans 1996). Identification of the Eps-15 homology domain protein EHD2, a protein that binds to actin filaments in differentiated 3T3-L1 adipocytes (Guilherme et al. 2004), provides additional evidence of a close link between LD and the cytoskeleton. The identification of calnexin in basal and lipolytically stimulated LD preparations, as confirmed by immunofluorescence microscopy, suggests that segments of endoplasmic reticulum come into close contact with LD. Finally, several mitochondrial proteins can be identified in both preparations, including a subunit of ATP synthase, prohibitin, and pyruvate carboxylase, indicating that mitochondria are closely associated with and difficult to separate

from LD. Several studies have illustrated the tight packing of mitochondria around LD in adipocytes (Cohen et al. 2004; Blanchette-Mackie et al. 1995).

In conclusion, surface proteins on adipocyte LD include enzymes involved in many aspects of lipid metabolism, as found on LD in other types of cells. However, unlike other types of LD, perilipins are a major protein component that controls lipid traffic and the association of adipophilin (Brasaemle et al. 1997; Tansey et al. 2001, 2003), HSL (Sztalryd et al. 2003), of CGI-58 (Yamaguchi et al. 2004; Subramanian et al. 2004), and likely of other proteins with the LD. The stimulation of lipolysis is accompanied by the fragmentation of large LD into numerous dispersed micro-LD accompanied by major changes in the overall protein composition. Although most of the changes appear to affect the relative mass of various proteins that also associate with basal LD, adipophilin and caveolin-1 selectively associate with LD in stimulated cells. It is expected that this type of analysis will be useful for the future characterization including elucidation of the mode of action of compounds/candidate drugs which affect lipid metabolism by either stimulating or inhibiting TAG synthesis or degradation and in consequence LD biogenesis in adipose as well as in nonadipose tissues.

Interaction of HSL and Perilipin

Purpose and Rationale

Recently, the differential effects of blocking perilipin phosphorylation on HSL translocation and lipolysis have been dissected (Miyoshi et al. 2006). Employing adenoviral and transgenic expression of a perilipin construct deficient in all 6 PKA sites (Peri A Δ1–6), PKA-dependent phosphorylation of perilipin has been demonstrated not to be required for HSL translocation to the LD, but is essential for the lipolytic actions of LD-associated HSL in adipocytes. Cross-linking and immunoprecipitation studies suggest that PKA-dependent phosphorylation of perilipin promotes close-range interaction with LD-associated HSL. It is conceivable that compounds/drug candidates promoting or abrogating TAG synthesis/LD biogenesis or their degradation modify the HSL-perilipin in negative or positive fashion, which can be monitored with the following assay systems.

Procedure

Construction of Perilipin KO Mice Expressing the Perilipin Mutant Transgene Lacking the 6 PKA Consensus Phosphorylation Sites in BAT

A targeted disruption of the perilipin gene is performed by replacing exon 3 with a neomycin cassette, thereby disrupting coding of all known perilipin mRNAs. The perilipin KO mice are viable and exhibit predicted reductions in adipocyte size and adipose mass (Martinez-Botas et al. 2000). Perilipin KO mice are backcrossed for ten generations to C57BL/6 mice. For generation of mice expressing a peri Δ1–6 transgene in adipose tissue, a mouse peri Δ1–6 cDNA, FLAG tagged at the carboxy-terminal using PCR is generated and then ligated into a SmaI site of pBluescript SK vector containing the aP2 enhancer/promoter region, the SV40 small tumor antigen splice site, and polyadenylation signal sequence. A fragment containing the entire aP2-perilipin A-FLAG transgene is microinjected into fertilized eggs of C57BL/6 mice using well-described procedures (Brinster et al. 1981). Transgenic mice expressing the peri Δ1–6 transgene almost exclusively in BAT are obtained. These mice are fully viable, exhibit no apparent adipose phenotype, and are mated with backcrossed perilipin KO mice to generate perilipin A Δ1–6 mice (Miyoshi et al. 2006).

HSL-Perilipin Cross-Linking and Coimmunoprecipitation

Differentiated adipocytes expressing GFP and wild-type or mutant (e.g., deletion of the 6 PKA consensus phosphorylation sites) FLAG-tagged perilipin A are incubated in differentiation medium containing palmitic and oleic acid (240 μM each) for 48 h. After overnight serum depletion, adipocytes are treated for 15 min with 200 nM phenylisopropyl adenosine (PIA, basal state) or 20 μM forskolin plus 0.5 mM IBMX

(stimulated state), washed with PBS, and then maintained for 10 min in hypotonic lysis buffer (20 mM HEPES/KOH, pH 7.5, 2 mM EDTA, 50 mM sodium fluoride, 100 μM sodium orthovanadate, protease inhibitor cocktail) and either PIA or forskolin plus IBMX. Cells are then harvested and homogenized (ten strokes with a Teflon-in-glass homogenizer on ice). 3,3′-Dithiobis sulfosuccinimidylpropionate (DTSSP), a cross-linker with 12A spacer arm length (Pierce, Rockford), is added to the homogenates and incubated with rotation at 22 °C for 30 min. The cross-linking reaction is terminated by addition of Tris/HCl (50 mM final). After addition of Triton X-100 (2 % final) and NaCl (75 mM final), samples are drawn through a 23 gauge needle and then centrifuged (10,000 × g, 20 min, 4 °C) to separate the LD (upper layer), infranatant (lower layer), and a membrane pellet. The LD are isolated with a tube cutter and placed in a fresh tube. The infranatant is subjected to multiple rounds of centrifugation and removal of the residual LD, until almost no LD are visible. The pooled LD fractions are washed with 1 ml of fresh lysis buffer, collected after centrifugation, and resuspended in fresh lysis buffer. Half of this LD suspension is used for TAG measurement. The other half is incubated with SDS lysis buffer (10 % final) at 37 °C for 30 min with vortexing and then centrifuged for 10 min, after which the final LD fraction is harvested. Portions of each fraction are loaded on SDS-PAGE gels and then immunoblotted for perilipin and HSL. This confirmed that more than 95 % of perilipin is removed from the LD and present in the infranatant fraction. The infranatant fraction is immunoprecipitated using the FLAG-tagged protein immunoprecipitation kit (Sigma) at 4 °C for 24 h with rotation. Immunoprecipitates are eluted from the pelleted anti-FLAG agarose beads by boiling in SDS-PAGE sample buffer containing 2-mercaptoethanol. The reducing conditions are sufficient to break the DTSSP-induced protein cross-links. Eluted samples are electrophoresed and immunoblotted for the LD-associated proteins, perilipin, and HSL. To assess potential nonspecific cross-linking of perilipin with membrane or cytosolic proteins, immunoblots are also probed with corresponding antibodies (e.g., toward clathrin, adiponectin).

Evaluation

With respect to the phosphorylation-dependent mechanism by which perilipin promotes lipolysis, the assay monitoring the interaction of HSL and perilipin reveals that the lipolytic action(s) of LD-associated HSL requires a novel event(s) mediated by PKA-dependent perilipin phosphorylation. The precise nature of this event(s) remains to be elucidated. However, it is likely to involve conformational changes in perilipin that bring LD-associated HSL into proximity with stored neutral TAG. The effected LD-associated HSL presumably includes the pool of HSL that is prepositioned at the LD in the basal state (Moore et al. 2005; Morimoto et al. 2001; Brasaemle et al. 2004). The cross-linking studies suggest that PKA-dependent phosphorylation of perilipin alters the spatial relationship between perilipin and LD-associated HSL in such a way as to facilitate close-range interaction between the two (or between HSL and a perilipin-associated moiety). This interaction may facilitate access of LD-associated HSL to stored neutral TAG, thereby initiating lipolysis. Recently, chronic PKA-dependent phosphorylation of perilipin A has been shown to induce LD remodeling (i.e., fragmentation and dispersion) independently of lipase action (Marcinkiewicz et al. 2006). This suggests that this remodeling promotes lipolysis and supports the concept that PKA-dependent phosphorylation induces dramatic conformational changes in perilipin.

Thus, perilipin seems to act as the critical component of a scaffold that stabilizes LD structure and composition for optimal TAG storage and regulated lipolysis (Moore et al. 2005; Brasaemle et al. 2004). Compound/drug candidates interfering with lipolysis may directly modify the perilipin–HSL interaction without affecting perilipin phosphorylation. Those compounds could be identified using the cross-linking assay.

Triacylglycerol (TAG) Lipase (HSL, ATGL) Activity

General Considerations

A direct effect of a compound on the activity of HSL rather than its translocation can be assayed in a cell-free system consisting of emulsified TAG rather than intact LD (see "Protein Composition of LD"). Lipases play a key role in human lipid metabolism as they degrade dietary nutritional as well as intracellular stored lipids and thereby initiate and regulate the release of FFA into the serum. Therefore, they represent potential targets for the development of drugs aimed at obesity, diabetes, and atherosclerosis (for a review, see Petry et al. 2004). Pancreatic lipase (PL), gastric lipase (GL), and HSL are responsible for the cleavage of dietary and stored lipids, respectively, to fatty acids and glycerol (and MAG) in the stomach and duodenum (GP; for a review, see Lengsfeld et al. 2004), enterocytes (PL; for a review, see Tiss et al. 2004), and adipocytes (HSL), which finally will appear in the blood stream directly or as lipoproteins after passage across and esterification/assembly into lipoproteins (chylomicrons) in enterocytes. In addition, lipases are responsible for the degradation of lipoproteins (TAG in chylomicrons) in the liver by hepatic lipase or of lipoproteins (VLDL, LDL from the liver, chylomicrons from enterocytes) in muscle and adipose tissue endothelium by lipoprotein lipase (LPL) and endothelial lipase (EL; for a review, see Badellino et al. 2004). The activities of LPL and HSL are under tight hormonal control by a complex interplay of transcriptional and posttranscriptional mechanisms.

The rate-limiting role of HSL in the breakdown of adipose tissue TAG, postulated for decades (Fredrikson et al. 1981; Holm 2003; Kraemer and Shen 2002; Yeaman 2004; Osterlund 2001), has been challenged by the data from HSL knockout mice (Hämmerle et al. 2002; Osuga et al. 2000; Wang et al. 2001; for a review, see Raben and Baldassare 2005). Catecholamine-induced lipolysis is abrogated, but residual basal lipolysis is observed in adipocytes from HSL-null mice, which surprisingly display a non-obese phenotype and the accumulation of DAG in their adipose tissues. These data suggest the existence of non-HSL lipases in adipose tissue (of rodents, at least) that preferentially hydrolyzes the first ester bond of the TAG molecules. To search for such TAG lipases, gene and protein databases for murine and human proteins with structural homologies to known lipases, i.e., the GXSXG motif for serine esterases and the α/β-hydrolase folds, have been screened. Candidates have been analyzed for TAG-hydrolase activity and expression in mouse adipose tissues and during differentiation of 3T3-L1 adipocytes. Only one previously undescribed enzyme fulfilled these requirements and has been named adipocyte TAG lipase (ATGL), desnutrin, or iPLA2ζ (Zimmermann et al. 2004; Jenkins et al. 2004; Villena et al. 2004). The 260 amino-terminal amino acids of this 54 kD protein contain a predicted esterase domain in the α-/β-hydrolase fold as well as a GXSXG site with a putative active serine (amino acid 47). Moreover, a "patatin" domain is present in the same domain. Patatin domain-containing proteins have been shown to exert acyl-hydrolase activity on phospholipid, MAG, and DAG substrates and are thought to be involved in the ATP and acyl-CoA-independent transacylation reaction. Consistent with this, ATGL catalyze the CoA-independent transfer of oleate from a mono-olein donor to a mono- or diolein acceptor, which generates DAG and TAG, respectively. Using antibodies directed against ATGL, it has been suggested that ATGL is responsible for 75 % of the cytosolic acyl-hydrolase activity in white adipose tissue of HSL-deficient mice. ATGL could therefore participate together with HSL in adipose tissue lipolysis. Although HSL catalyzes the rate-limiting step in TAG hydrolysis, the major physiological substrate for this enzyme is DAG, not TAG. Hammerle and coworkers generated ATGL-deficient mice (Haemmerle et al. 2006) and thereby confirmed that ATGL is the rate-limiting lipase for the initiation of TAG catabolism in adipose and many nonadipose tissues. The

association of ATGL deficiency with increased glucose tolerance, increased insulin sensitivity, and increased respiratory quotient during fasting provides compelling evidence that the decreased availability of FFAs promotes the use of glucose as metabolic fuel despite the presence of massive amounts of fat in adipose tissue and muscle. The inability to mobilize these fat stores leads to energy starvation, resulting in reduced energy expenditure, a decline in body temperature, and premature death when ATGL knockout mice are stressed by cold temperature or food deprivation. Thus, ATGL plays a crucial role in energy homeostasis in mice. The observations that ATGL contributes to adipocyte lipolysis in human adipose tissue (Kershaw et al. 2006) and that genetic variation in the human ATGL gene is associated with plasma FFA, TAG, and type II diabetes suggest that ATGL may be of similar relevance in humans. However, by comparing human adipose tissue gene expression of HSL and ATGL as well as total triolein lipase activity and lipolytic capacity in adipocytes from lean vs. obese human subjects and in addition the tri- and diglyceride hydrolase activities of Cos-7 cell transfected with HSL and ATGL cDNAs, Marial and coworkers (2006) found evidence for a downregulation by body fat and for association with in vitro hydrolysis of triolein by HSL rather than ATGL. Moreover, their data argue for a predominant role of HSL as both tri- and diglyceride-hydrolyzing lipase in human adipocytes. The findings reinforce the necessity for the use of appropriate human cellular assay systems for the identification and characterization of compounds/drug candidates interfering with lipid metabolism, in general, and lipolysis, in particular.

Purpose and Rationale

Since neutral lipases are water-soluble enzymes hydrolyzing insoluble long-chain TAG substrates and sometimes, in addition, phospholipids to a varying degree (LPL, EL), the cleavage reaction has to occur at the lipid-water interface (Petry et al. 2005; Hide et al. 1992; Verger 1997; Schmid and Verger 1998). This distinguishes lipases from other hydrolytic enzymes and makes them unique target proteins with regard to selectivity and mode of inhibition. The insolubility of neutral TAG has several striking consequences. Both in the serum compartment and in the cytoplasm of cells neutral lipids are never in direct contact with the aqueous milieu. They are organized as high-molecular-mass aggregates in emulsified droplets consisting of an interior TAG core and surrounded by a monolayer shell of phospholipids and embedded proteins (cytoplasmic LD and serum lipoproteins). Alternatively, lipids are organized in smaller complexes bound to serum or intracellular fatty acid-/lipid-binding proteins, such as albumin and caveolin. Lipases interact with these special substrate complexes or "super-substrates" using hydrophobic domains which are exposed/unmasked upon contact as a consequence of substrate-induced conformational change, which sometimes has been called "interfacial activation" (Petry et al. 2005; Hide et al. 1992; Verger 1997; Schmid and Verger 1998). The apparently "two-dimensional" reaction of lipases does not simply follow Michaelis–Menten kinetics of enzyme reactions in aqueous milieu and critically depends on the quality of the interface. In PL the free access of lipidic substrates to the catalytic site is hampered by a so-called lid domain. This blockade is released upon interaction of lipase with the water-lipid interface inducing a conformational change. However, there is considerable evidence that HSL lacks a similar lid domain (Ben Ali et al. 2004). The authentic, i.e., substrate-specific, measurement of lipase activity as well as the development of reliable lipase assay systems has to take these unique features into account.

Beisson and coworkers (2000) critically reviewed the lipase assay systems used most widely so far. The most sensitive and reliable assays are discontinuous "time-stop" assays using radiolabeled emulsified TAG or phosphoglycerides as substrates. The substrate has to be separated from the product by chromatographic methods or partitioning. Enzymatic fluorescence- or bioluminescence-based or chromatographic methods for determination of the unlabeled products of lipolysis are highly sensitive but are discontinuous, which is also true for even more sensitive radioactive methods. Titrimetric and potentiometric assays are continuous

but are relatively insensitive and may interact with acidic or basic assay constituents, such as test compounds. To overcome these handicaps, Beisson and coworkers (1999) have developed an elegant continuous lipase assay using naturally occurring fluorescent TAG isolated from *Parinari glaberrimum*. Synthetic octadeca-9,11,13,15-tetronic-3hydroxy-octadecyloxypropylester, a 1 acyl-2 alky glyceride from parinaric acid, represents as DAG analog an efficient substrate for HSL, but it is too sensitive toward oxidation to be used under routine conditions. An alternative ultraviolet spectrophotometric assay has been introduced by the same group which is less sensitive to oxidation using TAG from *Aleurites fordii* seeds (Pencreac'h et al. 2002).

Whereas continuous assays based on soluble fluorescent substrates are frequently and successfully being used for esterases, they have rarely been described for lipases. Some of these soluble substrates have been proposed for the measurement of lipolytic activity (Hendrickson 1994; Meshulam et al. 1992). These assay systems turned out to be simple, easy to handle, and of sufficient sensitivity. But one has to keep in mind that they do not account for the special features of the lipase–substrate interaction and therefore do not discriminate lipases from esterases. In consequence, lipase activity may be underestimated or even remain undetected, in particular in case of assays being performed with crude enzyme sources, such as body fluids or tissue samples. Moreover, the use of water-soluble substrates may lead to gross misinterpretations of the inhibitory profiles of compounds. Consequently, lipidic substrates resembling the native substrate with regard to chain length and amphiphilic nature, equipped with appropriate fluorophores, should be applied whenever feasible.

Importantly, even huge fluorophores, such as BODIPY (Meshulam et al. 1992), rhodamine (Agmon et al. 1993), or pyrene (Scholze et al. 1999), coupled to lipase substrates apparently do not interfere with their cleavage by lipolytic enzymes. Thus, the nature of the fluorescence label does not seem to be very critical. In general, it should be as small and of hydrophobic character as possible to guarantee optimal interaction with the lipase, should not interact with colored compounds, and should be insensitive toward oxidation. For these reasons, the p-nitrobenzodifurazan (NBD) moiety has often been chosen as fluorescence label. NBD is a relative small fluorophore and is well accepted by various lipid-handling enzymes/proteins without grossly influencing their recognition and catalytic mechanisms. For instance, NBD labeling has been used previously for the determination of phospholipase A2 activity in a time-stop assay design (Dagan and Yedgar 1987; Wittenauer et al. 1984). Müller and coworkers (1997) have shown that 12-(7-nitrobenzo[1,2,3]oxadiazol-4-ylamino)dodecanoic acid (NBD-FA) is taken up by adipocytes and incorporated into TAG in insulin-sensitive fashion. This demonstrates acceptance of NBD-modified fatty acid and lipid precursors as substrates by lipid-handling enzymes, in general.

Starting from NBD-FA, a non-soluble lipase substrate, NBD-MAG, has been introduced which upon presentation in mixed phospholipid-NBD-MAG micelles represents a "super-substrate" and is well accepted by a number of neutral lipases. Incorporation of NBD-MAG into mixed micelles and subsequent release of NBD-FA from the micelles in the course of lipase action can easily be followed by a change in extinction, thereby enabling the design of an assay system for a variety of neutral lipases in crude tissue samples on a routine basis.

Procedure

Preparation of Rat Adipocyte Extract

Adipocytes are isolated from epididymal fat pads of Wistar rats by digestion with collagenase and subsequent separation from undigested tissue using a nylon web as described (see K.5.1.2). Cells obtained from ten rats are washed three times with 50 ml each of homogenization buffer (25 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 μg/ml each of leupeptin, antipain, and pepstatin) by flotation (500 × g, 2 min, 25 °C, swing-out rotor), suspended in 10 ml of homogenization buffer, and then homogenized by 10 strokes at 1,500 rpm in a loosely

fitting Teflon-in-glass homogenizer (15 °C). The homogenate is centrifuged (3,000 × g, 10 min, 4 °C). The infranatant below the fat layer is aspirated and recentrifuged. This procedure is repeated three times for complete removal of residual lipid left at the top after the centrifugations. The final infranatant is centrifuged (48,000 × g, 45 min, 4 °C). The resulting fat-free supernatant is mixed with 1 g of heparin-Sepharose (washed five times with 25 mM Tris/HCl (pH 7.4), 150 mM NaCl), incubated at 4 °C for 1 h (under head-to-end rotation of the vial), and then centrifuged (1,000 × g, 10 min, 4 °C). The supernatant is adjusted to pH 5.2 and incubated (30 min, 4 °C). The precipitates are collected by centrifugation (25,000 × g, 10 min, 4 °C); suspended in 2.5 ml of 20 mM Tris/HCl (pH 7.0), 1 mM EDTA, 1 mM DTT, 70 mM NaCl, 13 % sucrose, and 10 μg/ml each of leupeptin, antipain, and pepstatin; and finally dialyzed (20 h, 4 °C) against 3 × 500 ml of 25 mM Tris/HCl (pH 7.4), 50 % glycerol, 1 mM EDTA, 1 mM DTT, and 10 μg/ml each of leupeptin, antipain, and pepstatin. Adipocyte extract is frozen in liquid N2 and stored at −70 °C for up to 4 weeks.

Preparation of Tissue Extracts

The tissues are surgically removed and washed in PBS containing 1 mM EDTA. Homogenization is performed on ice in lysis buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin) using an ULTRA-TURRAX. The infranatants are obtained after centrifugation (20,000 × g, 90 min, 4 °C). The reaction is performed in a water bath at 37 °C for 60 min with 0.1 ml of substrate and 0.1 ml of infranatant.

Method Based on Fluorescently Labeled Monoacylglycerol (NBD-MAG)

Procedure

Preparation and Immunoprecipitation of HEK293 Cell Lysates Ectopically Expressing Lipases

Expression constructs encoding only the open reading frame of the relevant lipase with or without epitope tags are constructed by subcloning PCR amplification products into a mammalian expression with CMV promoter or into the Gateway entry vector followed by recombination into the Gateway destination vector. Each expression vector is used to transiently transfect HEK293 cells. HEK293 cells are grown in DMEM supplemented with 10 % FCS at 37 °C in an atmosphere of 5 % CO_2. Ten centimeters of tissue-culture dishes with 90 % confluent HEK293 cells are transfected using Lipofectamine2000 (Invitrogen). 72 h after transfection, cells are washed with ice-cold TBS and harvested. Cells are pelleted by centrifugation (1,000 × g, 5 min) and stored at −80 °C until lysate preparation. For this, frozen cells are suspended in 1 ml of lipase reaction buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.53 % sodium taurodeoxycholate, 1.33 mM $CaCl_2$) containing the complete proteinase inhibitor mix. The resulting suspensions are then sonicated on ice with four bursts of 10 s from a probe sonicator. Homogenized lysate is centrifuged (1,000 × g, 10 min) for removal of cell debris. Fifty microliters of portions of the resulting cleared lysates are used for direct activity measurements. For immunoprecipitation, portions are supplemented with anti-V5 mouse monoclonal antibody (7.2 μg) and incubated at 4 °C overnight with tumbling. Next, 20 μl of protein A-bead suspension is added and tumbled at 4 °C for 2 h. Beads are then pelleted with gentle centrifugation and washed four times with 900 μl of lipase reaction buffer followed by resuspension in 100 μl of lipase reaction buffer.

Activity Measurement

Since HSL cleaves MAG in addition to DAG and TAG albeit a rather low rate, Petry and coworkers (2005) introduced the use of NBD-MAG as substrate for a convenient, nonradioactive, and homogeneous activity assay for HSL. For the preparation of the NBD-MAG substrate, 41.5 μl of a PC solution (6 mg/ml in chloroform), 83.5 μl of a PI solution (6 mg/ml in chloroform), and 100 μl of a NBD-MAG solution (10 mg/ml in chloroform) are added to plastic scintillation vials, dried over a stream of N2, and stored until

use at 4 °C for up to 3 days. The dried lipids are then resuspended in 20 ml of 25 mM Tris/HCl buffer (pH 7.4) and 150 mM NaCl and then subjected to an ultrasonic treatment in an ice bath using a Branson Sonifier (type II, standard microtip; 2 × 1 min at a setting 2 followed by 2 × 1 min at a setting 4 with 1 min intervals). During the ultrasonic treatment, the color of the substrate suspension shifts from yellow (maximum absorbance at 481 nm) to pink (maximum absorbance at 550 nm). It is used after a period of 15 min (minimum) up to 2 h (maximum). To start the assay procedure, 180 µl of NBD-MAG substrate solution is warmed up to 30 °C, supplemented with either 30 µl of an adipocyte extract (appropriately diluted with 25 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT containing 0.1 % BSA) or 20 µl of recombinant human HSL in the wells of 96-well microtiter plates.

Evaluation

The optical density (OD) at 481 nm is recorded continuously at regular time intervals (from 1 to 30 min) using a microplate scanning spectrophotometer. Buffer alone is used as a control experiment. Alternatively, the products generated in the reaction mixture are analyzed by TLC. For this purpose, 200 µl of the reaction mixture is transferred into 2 ml reaction vials and supplemented with 1.3 ml of a methanol/chloroform/heptane (10/9/7, by vol.) and then with 0.4 ml of 0.1 M HCl. After intense vortexing, phase separation is initiated by centrifugation (800 × g, 20 min, 25 °C); 200 µl aliquots of the lower organic phase are removed, then dried under vacuum (SpeedVac evaporator), and finally suspended in 50 µl of tetrahydrofuran (THF). Samples of 5 to 10 µl are separated by TLC on silica gel Si-60 plates using diethyl ether/petroleum ether/acetic acid (78/22/1, by vol.) as elution solvent system. The amount of NBD-FA acid released is evaluated by fluorescence imaging using a phosphorimager (Molecular Dynamics, storm 860 and ImageQuant software) with an excitation wavelength of at 460 nm and emission wavelength of at 540–550 nm.

Method Based on Fluorescently Labeled TAG

For substrate preparation, NBD-TAG (total mixture of NBD-MAG/DAG/TAG) are extracted with chloroform/heptane from adipocytes, which had been incubated with NBD-FA in the presence of insulin and purified as described (Müller et al. 1997). For 4 ml of substrate emulsion, 2 mg of dried NBD-TAG is emulsified with 0.2 mg of phosphatidylcholine/phosphatidylinositol, inositol (3/1, w/w) in chloroform. After evaporation of the solvent (SpeedVac), 2 ml of 0.1 M potassium phosphate (pH 7.0) is added and the substrate is sonicated (4 × 1 min, Branson Sonifier 250, setting 2–3, 22 °C). Subsequently, 1.6 ml of potassium phosphate is added and the sonication is repeated on ice. After the second sonication, 0.4 ml of potassium phosphate containing 10 % BSA is added. One hundred microliters of substrate emulsion is mixed with 100 µl of rat adipocyte extract as a source for crude HSL (prepared as above) in homogenization medium and incubated (1 h, 30 °C). The digestion is terminated by addition of 400 µl of chloroform/methanol (3/1; by vol.) and analyzed as described above for cell-free lipolysis (see "Cell-Free Lipolysis").

Method Based on Radiolabeled Trioleoylglycerol (TOG)

For assaying trioleoylglycerol (TOG = triolein) cleavage, the TOG droplet emulsion (final conc. 1.7 mM) is prepared by mixing 25–50 µCi glycerol-tri[3H]oleate (in toluene/ethanol, 1/1), 6.8 µmol TOG (in chloroform), 0.6 mg PC, and 0.2 mg PI, subsequent drying over a stream of N2, and finally suspending in 2 ml of 0.1 M KPi (pH 7.0) under ultrasonic treatment (Branson Sonifier B-12, standard microtip, setting 2, 2 × 1 min with 1 min intervals on ice). After addition of 1 ml of 0.1 M KPi (pH 7.0) and ultrasonic treatment (see above, 4 × 30 s with 1 min intervals on ice), the TOG substrate suspension is supplemented with 1 ml of 20 % BSA (in 0.1 M KPi) and stored until use at 4 °C (for up to 2 weeks). The reaction is started by addition of 10 µl of rat adipocyte extract (appropriately

diluted with 20 mM M KPi, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.02 % BSA, 10 μg/ml of each leupeptin, pepstatin, and antipain) to 10 μl of TOG suspension. After incubation (30 min, 37 °C), the reaction is terminated by addition of 325 μl of methanol/chloroform/heptane (10/9/7, by vol.). Phase separation is initiated by addition of 105 μl of 0.1 M K_2CO_3 and 0.1 M boric acid (pH 10.5), intense vortexing, and centrifugation (8,000 × g, 15 min). One hundred fifty microliters of the upper aqueous phase is transferred into scintillation vials and supplemented with 5 ml of scintillation cocktail (Beckman Ready Safe) for measurement of radioactivity by liquid scintillation counting. This method has been used by Vertesy and coworkers (2002) for the identification of novel inhibitors of HSL with putative antidiabetic activity from natural sources, such as fungi.

Method Based on Radiolabeled Tributyrin

For assaying tributyrin cleavage, 10 μl of [14C] tributyrin (20 mM, 0.1 μCi, in ethanol) is dried; then suspended in 25 μl of 0.1 M KPi (pH 7.25), 140 mM NaCl, 1 mM DTT, and 0.2 mM EDTA; and finally incubated (30 min, 37 °C) with 25 μl of rat adipocyte extract (appropriately diluted with the same buffer). The reaction is terminated by the addition of 325 μl of methanol/chloroform/heptane (10/9/7, by vol.) and 125 μl of 0.1 M NaOH and subsequently processed and described as above (see "Method Based on Radiolabeled Trioleoylglycerol").

Method Based on Resorufin Ester

The assay uses 1,2-dilauryl-rac-glycero-3-glutaricacid (6′-methylresorufin) ester (DGGR) as a substrate (Lehner and Verger 1997). DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester that is spontaneously hydrolyzed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 581 nm. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Hydrolysis of DGGR (final conc. 100 μM) is determined at 37 °C in a final volume of 1 ml. For this, substrate solution is prepared by mixing 40 μl of the ester (2 mg/ml dioxane) with 160 μl of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, and 0.05 % TX-100. Seven hundred ninety of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.53 % sodium taurocholate, and 1.33 mM $CaCl_2$ are added, and the reaction is started by addition of 10 μl of rat adipocyte extract. The release of resorufin is monitored at 572 nm. For high-throughput screening of compounds/drug candidates activating or inhibiting lipases, 10 μl of whole-cell lysate or immunoprecipitation beads is added to the wells of a 96-well plate. After adjustment of the samples to 125 μl in lipase reaction buffer, 125 μl of reaction buffer containing DGGR is added (final conc. of 36 μg/ml in a final assay volume of 250 μl per volume). After mixing, OD 581 is monitored at 5 min intervals for 2 h to assess lipase activity. This procedure has been used by Lake and coworkers (2005) for the analysis of the expression, regulation, and triglyceride hydrolase activity of the adiponutrin family members.

Method Based on p-Nitrophenylbutyrate

For assaying p-nitrophenylbutyrate (PNPB) cleavage according to the procedure described by Holm and Osterlund (1999) with modifications from Soni and coworkers (2004), 10 μl of 2 mM PNPB (in acetonitrile) is added to 890 μl of 0.1 M KPi (pH 7.25), 140 mM NaCl, 1 mM DTT, and 0.2 mM EDTA and then incubated (10 min, 37 °C) in the presence of 100 μl of rat adipocyte extract (appropriately diluted with the same buffer) in the absence or presence of 0.01 % TX-100. The reaction is terminated by adding 3.25 ml of methanol/chloroform/heptane (10/9/7), vigorously vortexed for 10 s, and then centrifuged (800 × g, 20 min, 21 °C). The upper phase is collected and thereafter subjected to incubation at 42 °C for 2 min. The absorbance of the released p-nitrophenol (extinction coefficient of 12,000) is measured at 405 nm.

Method Based on Potentiometry

For a potentiometric assay, the hydrolysis of short- and medium-chain TAG is determined at

pH 8.0 and 37 °C with a pH-stat (TTT 80 Radiometer) by titrating liberated fatty acids with 0.1 N NaOH. The standard mixture using tributyrin contains 14.9 ml of 150 mM NaCl and 100 µl of tributyrin (final vol. 15 ml). With TOG, the assay mixture is composed of 14.5 ml of 150 ml NaCl, 2 mM sodium taurodeoxycholate, 0.1 mg/ml BSA, and 0.5 ml of TOG.

Neutral Cholesterylester Hydrolase Activity

HSL possesses pronounced neutral cholesterylester hydrolase activity in addition to its neutral TAG-hydrolase activity in contrast to ATGL, which exhibits the latter, exclusively (Zimmermann et al. 2004; Jenkins et al. 2004). Consequently, assaying cholesterylester hydrolase activity can be used to discriminate between HSL and ATGL activity in crude cell lysates. Cholesterylester hydrolase is measured according to the procedure described by Holm and Osterlund (1999) with modifications from Soni and coworkers (2004) and Ben Ali and coworkers (2005). 1.17 mg of cholesteryl oleate, including 2 × 107 dpm of [14C]cholesteryl oleate, and a phospholipid mixture (71.4 µl, including phosphatidylcholine [15 mg/ml] and phosphatidylinositol [5 mg/ml], dissolved in chloroform) are placed in a 4 ml glass vial. Solvents are evaporated under a gentle stream of N2. 2 ml of 0.1 M potassium phosphate (pH 7.5) is added. After incubation (10 min, 37 °C), the mixtures are sonicated (twice for 1 min each, separated by a 1 min interval). An additional 1 ml of 0.1 M potassium phosphate (pH 7.5) is added and incubated (10 min, 37 °C). The mixtures are sonicated again (four times for 30 s each on ice, separated by 30 s intervals). After sonication, 1 ml of 20 % fatty acid-free BSA prepared in 0.1 M potassium phosphate (pH 7.5) is added. For each assay, 100 µl of substrate is mixed with 100 µl of sample and incubated (30 min, 37 °C). The reactions are terminated, extracted, and counted for radioactivity as described above for the method based on radiolabeled TOG.

Lipoprotein Lipase (LPL) Activity

For assaying LPL according to Dichek and coworkers (1993) and Iverius and coworkers (1985) with modifications, substrate is prepared by evaporation of 5 mg of unlabeled TOG together with 50 µCi glycerol-tri[3H]oleate and 0.24 mg PC from the solvent, toluene, under a stream of N2. The dried lipids are emulsified in 1 ml of 1 M Tris/HCl (pH 8.5), 0.1 M NaCl, and 2.5 % BSA in the absence or presence of 1 M NaCl and in the presence of 8 % heated rat serum under sonication (Branson Sonifier B-12, standard microtip, setting 2, 10 s on, 10 s off, for 10 cycles on ice). Affinity-purified LPL from bovine milk in 15 µl of 10 mM Tris/HCl (pH 7.4) and 0.5 mM DTT is incubated (60 min, 37 °C) with 5 µl of substrate emulsion. Reactions are terminated by addition of 325 µl of methanol/chloroform/heptane (10/9/7, by vol.) and subsequently of 105 µl of 0.1 M K_2CO_3 and 0.1 M boric acid (pH 10.5). After vortexing, the phases are separated, and 100 µl portions of the upper aqueous phase are mixed with 5 ml of scintillation cocktail (Beckman, Ready Safe) and counted for radioactivity. LPL activity is determined by subtracting the non-LPL-dependent lipolytic activity (presence of 1 M NaCl) from the total activity (absence of 1 M NaCl).

Analysis of Lipolysis Products

TAG Hydrolysis Rate

For the quantitation of DAG and MAG formed by the TAG lipase actions, the reaction is terminated by adding 1 ml of chloroform/methanol (2/1, by vol.) containing 1 % acetic acid, oleic acid (10 µg/ml), and standards for monooleoylglycerol (MOG) and dioleoylglycerol DOG (10 µg/ml, sn-1,2 and sn-1,3) (Folch et al. 1957). The mixture is vortexed vigorously three times over 15 min. After centrifugation (4,000 × g, 10 min), 0.5 ml of the lower phase is collected and evaporated under nitrogen. The lipid pellet is dissolved in chloroform and applied onto TLC (Merck, silica gel 60) with chloroform/acetone/acetic

acid (96/4/1, by vol.) as solvent. Lipids are visualized with iodine vapor, and the bands corresponding to MOG, DOG, and TOG and oleic acid are cut out. The comigrating radioactivity is determined by liquid scintillation counting.

Alternatively, for the determination of the rate of DAG formation, the lipolysis reaction is terminated by adding 25 µl of 1 M HCl and 1 ml of hexane/isopropyl alcohol (3/2, by vol.) containing oleic acid (10 µg/ml) and standards for MOG, DOG (sn-1.2 and sn-1.3, Sigma), and TOG (10 µg/ml each). The mixture is vortexed vigorously three times over a period of 15 min. After centrifugation (4,000 × g, 10 min), 0.4 ml of the upper phase is collected and evaporated under nitrogen. The lipid pellet is dissolved in chloroform and loaded onto a TLC plate (Merck, silica gel 60). The TLC is developed with chloroform/acetone/acetic acid (96/4/1) as solvent. The lipids and FFA are visualized with iodine vapor, and the bands corresponding to MOG, DOG, and TOG and oleic acid are cut out. The comigrating radioactivity is determined by liquid scintillation counting, and the molar concentrations of the products are calculated.

Evaluation

The specific TAG-hydrolase activity (TAG to DAG conversion) is calculated from the total AG lipolytic activity and the DAG formed during the reaction (considering that MAG does not accumulate) by the equation given by Hämmerle and coworkers (2002).

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Affinity Labeling of TAG Lipases

Purpose and Rationale

Compounds which interfere with lipolysis by direct blockade of TAG cleavage can be directed to various lipases in differential fashion. So far, in addition to HSL, ATGL, GS-2, and adiponutrin have been identified as putative TAG-degrading lipases in rodent adipocytes. Their differential targeting by compound/drug candidates can be analyzed by activity tagging (for a review, see Petry et al. 2004). A fluorescence technology for selectively screening for lipolytic enzymes developed by Schmidinger and coworkers (2005) is based on the so-called click chemistry utilizing the copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition reaction (Speers and Cravatt 2004a, b; Wang et al. 2003). Lipase- and esterase-mediated hydrolysis of acyl esters is based on a mechanism involving a nucleophilic serine, which in most cases is part of a catalytic triad with histidine and asparate. In the first reaction step, the active serine attacks the carbonyl group of the scissile fatty acid to give a tetrahedral transition state. This intermediate state is cleaved to yield the free fatty acid, the alcohol component, and the nucleophilic serine. Although many lipases show strong structural and sequential similarities (Pleiss et al. 1998; Arpigny and Jaeger 1999), their substrate and stereospecificities can vary significantly (Martinelle et al. 1995). It is known that p-nitrophenylesters of alkylphosphonic acids are irreversible and stoichiometrically react with the nucleophilic serine of lipases and esterases, freezing the reaction at the point of the tetrahedral transition state. In the past, they have been applied to the determination of serine hydrolase activity by using different approaches (Oskolkova et al. 2003; Rotticci et al. 2000). If fluorescent inhibitors are used, the tagged enzyme becomes visible (Manesse et al. 1995). Thus, it can be detected and quantified on the basis of its fluorescent signal (Scholze et al. 1999). Fluorescently labeled alkyl phosphonates detect active enzymes in electrophoretically pure proteins and in complex proteosome samples (Speers and Cravatt 2004a; Adam et al. 2002; Greenbaum et al. 2002).

Procedure

Activity tagging of lipases contained in adipocyte tissue can be performed according to the protocol of Birner-Grünberger and coworkers (2005). For this, gonadal fat pads (white adipose tissue) of fed and fasted mice are surgically removed and washed in PBS. Homogenization is performed on ice in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin) using a motor-driven Teflon-in-glass homogenizer (eight strokes, 1,500 rpm). Cell debris is removed by centrifugation (1,000 × g, 15 min) to obtain cytoplasmic extracts. Protein concentrations are determined using the BIORAD protein assay based on the method of Bradford. Fifty microliters of protein sample dissolved in lysis buffer, 10 μl of 10 mM Triton X-100 in methanol (final conc. 1 mM), and 20 μl of activity tag (NBD-labeled single-chain phosphonic acid ester, TAG phosphonates, and cholesteryl phosphonates which react with the nucleophilic serine in the active site of lipolytic enzymes due to the active phosphonate moiety with the good leaving PNPB group and then remain covalently attached leading to irreversible inactivation of the lipases) dissolved in methanol (1 nmol/10 μl, final conc. 20 μM) are mixed, and the organic solvent is evaporated under a stream of argon. One hundred microliters of protein sample (0.5 mg protein/ml) is added, and the resultant mixture is incubated (2 h, 37 °C) under light protection. Proteins are precipitated in 10 % ice-cold TCA (1 h, on ice) and collected by centrifugation (10,000 × g, 15 min, 4 °C). The pellet is washed once with ice-cold acetone and resuspended in 1D sample buffer (20 mM KH_2PO_4, 6 mM EDTA, 60 mg/ml SDS, 100 mg/ml glycerol, 0.5 mg/ml bromophenol blue, 20 μl/ml mercaptoethanol, pH 6.8) or 2D sample buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 60 mM DTT, 2 % Pharmalyte pH 3–10,0.002 % bromophenol blue). SDS-PAGE is performed in a Tris/glycine buffer system by aligning proteins (50 or 100 μg protein/lane) in a 5 % stacking gel and separating them in a 10 % resolving gel at 20–50 mA constant current. Two-dimensional gel electrophoresis is performed in the first dimension by isoelectric

focusing of 50–500 µg protein in 7 or 18 cm immobilized nonlinear pH 3–10 gradients at 6.5/12 kVh and in the second dimension by SDS-PAGE on 7 or 20 cm gels as described by Gorg and coworkers (1985, 1988). Gels are fixed in 7.5 % acetic acid and 10 % ethanol and scanned at a resolution of 100 pm. NBD fluorescence is detected at 530 nm and an excitation wavelength of 488 nm. For visualization of the whole protein pattern, gels are stained with SYPRO Ruby following the manufacturer's instructions (Molecular Probes) and scanned at 605 nm and an excitation wavelength of 488 nm. For LC-MS/MS analysis, the fluorescent protein spots are excised and tryptically digested according to the method by Shevchenko and coworkers (1996). Peptide extracts are dissolved in 0.1 % formic acid and separated on a nano-HPLC-system. Twenty microliters of samples are injected and concentrated on the loading column (LC packings PepMap™ C-18, 5 µm, 100 A, 300 µm ID × 1 mm) for 5 min using 0.1 % formic acid as isocratic solvent at a flow rate of 20 µl/min. The column is then switched into the nano-flow-circuit and the sample is loaded on the nanocolumn at a flow rate of 300 nl/min and separated using a gradient from 0.3 % formic acid and 5 % acetonitrile to 0.3 % formic acid and 50 % acetonitrile over 60 min. The sample is ionized in a Finnigan nano-ESI source equipped with NanoSpray tips and analyzed in a Thermo-Finnigan LCQ Deca XPplus iontrap mass spectrometer. Acceptance parameters are two or more identified distinct peptides (Carr et al. 2004). Identified protein sequences are subjected to BLAST and motif search for identification of potential serine hydrolases.

Evaluation

In pilot experiments, 50 µg of cleared cell lysate (see above) is incubated with 1 nmol of fluorescently labeled lipase inhibitor O-((6-(7-nitrobenz-2-oxa-1,3-dia-zol-4-yl)amino)hexanoyl)-aminoethyl-O-(n-hexyl)phosphonic acid p-nitrophenyl ester () and 1 mM TX-100 at 37 °C for 2 h under shaking. Total protein is then precipitated with 10 % TCA for 1 h on ice, washed once with acetone, and subjected to 10 % SDS-PAGE. After treatment of the gels with 10 % ethanol and 7 % acetic acid, the fluorescent signals are detected with laser scanner (excitation 488 nm, emission 530 nm).

Interaction of ATGL and CGI-58

Purpose and Rationale

In contrast to HSL, which undergoes PKA-dependent phosphorylation and translocation from the cytoplasm to LD upon β-adrenergic stimulation of adipocytes, the regulation of ATGL may rely on protein–protein interaction. It has been demonstrated by Lass and coworkers (2006) that efficient enzymic activity of ATGL depends on interaction with and activation by CGI-58. Mutations in the human CGI-58 gene are associated with Chanarin–Dorfman syndrome (CDS), a rare genetic disease where TG accumulates excessively in multiple tissues (Chanarin et al. 1975; Dorfman et al. 1974; Lefevre et al. 2001). CGI-58 interacts with ATGL, stimulating its TAG-hydrolase activity up to 20-fold. Alleles of CGI-58 carrying point mutations associated with CDS fail to activate ATGL. Moreover, CGI-58/ATGL coexpression attenuates lipid accumulation in COS-7 cells. Antisense RNA-mediated reduction of CGI-58 expression in 3T3-L1 adipocytes inhibits TAG mobilization. These data establish an important biochemical function for CGI-58 in the lipolytic degradation of TAG and raise the question whether defects in CGI-58 may contribute to the pathogenesis of type II diabetes and obesity. The following assays allow the analysis of the functional interaction of ATGL with CGI-58 and its modulation by compounds/drug candidates.

Procedure

Preparation of ATGL

Monkey embryonic kidney cells (COS-7, ATCC CRL-1651) are transfected using Metafectene (Biontex, Germany) as described (Zimmermann et al. 2004). For the preparation of cell extracts, cells are collected by trypsin treatment, washed three times with PBS, and disrupted in buffer A

(0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 µg/ml leupetine, 2 µg/ml antipain, 1 µg/ml pepstatin, 20 mM Tris/HCl, pH 7.0) by sonication. Nuclei and unbroken cells are removed by centrifugation (1,000 × g, 4 °C, 5 min). The expression of the His-tagged proteins is detected using immunoblotting as described (Zimmermann et al. 2004). COS-7 cells expressing His-tagged mouse ATGL are lysed in buffer C (25 mM NaPO4, pH 7.0, 20 % glycerol, 2 mM β-mercaptoethanol, 0,01 % NP-40) and incubated with TALON Co_2+ resin (BD Biosciences). The resin-cell extract suspension is then poured into a micro bio-spin column (Bio-Rad) and washed with ten column volumes of buffer C containing 500 mM NaCl. Recombinant His-tagged ATGL is eluted by a step gradient of imidazole (200 mM final concentration) in buffer C. For determination of the purity of the protein, fractions are subjected to SDS-PAGE gel electrophoresis and immunoblotting.

Preparation of GST-Tagged CGI-58

The coding sequence of mouse CGI-58 is cloned into pYex4T-1 vector and transformed into the *S. cerevisiae* BY4742 (Matα; his3; leu2; lys2; ura3) strain. Large-scale overexpression of GST-CGI is achieved by maintaining transformed *S. cerevisiae* carrying in YNB-urea containing 0.5 mM CuSO4 to induce copper promoter-driven expression of the fusion cassette. After induction, cells are harvested and protoplasts are generated with zymolyase and disrupted by sonication in the presence of 0.2 % NP-40. The supernatant containing the GST-fusion protein is purified using glutathione-Sepharose beads. Purified GST-CGI is dialyzed overnight with 150 mM KCl, 10 mM potassium phosphate buffer (pH 7.4), and 0.01 % NP-40.

Interaction Assay

For the detection of interacting proteins, ELISA plates are coated with 3 µg GST-CGI or GST in buffer D (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM $CaCl_2$). The wells are blocked with 5 % BSA in buffer D and incubated with 50 µg protein/well of COS-7 cell extracts containing His-tagged ATGL in 50 mM potassium phosphate buffer (pH 7.0) in the absence or presence of compounds/drug candidates. After washing with buffer D containing 0.05 % Tween 20, the mouse anti-His antibody is added in the same buffer containing 0.5 % BSA. Subsequent to three further washes, horseradish peroxidase-conjugated anti-mouse antibody is added. After washing three times with buffer D containing 0.05 % Tween 20, the absorbance of tetramethylbenzidine is determined at 450 nm using 620 nm as reference wavelength. The absorbance of GST coated wells is subtracted from that coated with GST-CGI.

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Measurement of cAMP Levels

Purpose and Rationale

Activation of lipolysis in adipocytes by β-adrenergic stimuli and its inhibition by insulin critically depend on the generation by adenylate cyclase and degradation by

phosphodiesterase 3B (PDE3B), respectively (for a review, see Müller and Petry 2004). The intracellular cAMP levels can be determined in adipocytes which have been exposed to isoproterenol prior to incubation with insulin/compounds/drug candidates.

Procedure

Four hundred microliters of adipocyte suspension (lipocrit 10 %) is suspended in 600 µl KRH containing 0.75 % BSA and 1.5 mM glucose and incubated in the absence or presence of lipolytic stimuli (isoproterenol, adenosine deaminase) in combination with insulin or compound/drug candidates at 37 °C in a mildly shaking water bath. Two hundred microliters of aliquots are transferred into Eppendorf cups containing 20 µl HClO4 (70 %), vortexed vigorously, and centrifuged (12,000 × g, 15 min, room temperature). One hundred ten microliters of the infranatant is removed, taking care not to aspirate any of the precipitate, supplemented with 60 µl of 1 M Tris/HCl (pH 7.5), incubated for 15 min on ice, and then centrifuged (13,000 × g, 15 min, 4 °C). One hundred fifty microliters of the infranatant is removed for glycerol determination, frozen in liquid N2, and stored at −20 °C prior to the determination of glycerol and cAMP levels. cAMP is determined by a modification of the protein kinase binding procedure. Samples and standards are incubated with 8 µg of R-subunit of PKA and 175 nCi [8-3H]cAMP in a buffer composed of 50 mM Tris/HCl (pH 7.4) and 4 mM EDTA in a final volume of 200 µl for 4 h at 4 °C to reach equilibrium. Charcoal suspension (30 g/l) is added, and after vortexing, the mixture is centrifuged (16,000 × g, 2 min, 4 °C). The supernatant is transferred into scintillation vials, supplemented with 5 ml water-compatible scintillation fluid and counted for radioactivity using a liquid scintillation counter.

Evaluation

The concentration of endogenous unlabeled cAMP in the samples is determined from a linear standard curve.

cAMP-Specific Phosphodiesterase (PDE) Activity

Purpose and Rationale

Membrane-bound phosphodiesterase of rat epididymal adipose cells is stimulated when intact cells are exposed to insulin. The localization at the endoplasmic reticulum (corresponding to the low-density microsomal fraction) and properties of the insulin-sensitive particulate cGMP-inhibitable cAMP phosphodiesterase have been described by Kono and coworkers (1975), Osegawa and coworkers (1982), Saltiel and Steigerwalt (1985), and Solomon and coworkers (1986). Recently, the phosphodiesterase isoform, PDE3B, has been identified as target for phosphorylation and activation by protein kinase PKB/Akt, which is a key element of the downstream insulin signaling cascade.

Procedure

The low-density microsomal (LDM) fraction of primary or cultured adipocytes (pellet of a 100,000 × g/60 min centrifugation of the post-mitochondrial supernatant prepared from the homogenate of adipocytes exposed to lipolytic stimuli in the absence or presence of compounds; see above) is incubated in the absence or presence of compounds at various concentrations for 20 min. For the assay of cAMP-specific PDE, up to 50 µg of LDM protein is incubated (5 min, 30 °C) with 500 nM [2,8-3H]cAMP (100 nCi) in 50 mM Tris/HCl (pH 7.4), 0.5 mM DTT, 5 mM $MgCl_2$, and 50 µM PMSF in a total volume of 0.25 ml. The incubation is terminated by the sequential addition of 30 µl of 10 mM IBMX and 120 µl of 0.1 N HCl and heating (5 min, 95 °C). After neutralization (120 µl of 0.1 N KOH, 80 ml of 250 mM Tris/HCl (pH 7.4), 10 µl of crude 5′-nucleotidase (5 mg/ml) (*Crotalus atrox*) is added to the mixture. The reaction (30 min, 37 °C) is terminated by the addition of 60 µl of 200 mM EDTA and 5 mM adenosine. Unreacted cAMP is removed by the addition of 1 ml of a 1:3 slurry of Dowex AG-1X8 (Biorad). Solutions are shaken (5 min, 4 °C) and centrifuged (1,000 × g, 5 min).

Evaluation

Radiolabeled adenosine left in the supernatant after enzymic degradation of 5′-AMP and chromatographic removal of cAMP is determined by liquid scintillation counting. The assay is proportional to up to 100 μg of protein and linear throughout 20 min. It monitors the putative insulin-like effects of compounds on the regulation of cAMP degradation via the insulin signaling cascade (or alternative pathways) as well as directly on the activity state of PDE3B depending on whether or not the compound is present during the incubation of the adipocytes or LDM.

Activity State of Protein Kinase A (PKA)

Purpose and Rationale

The cAMP levels generated in response to β-adrenergic stimulation in the absence or presence of insulin or compounds in adipocytes determine the activity of PKA phosphorylating both HSL and perilipin which play key roles in the regulation of lipolysis in adipocytes (see "Cellular Lipolysis" and "Interaction of HSL and Perilipin"). Thus, the PKA activity ultimately determines the lipolytic state of the adipocytes in response to insulin/compounds/drug candidates. The assay monitors the activity state of PKA in adipocytes in response to β-adrenergic stimuli in the absence or presence of insulin/compounds/drug candidates at the time point of homogenization. This activity ratio is a parameter for the portion of PKA active in vivo toward total cellular PKA at the time point of homogenization.

Radioactive Method

For the determination of the activity state of PKA, 500 μl adipocyte suspension (5 × 106 cells/ml) is transferred to 2 ml microfuge tubes and precooled to 4 °C, containing at final concentrations 20 mM Tris/HCl (pH 7.4), 10 mM EDTA, and the cAMP PDE inhibitor, Ro 20–1,724 (100 μM). The mixture is vortexed briefly, decanted into a 5 ml Teflon-in-glass homogenizer, and homogenized with 10 strokes of the rotating (1,500 rpm) Teflon pestle. The homogenate is transferred to precooled microfuge tubes and centrifuged (16,000 × g, 10 min, 4 °C). The infranatant is removed and immediately assayed for PKA activity. 25 μl of assay medium (20 mM MOPS/KOH, pH 7.0, 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mg/ml histone H1, 5 mM DTT, 50 μCi/ml [γ-32P]ATP) is added to 25 μl infranatant. After vortexing and incubation for 20 min at 30 °C, the reaction is terminated by addition of 1 ml of ice-cold 20 % TCA containing 5 mM sodium pyrophosphate and incubation (1 h, 4 °C). The precipitates are sedimented by centrifugation (16,000 × g, 5 min, 4 °C) and, after removal of the supernatant, dissolved in 100 μl of cold 1 N NaOH and re-precipitated with 20 % TCA and 5 mM sodium pyrophosphate. The precipitate is then filtered under vacuum over GF/C-glass microfiber filter that had been pre-wetted with 5 % TCA and 5 mM sodium pyrophosphate. The filters are washed with 4 ml of the same medium, dried, and counted for radioactivity.

Evaluation

Each adipocyte infranatant is assayed in quadruplicate under four separate conditions: (I) with no further additions, (II) in the presence of 15 μM cAMP, (III) in the presence of 2 μM PKA inhibitor synthetic peptide, and (IV) in the presence of 15 μM cAMP and 2 μM PKA inhibitor synthetic peptide. The results are expressed as corrected PKA activity ratios (I)–(III)/(II)–(IV) according to Honnor and coworkers (1985a, b) and Londos and coworkers (1985).

Fluorescent Method

Procedure

The method based on fluorescently labeled kemptide has been introduced by Müller and coworkers (2000) and Schölch and coworkers (2004) and can be used for determination of the activity ratio in cells or tissues from rats treated with compounds/drug candidates. For the ex vivo experimental design, frozen tissue samples are minced under liquid N2 in 2 ml of 25 mM MES

(pH 6.0), 140 mM NaCl, 2 mmol/l EDTA, 0.5 mM EGTA, 0.25 M sucrose, 50 mM NaF, 10 mM sodium pyrophosphate, 20 mM glycerol-3-phosphate, 1 mM sodium orthovanadate, 2 μM microcystin, 1 μM okadaic acid, and protease inhibitors per gram of wet weight and then homogenized by sequential use of an ULTRA-TURRAX homogenizer (30 s, maximum speed) and a Teflon-in-glass homogenizer (tightly fitting, five strokes, 3,000 rpm, on ice). After centrifugation (500 × g, 5 min, 4 °C), the infranatant below the fat layer is carefully removed, adjusted to 0.5 % Triton X-100, and after incubation (30 min, 4 °C) and homogenization (Teflon-in-glass, 10 strokes, 500 rpm, 4 °C) centrifuged (10,000 × g, 15 min, 4 °C). The supernatant is recentrifuged (100,000 × g, 1 h, 4 °C). Twenty-five microliters of the supernatant (cytosol) are incubated (30 min, 30 °C) with 12.5 μl of assay buffer (200 mM Tris/HCl, pH 7.5, 40 mM $MgCl_2$, 4 mM ATP, 400 μM rhodamine-labeled kemptide [PepTag A1 peptide obtained from Serva/Promega Heidelberg, Germany]) in the absence or presence of 10 μM mono-butyryl-cAMP and 50 μM heat-stable PKA inhibitor peptide (Sigma, Germany) in a total volume of 50 μl. The reaction is terminated by placing the tube into a boiling water bath for 2 min, and the mixture is stored at −20 °C for further analysis. Portions of this mixture (10 μl) supplemented with 1 μl of 80 % glycerol are separated by agarose gel electrophoresis (0.8 % in 50 mM Tris/HCl, pH 8.0, 15 min at 100 V). Phosphorylated kemptide (net charge −1) migrating toward the anode and separated from the nonphosphorylated species (net charge +1) moving toward the cathode is visualized under UV light. For quantitative evaluation, the gel material that contained the phosphorylated band is excised (125 μl), heated (95 °C), and supplemented with 75 μl of gel solubilization solution and 50 μl of glacial acetic acid, and finally its absorbance is determined at 570 nm using a 96-well plate reader (normalized for liquefied agarose containing no kemptide).

Evaluation

The PKA activity ratio is calculated as the ratio between the absorbance in the absence and presence of cAMP corrected in each case for unspecific phosphorylation in the presence of the PKA inhibitor (which typically is <15 % of total) and reflects the portion of PKA activity (i.e., cAMP concentration) present at the time point of removal of the tissue or cell samples. Control experiments demonstrated that the activity ratio is independent of dilution of the tissue or cell homogenates (and protein concentration) from 2 g wet wt/ml to 0.03 g/ml (limit of detection) corresponding to 20–0.02 mg protein/ml and of incubation period (up to 2 h), thereby excluding formation of inactive PKA dephosphorylated holoenzyme during homogenization and subsequent assay.

PKA Catalytic Activity

Purpose and Rationale

In addition to the regulation of the PKA activity by modulation of the cytosolic cAMP levels, insulin and insulin-like compounds/drug candidates may affect PKA by modulating its regulation via signaling mechanisms as provoked by insulin or by its direct allosteric activation or blockade by small molecule inhibitors.

Procedure

PKA activity is assayed according to Roskoski and coworkers (1983) and Honnor and coworkers (1985a). The reaction mixture contains 25 μl of rat adipocyte cytosol, 0.4 μM kemptide (consensus substrate peptide), 40 mM Tris/HCl (pH 7.2), 2 mM DTT, 12.5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM IBMX, and 100 μM [γ-32P]ATP (1 μCi) with or without 1 μM cAMP in a total volume of 100 μl. After incubation (10 min, 30 °C), the reaction mixture is chilled on ice; then supplemented with 3 ml of 75 mM phosphoric acid, 100 mM NaF, and 10 mM ATP; and immediately spotted on phosphocellulose filters (Whatman P18). After extensive washing with 75 mM phosphoric acid, the filters are dried and counted for radioactivity.

Modifications of the Method

The inhibition of isoproterenol-stimulated PKA by sulfonylureas in rat adipocytes was tested by

Müller and coworkers (1994). The direct effect of tolbutamide and glyburide on the activity of PKA in rat liver cytosol was investigated by Okuno and coworkers (1988).

Protein Phosphatase (PP) Activity

Purpose and Rationale

Insulin inhibits lipolysis by decreasing the phosphorylation and thus the activation state of HSL in adipose tissue via both the inhibition of PKA and the stimulation of protein phosphatase 2A (PP2A) via ill-defined mechanisms. They may involve the phosphoinositol-3′-kinase (PI3K) signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway or soluble mediators of the phosphoinositolglycan (PIG) class (Vila et al. 1990; see K.6.3.6.4).

Procedure

For measurement of PP2A activity, the adipocytes (3 × 107 cells) are washed once with 25 ml of ice-cold 100 mM Tris/HCl (pH 8.5), 10 mM EDTA, and 25 mM DTT and immediately resuspended in 0.5 ml of 100 mM Tris/HCl (pH 7.0), 2 mM EDTA, 10 mM DTT, 0.5 mM benzamidine, 0.2 mM PMSF, 2 μg/ml leupeptin, and 5 μg/ml pepstatin. After addition of the same volume of ice-cold glass beads, cell extracts are prepared by vigorous vortexing (five times for 5 s each with cooling intervals on ice) and centrifuged (13,000 × g, 5 min, 4 °C). The supernatant (S13) is diluted with three volumes of the same buffer containing 1 % BSA. Protein phosphatase activity is assayed as the ability to dephosphorylate 32P-labeled myelin basic protein (MBP). Ten microliters of S13 is added to an assay mixture (total volume 50 μl) containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, and 0.2 nmol 32P-labeled myelin basic protein (900 dpm/pmol) for 20 min at 30 °C in the absence or presence of 2 nM okadaic acid. The reaction is terminated by the addition of 50 μl of ice-cold 10 % TCA. After incubation for 15 min on ice and centrifugation (12,000 × g, 5 min, 4 °C), the supernatant is neutralized with NaOH and measured for radioactivity by liquid scintillation counting (10 ml Aquasol).

Evaluation

PP2A activity is determined as the difference between 32P-radiolabeled MBP measured in the presence of okadaic acid (10 nM) corresponding to the PP2A independent phosphatase portion and radiolabeled MBP determined after uninhibited incubation corresponding to the total phosphatase activity. Under these conditions, release of 32Pi is linear for up to 10 min. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of 32Pi-radiolabel from labeled myelin basic protein in 1 min under conditions of the standard assay (Müller et al. 2000).

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Thermogenesis and Mitochondrial Function

Purpose and Rationale

Adipose tissue in mammals is distinguished as being brown or white. Brown adipose tissue functions to produce heat and, thus, has a high oxidative capacity, evidenced by the extraordinarily high density of mitochondria in the cells. White adipose tissue is primarily an energy-storing tissue with low oxidative capacity. However, studies of metabolic activity are relevant for both tissues. Because thermogenesis (heat production) is the function of brown adipose tissue, it would be a natural choice to measure this directly. However, this has only been done a few times, mainly because of technical limitations (microcalorimetry is still not a widespread equipment in biological laboratories). However, it has been calculated that respiratory determinations are indeed satisfactory measures of heat production (Nedergaard et al. 1977). This is probably true for most mammalian organs with good blood supply. It is therefore a routine procedure to perform respiratory measurements and equate the result with that of thermogenesis and metabolic activity in general (Cannon and Nedergaard 2001).

Respiratory measurements can be performed on isolated mitochondria, isolated cells, and tissue pieces. To obtain sound values, it is essential that the measurements are made under the most optimal conditions possible. This includes provision of an adequate oxygen supply throughout the experiment and also the use of a substrate for respiration that is not limiting. Because of the requirement for sufficient oxygen, tissue pieces can often be problematic, as oxygen supply may be limited by the diffusion of oxygen through the piece of tissue. Dispersed cells and mitochondria can be more easily oxygenated but are obviously more artificial in other respects.

The supply of a suitable substrate for respiration is often difficult. To estimate maximal capacity, the rate of substrate supply must exceed that of the ongoing respiration. For brown adipose tissue, respiration is uncoupled from phosphorylation under conditions when thermogenesis is

activated, and the rate is thus limited by the capacity of the uncoupling protein (UCP1) or by the respiratory chain. In white adipose tissue, respiration is normally coupled to ADP phosphorylation, and the rate is therefore determined by the rate of utilization of ATP. The choice of a nonoptimal substrate, the transport of which is rate limiting, can provide spurious results, leading to erroneous conclusions.

Isolated mitochondria from brown adipose tissue are relatively easy to study, because the mature cells contain such high mitochondrial density that the mitochondrial population isolated after homogenization of whole tissue is statistically representative for the mature adipocytes. For white adipose tissue, the mitochondrial density in the adipocytes is low. A mitochondrial preparation from total tissue may therefore not be representative for white adipocyte mitochondria, and it can therefore be necessary to isolate mitochondria from isolated cells, which leads, however, to very low yields. Mature adipocytes can be conveniently isolated from both tissues based on Rodbell's classical collagenase digestion procedure (Rodbell 1964), as the fat-containing cells readily float and can thus be separated from tissue debris in aqueous media.

Procedure

Isolation of Brown Adipose Tissue Mitochondria

For a routine preparation of brown adipose tissue mitochondria, five mice are used that have been living at normal animal facility temperatures. All procedures are conducted at 0–4 °C. The mice are anesthetized for 1–2 min in 79 % CO_2 and 21 % O_2 and decapitated. The periaortic, cervical, interscapular, and axillary brown adipose tissue is carefully dissected out into a small volume of ice-cold 0.25 M sucrose on a square of parafilm and then rinsed in the same solution. (Some researchers use a slightly buffered sucrose solution containing, e.g., 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 100 mM KCl, abbreviated K-TES. However and in contrast to the preparation of mitochondria from skeletal muscle and liver, the beneficial effect of the use of a chelator, such as EDTA or EGTA, remains questionable.) Excess sucrose is absorbed with a medical wipe. The tissue is minced with scissors and homogenized in about 40 ml of 0.25 M sucrose solution (about 5 % w/v) using five to six strokes with a loosely fitting Teflon pestle in a glass homogenizer. Thereafter, the homogenate is filtered through two layers of gauze and centrifuged at 8,500 × g for 10 min at 4 °C. The fat layer at the top of the tube and the infranatant is removed by rapidly inverting the tube and wiping the walls of the tube clean with a medical wipe. The pellet harboring the cell debris, nuclei, and mitochondria is resuspended in a small volume of sucrose solution and centrifuged at 800 × g for 5 min. The supernatant harboring the mitochondria is recovered and transferred into a new tube and then centrifuged at 8,500 × g for 10 min at 4 °C. The resulting mitochondrial pellet is resuspended in 5 ml of sucrose solution containing 0.3 % fatty acid-free BSA (fraction V, Sigma). Thereafter, the mitochondria are collected by centrifugation at 8,500 × g for 10 min at 4 °C. The resulting mitochondrial pellet is resuspended in 15 ml of K-TES. After centrifugation under the same conditions, the last pellet is resuspended in a minimal volume of K-TES by hand homogenization using a small hand homogenizer. Finally, the protein concentration is determined, and the mitochondrial suspension is diluted with K-TES to a stock solution of 10–20 mg per milliliter for storage on ice.

Isolation of Brown Adipocyte Mitochondria

For a routine preparation of brown adipocytes, two adult (10- to 30-week-old) Syrian hamsters (*Mesocricetus auratus*) of either sex kept at 20–22 °C one to three per cage with food and water ad libitum are anesthetized by 79 % CO_2 and 21 % O_2 and then decapitated. The cervical, interscapular, and axillary brown adipose tissue is dissected out into small volume of ice-cold Krebs–Ringer–phosphate buffer (KRPB: 148 mM NaCl, 6.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM MgSO4, 5.6 mM KH_2PO_4, 16.7 mM

K_2HPO_4, 10 mM glucose, and 10 mM fructose containing 4 % BSA) and adjusted to pH 7.4 with Tris/NaOH to 7.4 on a square of parafilm and carefully cleaned from contaminating tissues. The brown adipose tissue is placed into a polyethylene vial containing 3 ml of KRPB containing 0.83 mg/ml of collagenase (crude, type I, clostridiopeptidase A) and then incubated at 37 °C for 5 min in a 1.7 Hz shaking water bath. After addition of 7 ml of KRPB and vortexing the vial for 5 s, the mixture is filtered onto silk filter cloth (Joymar Scientific). The first filtrate is discarded. The collected tissue pieces are transferred into a small volume of KRPB on a square of parafilm and minced with scissors. The mince is incubated in 3 ml of KRPB containing fresh collagenase as above for 25 min with 5 s vortexing intervals every fifth minute. After addition of 7 ml of KRPB and vortexing for 15 s, the mixture is filtered as above. The filtrate is collected and centrifuged at 65 × g for 5 min. The infranatant consisting of the collagenase incubation medium is discarded by suction with a Pasteur pipette with a plastic tubing on the tip connected to a water suction pump. After addition of 10 ml of KRPB, the cells are allowed to stand at 4 °C. The tissue pieces remaining on the silk filter are incubated for 15 min as above. The filtrate containing cells is collected. The tissue pieces now remaining are incubated for additional 10 min and the cells of the filtrate are collected, in order to increase the yield. The infranatants consisting of the collagenase incubation media (and undifferentiated preadipocytes) in the three tubes are discarded. (The preadipocytes can be collected, cultured in primary culture, and differentiated into mature brown adipocytes as described in the previous chapter.) The three filtrates harboring the brown adipocytes are combined, supplemented with 10 ml of KRPB, and centrifuged at 65 × g for 2 min. The infranatant is discarded. The brown adipocytes contained in the layer between the lipid layer at the top of the tube and the pellet are recovered and counted in a Bürker chamber. The cells are diluted with KRPB to a concentration of 1–3 × 106 cells/ml and stored on ice until use. Hamster brown adipocytes display very little loss of cell response during a working day, whereas those from rats and mice break easily and should be aliquoted into Eppendorf tubes, kept at room temperature, and used as soon as possible. Hamster cells can be stored overnight at 4 °C in 10 ml of KRPB, in which case the cells are washed again the next day by centrifugation.

Measurement of Respiratory Rate

The rate of oxygen consumption of both isolated brown-fat mitochondria and cells can be readily measured polarographically with a Clark-type oxygen probe (e.g., Rank Bros. or Hansatech as complete systems with measuring chamber and magnetic stirrer or Oroboros Oxygraph 2 k for smaller samples) (Robinson 1994). Such a probe determines oxygen concentration in aqueous solutions. The current produced by the electrode is proportional to the oxygen tension in the solution. The electrode chamber must be continuously stirred (most practically magnetically). Preferably, the electrode chamber must also be temperature controlled (e.g., by circulating water from a water bath) and calibrated as follows: The electrode chamber is filled with H_2O bidest at the experimental temperature and allowed to equilibrate with atmospheric oxygen until the output is stabilized. Following incubation at 37 °C, this corresponds to 217 nmoles O_2/ml, at 25 °C (a traditional but clearly less physiological temperature typically used for mitochondrial experiments) 253 nmoles O_2/ml. (These values are, of course, only valid for normal atmospheric pressure. However, the effects of normal fluctuations in atmospheric pressure are normally ignored.) Thereafter, a few crystals of sodium dithionite are added to the solution for reduction of oxygen as a prerequisite for determination of zero oxygen levels. After removal of the calibration solution, the chamber is carefully washed with H_2Obidest and filled with the relevant buffer. The output may not fully return to the level it had with H_2O bidest. that is correct, as salt-containing media dissolve less oxygen than H_2O bidest. For mitochondrial studies, a medium consisting of 100 mM KCl, 20 mM K-TES, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EDTA, and pH 7.2 contains the appropriate substrate (see under “EVALUATION”). For cellular studies, Krebs–Ringer bicarbonate buffer

(145 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2$, MgSO4, 25.3 mM NaHCO3, 1.2 mM NaH_2PO_4, 10 mM glucose, and 10 mM fructose containing 4 % fatty acid-free BSA adjusted to pH 7.4 with Tris/HCl under continuous bubbling with a small stream of 5 % CO_2 in air at 37 °C until use) is used instead. Thereafter, 0.2–0.5 mg mitochondrial protein or 50,000–80,000 cells per ml buffer are added to the electrode chamber. After closure of the chamber and stabilization of the system, further additions are made through a small hole in the cover of the chamber using a Hamilton syringe (the addition of ethanol as solvent for some compounds may lead to a small baseline shift). Finally, the electrode output is connected to a computer via an analog-to-digital converter and PowerLab 4/30 data acquisition and analysis system with ChartPro software for Windows or MacIntosh. Alternatively, a regular chart recorder can be used.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential is determined by a number of methods that rely upon distribution of a cationic dye within or across the mitochondrial inner membrane in accordance with the membrane potential. Several compounds can be used for this purpose, such as triphenylmethylphosphonium (i.e., TPMP), tetraphenylphosphonium (TPP, which can also be used in ion-selective electrodes), and rhodamine 123 or safranine O, as is used in the method described here. The conditions and media should be identical to those described above for oxygen consumption measurements in mitochondria, so that the results between the two types of determinations can be compared. The changes in absorbance of 5 μM safranine O at 511–533 nm are followed. Increased quenching of the color gives an upward deflection.

One milliliter of medium and 0.5 mg of mitochondrial protein are added to a suitable cuvette together with 5 μM safranine. The changes in absorbance are recorded after the addition of compounds of interest. At the end of the experiment, 25 μM FCCP, followed by 7 × 10 mM NaOH are added until the mitochondria are solubilized. For each preparation, the membrane potential is calibrated by initially transferring the mitochondria into a state of energy conservation as noted in the EVALUATION; then addition of 9 μM valinomycin followed by KCl, at concentrations between 0.1 and 50 mM to the mitochondria in the cuvette; and finally addition of 25 μM FCCP, followed by 7 × 10 mM NaOH. The change in absorbance is plotted against log KCl concentration in order to extrapolate to the internal K+ concentration in the mitochondria and the initial concentration in the medium. The Nernst equation and the values obtained in the calibration are used to calculate the membrane potential under each condition.

Brown Adipose Tissue

When brown adipose tissue mitochondria are isolated, they are uncoupled (Smith et al. 1966; Lindberg et al. 1967) and typically have a collapsed membrane potential. They demonstrate high permeability to many monovalent ions. Presumably, as a consequence of this, they have lost the ability to retain osmotic support in the mitochondrial matrix. The matrix is therefore highly condensed after preparation and oxidation of substrates in the matrix is markedly inhibited (Nicholls et al. 1972, 1973; Nicholls 1974). To re-expand the matrix, the mitochondria may be incubated in an iso-osmotic medium of permeant ions (such as KCl). Matrix expansion can also be achieved with low osmolarity sucrose (100 mM), although this probably gives a less controlled expansion.

Brown adipocytes can also be prepared by the same method from rats (Fain et al. 1967; Zhao et al. 1998a) and mice (Zhao et al. 1998b), with the cells from Syrian hamsters apparently being the most robust and most appropriate for many studies. The robustness of the latter also means that they are a suitable choice for researchers learning the technique.

The details given here for the preparation of brown adipocytes are examples of incubation times with collagenase and its concentration (and type) and centrifugation times (or flotation without centrifugation), which can be applied. Different researchers tend to develop personal modifications of these, particularly at times and

under circumstances when, for unclear reasons, the preparations are less successful. It is difficult to find convincing evidence that these modifications are of major significance, but this idiosyncrasy also indicates that the details specified here are for guidance and need not be adhered to exactly.

The preparation technique is dependent upon the property of the cells to float on top of an aqueous medium. If cells are isolated from animals that are cold exposed, the triglyceride concentration may be so low that the cells sink in the medium and can therefore not be separated. The yield of cells will thus be lower than normal (Nedergaard 1982; Svartengren et al. 1982). In general, the yield of mature adipocytes as a percentage of total adipocytes in the tissue is not very high. The representativeness of the cell population can perhaps therefore be discussed. Also, if cells are prepared from animals living at thermoneutrality, the cells are very replete with triglycerides and their diameter larger than of cells from animals at room temperature. This large cell size seems to make the cells more fragile and sensitive to mechanical manipulation.

To transfer the mitochondria into a state of energy conservation, incubation should be performed in the presence of fatty acid-free albumin (0.1–0.5 %) to remove fatty acids and related substances and of purine nucleotides. The nucleotides bind to the brown-fat-specific uncoupling protein, UCP1, and in doing so close, the proton leaks through this protein (Nicholls 1974). The most commonly used nucleotide is GDP, which is used at a concentration of 0.1–1 mM. Other di- and triphosphate purine nucleotides are also more or less efficient. The nucleotide-binding site is on the outer side of the inner mitochondrial membrane.

When respiratory studies are performed on isolated mitochondria, it is of great importance that a suitable substrate is used. The most relevant is a fatty acid or its derivative, such as long-chain acyl-coenzyme A or acyl-carnitine. In all cases, to permit complete fatty acid oxidation, 5 mM malate must be added to the buffer to replenish citric acid cycle intermediates which have been lost during isolation (Cannon 1971). In some species, the reuptake of malate is low and this may even limit fatty acid combustion. When acyl-carnitine esters are used (50 μM), no further additions (except malate) are required. For acyl-CoA derivatives at similar concentrations, a further addition of 2 mM L-carnitine guarantees that availability of this compound does not limit oxidation. If free fatty acids are used, further additions of 100 μM ATP and 5 μM coenzyme A, in addition to carnitine, allow unlimited fatty acid oxidation. A further NADH-coupled substrate that demonstrates fairly high rates of respiration is pyruvate, used at 5 mM concentration together with 5 mM malate. Glutamate is inappropriate. Brown-fat mitochondria also demonstrate a high rate of oxidation of glycerol-3-phosphate (used at mM concentrations), a flavoprotein-coupled substrate, which is oxidized on the external face of the inner membrane and thus does not require transport (Bukowiecki and Lindberg 1974). In many species, succinate (which is a classical substrate for studies of liver mitochondria) permeates only poorly into the mitochondria, and its use may therefore lead to severe underestimates of oxidative capacity. This is also often the case for other potential substrates, mainly the intermediates of the citric acid cycle, which have a low rate of permeation in certain species (Cannon et al. 1984).

Respiration in the brown adipocytes is most notably stimulated by the physiological agent norepinephrine, in which case endogenous lipolysis provides the substrate and also permits uncoupling of respiration from the constraints of a requirement for ATP utilization (Fain et al. 1967; Prusiner et al. 1968). This uncoupling is entirely dependent upon the presence of UCP1 (Matthias et al. 1999, 2000). Free fatty acids can also be added to the cell suspension and provide an adequate substrate (Prusiner et al. 1968). Their combustion is also fully dependent on the presence of UCP1, and this demonstrates that fatty acids can directly or indirectly activate UCP1 (Matthias et al. 1999; Shabalina et al. 2004). If other substrates are utilized, there is a transport requirement into the cells, in addition to which rates of

respiration are generally low unless respiration is artificially uncoupled with, e.g., 20 μM FCCP. The maximum respiratory rates then seen are usually much lower with exogenous substrates, such as pyruvate (5 mM) than with the endogenously generated or exogenously added fatty acids.

White Adipose Tissue

To obtain mitochondria representative for the mature adipocytes in the tissue, isolated adipocytes, prepared essentially as described for the brown adipocytes, can be used as the starting material. The cells are homogenized and the mitochondria isolated by routine differential centrifugation, as described. The yields are very low. The mitochondria are well coupled and can be stimulated to respire on citric acid cycle intermediates, in the presence of ADP (Marshall et al. 1984).

Few respiratory studies have been performed on isolated primary white adipocytes that are easily prepared (Rodbell 1964), but due to their appearance (one unilocular lipid droplet filling most of the cell volume) are not easily distinguished from large lipid droplets. Their rate of respiration is very low and high cell densities must be used. Basal metabolic/respiratory rates can be estimated and hormonal stimulation can be performed, but this is generally evaluated in terms of metabolic changes other than respiration. A number of microcalorimetric studies have been performed on isolated white adipocytes from humans. Basal metabolism has been determined (Monti et al. 1980) and comparisons made between tissue taken from obese and lean (Olsson et al. 1986) or hypo-/euthyroid (Valdemarsson et al. 1985) individuals. The effects of hormone stimulation can be determined.

Evaluation

Brown and white adipose tissues in mammals have a number of similar properties, such as lipid storage and adipokine production, but also distinctive properties. The energy-storing white adipose tissue has few mitochondria and low oxidative capacity. The heat-producing brown adipose tissue has a high density of mitochondria and high oxidative capacity. Mitochondrial function can be investigated in cells and organelles isolated from both brown and white adipose tissues. The methods described here may be helpful for successful isolation of suitable preparations of adipose tissues and their subsequent use. Questions concerning thermogenic capacity of the tissues, their potential influence on whole body metabolism, and specific properties of the mitochondria and their mode of function may be addressed using these approved methods.

Because many studies are now performed on genetically modified mice, the method described is also for such animals. However, mitochondria can also be isolated by the same method from the brown adipose tissue of other mammals (most commonly rats and the Syrian hamsters). They can be isolated from tissues taken from animals kept at colder or warmer environmental temperatures. In these cases, the tissue contains less or more triglyceride, and the number of mitochondria per gram tissue is higher in cold-acclimated animals. Triglycerides disturb the homogenization, and a lower relative yield of mitochondria will generally be obtained from animals kept at higher temperatures and similarly higher from animals kept a lower temperatures.

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Assays for Insulin and Insulin-Like Regulation of Energy Metabolism

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Contents

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Determination of Oxygen Consumption and Extracellular Acidification Rates

Purpose and Rationale

Accumulating experimental evidence indicates that dysregulation of energy metabolism is a fundamental process that is associated with the phenotype of metabolic disorders, in particular type II diabetes and obesity. Many current antidiabetic drugs (metformin, glitazones) target the pathways that control glucose metabolism. Very recently, technologies (Bionas Inc., Germany; Seahorse Bioscience Inc., USA) have been developed that manage to rapidly profile the bioenergetic pathways in a variety of cell types. These include alterations caused by drugs that target the specific metabolic pathways the cell uses to ensure its energy demands, normal metabolic functions, and survival. These technologies allow a more detailed analysis of the link between glucose/lipid metabolism and energy metabolism, including interference of one or the other by compounds/drug candidates, as well as the screening for or characterization of drug candidates affecting or leaving unaffected those pathways. For this, both the oxygen consumption rate (OCR), which primarily reflects mitochondrial respiration, and the extracellular acidification rate (ECAR), which primarily reflects lactic acid production (glycolysis), are measured using the Bionas or Seahorse technology. Furthermore, the OCR and ECAR

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readings allow to profile the metabolic sensitivities and degree of inhibition/stimulation of a number of cell lines relevant for the study of glucose and lipid metabolism and its regulation toward modulators of anaerobic and aerobic energy metabolism (e.g., phloretin, 2-deoxyglucose, dinitrophenol). Analysis of the sensitivities of the cell lines to these modulators provides insights into their bioenergetic preferences/dependencies and their global physiological responses to the modulation. This characterization may be useful for the selection of cell lines appropriate for use in screening for compounds with antiproliferative activity (ECAR for anaerobic energy metabolism; Boros et al. 2002) as well as insulin-like metabolic activity (OCR for aerobic energy metabolism; Wolf et al. 1997), with regard to sensitivity and responsiveness of their energy metabolism and the relevant bioenergetic pathways (Ehret et al. 2001). During the last decade, multiparametric cellular microelectronic interdigitated biosensor chips for microphysiological and screening applications with living cells have been developed (Ehret et al. 2001; Lehmann et al. 2000).

Procedure

Bionas 2500 Analyzing System

The Bionas 2500 analyzing system is an automated system suitable for online cell-based compound/drug candidate identification and profiling. The innovative aspect of this bioanalytical device is the adaptation of electronic chip design for a completely noninvasive and multiparametric sampling of metabolically relevant readouts in long-term tissue and cell culture. The system uses a silicon-based technology to continuously monitor minute changes in (i) solution pH resulting from the output of acid metabolites excreted by living cells into their immediate microenvironment (Lehmann et al. 2001), (ii) oxygen content resulting from oxygen consumed by living cells from their immediate microenvironment, and (iii) electrical impedance resulting from the electrical isolation of intact cell membranes close to the sensor surface (adhesion/confluence) (Ehret et al. 1998). The Bionas 2500 analyzing system works in real time, allowing the rapid and continuous monitoring of cellular activity and the measurement of recovery from a particular challenge and the process of desensitization.

Cells are seeded onto Bionas metabolic chips the day prior to the experiment and left to attach to the sensor surface overnight. The sensor chips are then transferred into the Bionas 2500 analyzing system. The system has the capacity to measure the acidification, respiration, and adhesion/confluence rate from six separate sensor chips. Modified, low-buffered medium is pumped across the cells at a rate of 56 µl/min during which time the pH and the oxygen content of the microenvironment surrounding the sensor are maintained constant. To measure the rates for acidification and respiration from cells, the fluid flow is periodically halted, allowing a buildup of acid metabolites and a depletion of oxygen in the chamber and, therefore, an alteration in the fluid pH and oxygen content. Flow is resumed and the acid- and oxygen-depleted fluid flushed out of the chamber. This flow-on, flow-off cycle is repeated throughout the experiment. To measure the adhesion/confluence, no fluid perfusion is necessary. After collecting baseline acidification, respiration, and adhesion rates, the compound/drug candidate is introduced, and the effect on acidification, respiration, and adhesion rates is monitored.

Seahorse XF Instrument

Adherent 3T3-L1 adipocytes, L6 myoblasts/myotubes, or HepG2 cells are seeded in 24-well Seahorse cell culture microplates at various cell densities per chamber per well. Approximately 45 min prior to the assay, the culture medium is exchanged with a low-buffered RPMI assay medium to ensure accurate ECAR readings. For detection of acute drug responses, OCR and ECAR are measured for 5 min in each well to establish a baseline. The compound/drug solution is then added followed by measurement of OCR and ECAR. For time-resolved measurements, test measurements are made at a series of time points. Nonadherent cells (primary hepatocytes and adipocytes) are seeded onto Cell-Tak-coated Seahorse cell culture microplates either before or

after the compound/drug treatment. Cell-Tak treatment of plates is performed according to manufacturer's instruction. Cells remain attached to the surface of the well after the measurement as determined by light microscopy. Cells are lifted from the culture surface by simple pipetting without trypsin treatment.

A unique feature of the Seahorse instrument is its ability to make accurate and repeatable measurements in as little as 5 min. This is accomplished by isolating an extremely small volume (less than 10 μl) of medium above the cell monolayer. Cellular metabolism causes rapid, easily measurable changes to the "microenvironment" in this small volume. Typically, a measurement cycle is performed for 2–10 min. During the time, analyte levels are measured every 8 s until oxygen concentration drops approximately 10 % and medium pH declines approximately 0.1 unit. The measurement is performed using fluorescent biosensors embedded in a sterile disposable cartridge that is placed above a 24-well tissue culture microtiter plate.

Evaluation

Baseline metabolic rates are typically measured twice and are reported in nmol/min for OCR and mpH/min for ECAR. The compound/drug is then added to the medium and mixed for 5 min, and then the posttreatment OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR changes. Because these measurements are nondestructive, cells can be profiled over a period of minutes, hours, or days.

Omics Technologies

General Considerations

The flow of information from DNA to cellular building blocks is the central dogma of biology. DNA is transcribed into RNA and then translated to proteins, which then make small molecules. While there may be over 25,000 genes, 100,000–200,000 transcripts, and up to 1,000,000 proteins, it is estimated that there may be as few as 2,500 small-molecule species in the human metabolome. Metabolomics is the study of the metabolome, i.e., of the repertoire of these non-proteinaceous, endogenously synthesized small molecules present in an organism. Representative small molecules include well-known compounds such as glucose, cholesterol, ATP, and soluble signaling molecules, such as cAMP, AMP, and diacylglycerol. These molecules are the ultimate product of cellular metabolism. The metabolome refers to the catalogue of those roughly 2,400 known endogenous metabolites in a specific organism. Most important, the normal physiological and disease states are ultimately manifest at the level of biochemistry and more precisely at the level of the metabolome. It is more clear-cut and more quantitative because it is known where metabolites fit in biochemical pathways and the metabolites' relative concentrations are measurable. This makes a clear difference to many of the other "omics" sciences. It seems very likely that intelligent application of this methodology in appropriate animal disease models will considerably facilitate the identification and characterization of compounds/drugs for most indications and for metabolic diseases, in particular, with regard to potency, safety, accuracy, predictability, and the time required. The putative "systems" contribution of metabolomics to pharmaceutical drug discovery and development has been reviewed by Harrigan (2006).

Purpose and Rationale

Metabolomics technology determines the small-molecule repertoire of a cell or a biological sample typically using a proprietary approach of simultaneous, multidimensional molecular analysis, followed by sophisticated data analysis and visualization. Biological samples are extracted using special protocols, and the resulting extracts are separated and analyzed using a variety of detection techniques. Chromatographic outputs are analyzed using multiple mass spectrometers, which accurately identify the mass for a particular

molecular species. Electrochemical detectors monitor additional broad classes of compounds. By choosing appropriate types of separation and detection technologies and mass spectrometers, the cellular metabolites are analyzed according to their various properties. Modified mass spectrometers produce data streams that are automatically processed and stored in proprietary information management systems. The data are quality checked, reduced, refined, and abstracted using custom algorithms and software. Data visualization is then used to compare experimental data to a large database of control information. Hundreds of compounds of known identity are measured in a sample and contrasted to controls. Unique unknowns are brought to light for structure elucidation.

Procedure

Metabolomics research is characterized, at least in part, by two different (but not necessarily mutually exclusive) conceptual approaches broadly defined as "targeted" and "nontargeted" (Harrigan and Goodacre 2003; Goodacre et al. 2004; Vaidyanathan et al. 2005). Nontargeted approaches provide a hypothesis-free global overview of high-abundance metabolites most affected by experimental perturbation or disease. Targeted approaches which highlight identified and preselected metabolic pathways may prove more relevant in evaluating the impact of a compound/drug candidate on metabolic regulation. Technology platforms for targeted approaches are based on discrete optimized analytical strategies for different classes of metabolites or pathways. This approach represents an accommodation with the wide differences in physicochemical structure, stability, and differential abundance of metabolome components. It clearly facilitates greater evaluation of low-abundance, biologically important metabolites, such as eicosanoids, other signaling lipids, and hormones. A key challenge for vendors providing instrumentation to support metabolomics is in establishing an optimal balance between the accuracy and range of metabolite measurements. Targeted metabolomics approaches additionally allow adoption of flux-based methodology where specifically designed tracer-labeled substrates can be incorporated into test biological systems and their distribution and metabolic fate recorded. Metabolic flux analyses provide an operational "moving picture" rather than a compositional "snapshot" of a biological system. At present, this approach is somewhat underrepresented in metabolomics, but it may be pointed out that the data acquisition technologies used here are essentially that used in compositional studies. The data acquisition technologies utilized in metabolomics, primarily nuclear magnetic resonance spectroscopy and mass spectrometry, have been extensively reviewed (Weljie et al. 2006).

Use in Drug Discovery

Applications in Early Drug Discovery

There is considerably emphasis on metabolomics applications in later stages of drug discovery, including clinical trials. However, strategies to reduce attrition may be most cost-effectively implemented at the earliest stages of drug discovery, which encompasses disciplines such as target validation and high-throughput screening. In current drug discovery projects, substrate/product ratios are used as mechanistic markers of enzyme function in increasing number. It is critical that a drug candidate be shown to act on its target throughout all stages of the value chain and that modulation of that target does indeed impact disease progression.

Applications in the Analysis of Animal Models

The development of drugs for the regulation of metabolic diseases, such as cardiovascular diseases, obesity, and type II diabetes, clearly requires considerable efficacy and toxicological testing in animal models. Efficacy assessments also require that regulation of metabolism in

animal models is reasonably well understood. A metabolomics study utilizing an analytical platform developed by Lipidomics Technologies Inc. that allows quantitative measurement of more than 500 lipid metabolites in blood and tissue samples has recently been employed to probe metabolic regulation in the low-density lipoprotein receptor null (LDLrKO) mouse, a model for atherosclerotic progression (Krul et al. 2006).

Correlation of Metabolome, Proteome, and Transcriptome Data Sets

An increasing area of interest is correlation of metabolomic data sets with proteomic and transcriptomic data sets (Davidov et al. 2004). While the goal of such studies is presumably to provide a deeper understanding of biological systems, it may be worth pointing out that if the metabolome were truly closer to phenotype, then the use of complementary data from genomics, transcriptomics, and proteomics could be perceived as merely ancillary or even potentially confounding. In an integrated transcriptomic and NMR-based metabolomics study of fatty liver in the rat, an inverse correlation between stearoyl-CoA desaturase mRNA levels and levels of unsaturated fatty acids described as "surprising" by the authors was revealed (Griffin et al. 2004). This observation is somewhat less surprising when placed in the context of a metabolomic demonstration that gene expression patterns in the adipose tissue of obese mice resulted in assessments of rates of fatty acid synthesis and lipogenesis that are inconsistent with direct measurements of the metabolic fluxes (Hellerstein 2003).

Future Metabolomics Technologies

The development of a new methodology for determining the concentration of protein-unbound FFA in intracellular and extracellular milieu entitled "Fluorescent Probes for Hydrophobic Metabolites" represents a particularly intriguing program. Although FFA levels are clearly critical in many diseases, monitoring of unbound FFA profiles remains technically challenging, and improved detection methodologies can only help facilitate drug candidate progression in a range of therapeutic areas. The use of fluorescently labeled metabolite probes is also a theme in a proposal "Glycolipid Metabolism in Single Cells" to dissect glycolipid metabolism in single neurons. This proposal will focus on the development of different fluorescently labeled substrates within two different glycolipid metabolite pathways and offers particular promise in pharmacological evaluations of neuroactive agents. Another cellular neurometabolomic proposal "Technologies for Cellular Neurometabolomics" addresses the use of a suite of technology developments including unique sampling protocols and microfluidically based sample conditioning unit with integrated electrophoretic separations, followed by native fluorescence and mass spectrometric detection and capture of appropriate metabolites into nl-volume capillaries for nl-volume NMR spectroscopic characterization.

Evaluation

Companies (e.g., Metabolon Inc.) have developed proprietary software to link the resulting profile directly to metabolic pathway maps. This linkage greatly facilitates the identification of particular pathways that are affected in the biological sample relative to controls. In this way, the genes, RNAs, and proteins involved in a disease can be quickly and efficiently elucidated. By analyzing a sample using multiple mass spectrometry-based technologies, integrating the data, and analyzing through proprietary software and algorithms, the characterization of compounds/drugs in animal disease models, in general, for metabolic diseases such as type II diabetes, in particular, can be achieved much faster and more accurately than previously possible. Moreover, academic efforts have recently been initiated supporting well-conceived and technically feasible innovations that have the potential to drive metabolomics as a biologically informed science and provide metabolomic data that can be integrated into early drug candidate evaluation and also guide subsequent biomarker-enabled in vivo studies.

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Metabolomics and Lipidomics of Adipose Tissue

Purpose and Rationale

Metabolomics is a discipline dedicated to the global study of metabolites, their dynamics, composition, interactions, and responses to interventions or to changes in their environment, cells, tissues, and biofluids (Oresic et al. 2006). Metabolites are known to be involved as key regulators of systems homeostasis. Concentration changes of specific groups of metabolites may be descriptive of system responses to environmental or genetic interventions, and their study may therefore be a powerful tool for characterization of complex phenotypes as well as for development of biomarkers for specific physiological responses.

The role of specific reactive lipids as well as amino acids in the control of insulin signaling in adipose tissue is well recognized. For example, two mechanisms have been proposed to explain how expansion of the adipose tissue stores affects insulin sensitivity. One mechanism suggests that increased adiposity induces a chronic inflammatory state characterized by increased cytokine production by adipocytes and/or macrophage infiltrating adipose tissue. Cytokines produced by these adipocytes or macrophages may directly antagonize insulin signaling. In this context, the role of eicosanoids as key mediators of inflammatory signaling is well recognized (Funk 2001). A second nonexclusive mechanism is the lipotoxic hypothesis. It states that if the amount of fuel entering a tissue exceeds its oxidative or storage capacity, toxic metabolites that inhibit insulin action are formed. For example, lipid metabolites, such as ceramides and diacylglycerol, or reactive oxygen species generated from hyperactive oxidative pathways have been shown to inhibit insulin signaling (Summers 2006; Medina-Gomez et al. 2005). There is also increasing evidence that branched chain amino acids affect the insulin signaling in adipose tissue (Lynch et al. 2002; Hinault et al. 2006; Um et al. 2006). The increased amino acid levels in the circulation as a result of nutrient overload may therefore lead to insulin resistance in peripheral tissues (Um et al. 2006).

It is practically impossible to measure the levels of all metabolites in the biological sample simultaneously with a single analytical platform. The reason for this is that metabolites are (bio) chemically diverse and can cover a dynamic range of over ten orders of magnitude in concentration; therefore, a single extraction and detection method for all metabolites from biological matrices is unfeasible (Oresic et al. 2006). Additionally, the complexity of biological samples may also affect the efficiency and reliability of detection, for example, due to ion suppression effects in mass spectrometry-based approaches (de Hoffmann and Stroobant 2001). Multiple analytical platforms are commonly applied in parallel to cover the broad range of metabolites and typically include different extraction methods for specific groups of metabolites (Oresic et al. 2006). Analytical technologies based on gas chromatography coupled to mass spectrometry, liquid chromatography-mass spectrometry, capillary electrophoresis coupled to mass spectrometry, as well as nuclear magnetic resonance have most commonly been applied (von der Greef et al. 2004; Lindon et al. 2004).

To cover the metabolites most relevant to adipose tissue metabolism, typically four different platforms can be used, two screening platforms covering a broad range of lipid molecular species (UPLC/MS-based lipidomics platform) as well as organic acids and sterols (GCxGC-TOF platform) as well as a targeted platform for amino acids (UPLC).

Procedure

Lipidomics Profiling Platform (UPLC/MS)

Weighed for extraction are 20-mg aliquots of tissue samples. Samples are extracted with a mixture of chloroform and methanol (2:1 v/v; 100 ul, HPLC-grade). A standard mixture is added after the extraction containing three labeled lipid compounds (GPCho 16:0/0:0-D3, GPCho 16:0/16:0-D6, and TG 16:0/16:0/16:0-13C3). The UPLC/MS system consists of an Acquity Ultra Performance LCTM (UPLC) combined with a Waters Q-TOF Premier mass spectrometer. A sample organizer is used for the automatic sampling. The column used is an Acquity UPLC BEH C18 column 1.0 × 50 mm with 1.7-um particles from Waters Inc. The temperature of the column is 50 °C. The solvent system includes A (water/1 % 1M NH4Ac, 0.1 % HCOOH) and B (AcCN/2-propanol 5:2 and 1 % 1M NH4Ac, 0.1 % HCOOH). The gradient is started at 65 % A/35 % B and set to reach 100 % B in 6 min for maintenance for further 7 min. The total running time including a 5-min re-equilibration step is 18 min. The flow rate is 0.2 ml/min and the injected amount of lipid extract is 0.75 µl. The temperature of the sample organizer is 10 °C. Lipid compounds are detected by using electrospray ionization in positive ion mode. The following internal standard mixture is used: GPCho (17:0/0:0), GPCho (17:0/17:0), GPEtn (17:0/17:0), GPGro (17:0/17:0),

Cer (d18:1/17:0), GPSer (17:0/17:0), GPA (17:0/17:0), D-erythro-sphingosine-1-phosphate (C17 base), MG (17:0/0:0/0:0), DG (17:0/17:0/0:0), and TG (17:0/17:0/17:0). The data is collected in continuum mode using extended dynamic range at mass range of m/z 300-1,200 with a scan duration of 0.2 s. Data are processed using the MZmine software (Katajamaa and Oresic 2005; Katajamaa et al. 2006).

Metabolomics Platform (GC/GC-TOF)

Tissue samples of 10–20 mg are weighed into Eppendorf tubes. 10 µl of 500 ppm of labeled palmitic-16,16,16-d3 acid (1 g/l in methanol) is added as internal standard. Samples are extracted with 500 µl of methanol (HPLC-grade) by vortexing for 2 min and incubating for 30 min and then centrifuging at 10,000× g for 3 min. The separated supernatants are evaporated to dryness under a stream of nitrogen. The residues are derivatized as follows: 25 µl of 2 % methoxyamine hydrochloride in pyridine and N-methyl-N-(trimethylsilyl)trifluoroacetamide are added. After heating to 30 °C for 90 min, 50 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide is added, and the incubation is continued for 30 min at 37 °C. The samples are run on a GCxGC-TOF instrument (Agilent 6890N gas chromatograph and LECO Pegasus 4D mass spectrometer).

Amino Acid Platform (UPLC)

Tissue samples of 10 mg are weighed and supplemented with 250 µl of 1:10 diluted borate buffer supplied in derivatization kit and 50 µl of 10-µM alpha-aminobutyric acid in water. 50 mg of glass beads (0.45–0.75-mm diameter) is added. An external standard mixture consisting of D-leucine, D-isoleucine, and D-valine in borate buffer at concentrations ranging from 12 to 0.3 µM and an internal standard of 10-µM alpha-aminobutyric acid in water are added. The sample is heated to 45 °C for 5 min and then homogenized in a Sartorius Dismembrator at 3,000 cps for 3 min. After cooling in an ice bath for 10 min, the sample is centrifuged (centrifugal filters Nanosep MF GHP 0.45 um, Pall Life Sciences) at 10,000× g for 3 min. The supernatant is taken off and saved. The pellet is washed with 200 µl of 1:10 diluted borate buffer supplied in AccQ Tag reagent kit (Waters Inc.) and centrifuged again. The supernatant and washing fluids are combined and then dried under nitrogen. The dried residues are dissolved in 25 µl of borate buffer supplied in AccQ Tag reagent kit. After addition of 5 µl of AccQ Tag reagent, 4 µl of the sample is injected into UPLC instrument equipped with the BEH C18 column (Acquity UPLC, 1.0 × 50 mm with 1.7-mm particles, Waters Inc.) using the chromatographic solvents A (1.2 % formic acid adjusted to pH 5.3–5.7 with 5N ammonia) and B (2 % formic acid in HPLC-grade acetonitrile) and running at 55 °C. UV detection is measured at 260 nm (Acquity 2996 detector).

Evaluation

The role of specific reactive lipids as well as amino acids in the control of insulin signaling in adipose tissue is well recognized. Since it is practically impossible to measure the levels of all metabolites in the biological sample simultaneously with a single analytical platform, multiple platforms have to be used to study the lipids and metabolites of relevance to adipose tissue metabolism and insulin signaling. Two screening platforms manage to cover a broad range of lipid molecular species (UPLC/MS-based lipidomics platform) as well as organic acids and sterols (GCxGC-TOF platform). The use of a targeted platform for amino acids (UPLC) may also be of advantage.

The method parameters are optimized for the specific analytical system used. The primary aim of the described method is rapid screening of lipid molecular species across a broad range of lipid classes. Although the use of positive ion mode is described, the same platform can also be applied using electrospray ionization in negative ion mode, which will lead to better sensitivity for specific phospholipid classes, such as phosphatidylinositols, phosphatidylserines, and phosphatidic acids. The method can be transferred to a different LC/MS system. Ideally, such system

should have MS/MS and/or accurate mass capabilities.

MZmine data processing parameters need to be optimized for a specific analytical system used in order to account for different peak shapes and lengths as well as different mass spectrometer resolution. Differential profiling of multiple samples requires steps such as peak detection, alignment (matching of peaks across multiple samples), and normalization using internal standards (Katajamaa and Oresic 2005; Katajamaa et al. 2006).

The gas chromatograph is operated in split mode (1:20) using helium as carrier gas in constant pressure mode. The injection volume is 1 µl. The first column is relatively nonpolar RTX-5 (10 m × 180 µm × 0.20 µm; Restek), and the second column is polar BPX-50 (1.10 m × 100 µm × 0.10 µm; SGE Australia). The temperature program is as follows: primary oven initial 50 °C, 1 min at 280 °C, 7 °C/min, 5 min; secondary oven initial 60 °C, 1 min at 290 °C, 7 °C/min, 5 min. The modulator is kept at 25 °C above the primary oven temperature. The second dimension time is set to 4 s. Data are acquired at 100 spectra s-1 from m/z 40 to 700.

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Proteomics of Adipose Tissue

Purpose and Rationale

Advanced mass spectrometry-based proteomics technologies have revolutionized the way of conducting biological studies during the last decade (Anderson and Mann 2006; Ong and Mann 2005; Domon and Aebersold 2006a, b). Although it is still a major challenge to determine global changes in protein expression, focused approaches on organelles and selected tissues are now feasible (Anderson and Mann 2006; Ong and Mann 2005; Domon and Aebersold 2006a, b; Chen et al. 2005). Proteomics technologies have also enabled scientists in the research fields of endocrinology, obesity, and diabetes to study complex changes, for instance, in the secretory

proteome of adipose cells through liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS)(Chen et al. 2005; Kratchmarova et al. 2002; Sidhu 1979; Spiegelman and Green 1980; Wilson-Fritch et al. 2003; Tsuruga et al. 2000; Wang et al. 2004). This concept is particularly interesting because adipose tissue plays a pivotal role as an endocrine organ in the regulation of energy metabolism and glucose homeostasis and because dysfunction of adipose tissue secretion is associated with obesity and its linked metabolic syndrome (Ahima and Flier 2000; Tilg and Moschen 2006; Rosen and Spiegelman 2006; Van Gaal et al. 2006). Because cultured adipose cells secrete their proteins directly into the medium, they are thus amenable to extensive 2D-LC-MS/MS studies.

To retain as much information in the intact proteins as possible, the intact proteins are separated in the first-dimensional reversed-phase chromatography into eight fractions. Adipokines of the individual fractions are then digested with Lys-C in the presence of urea and trypsin prior to LC-MS/MS analysis. Using 18O proteolytic labeling strategies, comparative differences after drug treatment such as insulin or rosiglitazone can additionally be assessed by measuring the isotopic ratios of the labeled and unlabeled peptides. With the use of this technique, autocrine and endocrine effects of drug treatment of adipose cells can be systematically studied. To eliminate false-positive proteins that may have leaked out of the damaged tissue, all identified proteins are checked for signal peptides, common to secreted proteins using SignalP predictions. Studies like this give significant insight into the complexity of signaling cascades and the multiple effects of a drug treatment on the global changes in the secretory proteome of adipose cells in healthy and diseased states.

Procedures

Rat Adipose Tissue Culture

Adipose tissue is removed from the epididymal fat pads of 150–200-g male Zucker fa/fa rats (Charles River Laboratories) treated with or without the corresponding antidiabetic drug, such as rosiglitazone (3 mg/kg body weight via gavage for 12 days, prepared freshly at 2 mg/ml by dissolving 8-mg pill in water) or insulin (173 μmol prepared in 0.1-M HCl via intraperitoneal injection). The tissue is finely minced and then washed by centrifugation at 1,200× g for 2 min, twice with Krebs-Ringer bicarbonate Hepes (KRBH) buffer (120-mM NaCl, 4-mM KH_2PO_4, 1-mM $MgSO_4$, 1-mM $CaCl_2$, 10-mM $NaHCO_3$, 200-nM adenosine, and 30-mM Hepes-KOH, pH 7.4) containing 0.1 % BSA, and twice with serum-free BSA-free DMEM medium containing 4.5-g glucose/l.

The tissue is cultured in serum-free BSA-free DMEM at 150 mg/ml under 5 % CO_2 at 37 °C for 48 h. The conditioned medium is collected and centrifuged at 1,200× g for 10 min to separate the cell debris. The supernatant medium is filtered through a 0.45-μm syringe-driven filter to remove remaining possible cell debris. Then the medium is desalted and concentrated using a Macrosep centrifugal device with a molecular weight cutoff of 1 kDa (Pall Life Sciences). The protein concentration is determined using a Pierce BCA protein assay and should finally result in approximately 650 ng/μl.

Primary Adipose Cell Culture

The rat adipose tissue removed and minced as described above is digested with collagenase (type I Worthington Biochemical) in KRBH buffer containing 0.1 % BSA at 2 mg/ml. Adipocytes are separated from the stromal-vascular cells by centrifugation at 1,200× g for 10 min. The floating isolated adipocytes are washed by centrifugation at 1,200× g for 2 min twice with KRBH buffer containing 0.1 % BSA and twice with serum-free BSA-free DMEM medium. The washed cells are cultured at a density of 0.5 × 105/ml in serum-free BSA-free DMEM at 37 °C for 48 h in 5 % CO_2. The conditioned medium is collected and centrifuged at 1,200× g for 10 min to separate cell debris. The supernatant medium is filtered through a 0.45-μm syringe-driven filter to remove the remaining adipocytes. The medium is desalted and concentrated using a Macrosep centrifugal device with a molecular weight cutoff of 1 kDa

(Pall Life Sciences). The protein concentration is determined using a Pierce BCA protein assay and should finally result in approximately 650 ng/µl.

HPLC Separation of Intact Proteins

One hundred microliters of concentrated extracts of the culture medium is injected into a Zorbax 300SB-C3 reversed-phase column (150 × 4.6 mm ID, 5 um) equipped with a guard column (C3 reversed phase) of the HPLC system (Agilent Technologies HP1100 system consisting of a quaternary pump, degasser, autosampler, and UV detector and connected with HP Chemstation for data acquisition). The HPLC is performed using solvent A (0.1 % (v/v) trifluoroacetic acid, 99 % glacial acetic acid) and a gradient of 5–40 % solvent B (acetonitrile, LC/MS quality) within 55 min at a flow rate of 700 µl/min. Fluorescence is measured with the UV detector at 254 nm. Eight fractions are collected in total and dried in a SpeedVac to remove excess solvents, finally resulting in a concentration of approximately 650 ng/µl.

Digestion with Lys-C and Trypsin

The dried fractions are dissolved in 20 µl of 5 M urea containing 5-mM dithiothreitol (DTT). Alkylation is initiated by the addition of 5 µl of 50-mM iodoacetamide (prepared in water). Thereafter, 0.5 µg of endoproteinase Lys-C (Roche Biochemicals) is added. After dilution with water resulting in a final concentration of 2-M urea, the digestion is continued in the presence of 1 µg of trypsin (modified sequencing grade, Roche Biochemicals).

18O Labeling Procedure

Aliquots of the tryptic peptides from basal and drug-treated samples are evaporated to dryness and then redissolved in H216O and H218O, respectively (Stewart et al. 2001; Mirgorodskaya et al. 2000; Yao et al. 2001; Reynolds et al. 2002; Krijgsveld et al. 2003; Gygi et al. 1999). It is important not to precipitate the proteins during the fractionation and evaporation of the solvent and to avoid full evaporation to complete dryness. Immobilized trypsin is added to basal (H216O) and drug-treated (H218O) samples. The samples are incubated in the presence of 0.1-M ammonium carbonate overnight. The above steps are repeated three times. To force the equilibrium toward the double label, two strategies are used by removing an excess of 16O-H2O and replacing it with 18O-H2O and the use of immobilized trypsin. After a 16-h period in total, the labeling procedure is terminated. Aliquots of untreated unlabeled control peptides and 18O-labeled treated peptides are mixed at a 1:1 ratio and subjected to LC/MS analysis.

Electrospray Mass Spectrometry

The samples are introduced with an autosampler into the electrospray mass spectrometry instrumentation (Hybrid LTQFT mass spectrometer, Thermo Fisher, equipped with a Waters CapLC, Waters) and concentrated on a Waters Symmetry300 C18 5-µm trap column. Other general settings are spray voltage of 3.0 kV, capillary temperature 100 °C, normalized collision energy using wide-band activation mode, 35 % for MS2, ion selection thresholds of 1,000 counts for MS2, activation q = 0.25, and activation time of 30 ms applied in MS2 acquisitions. After a 5-min delay, the flow is directed onto a Microtech Scientific C18 column (100 mm × 150 µm ID, 5 um). After preparation of solvent A (0.2 % formic acid, 1 % acetonitrile, and 98.8 % water) and solvent B (0.2 % formic acid, 1 % water, and 98.8 % acetonitrile), a gradient is run, ramped from 5 % solvent B to 95 % solvent B within 175 min at a flow rate 6 µl/min and a split ratio of 1:10. For protein identification, MS/MS data in a data-dependent mode are used to switch automatically between MS and MS/MS mode. Full spectra (m/z 400–20,000) are acquired in the FTICR cell with R = 25,000. Using collisionally induced dissociation with helium gas, the ten most abundant ions are subjected to MS/MS fragmentation. For protein quantification, MS data are used.

Database Searching

Data are processed using Bioworks to create .dta files (data files), which are merged to merged .mrg files. The program Bioworks comes with a version of Xpress that can be used for automatic

interpretation of the data. More sophisticated software such as MSQuantification (Schulz and Mann 2004) or ProRata (Pan et al. 2006) can be used by more advanced users. The .mrg files are used for subsequent database searching using a MASCOT search engine. The following parameters are used: MSDB; 1 missed cuts; iodoacetamidation of cysteines; deamidation; and charge states +2, +3, and +4. A window is chosen of 10-ppm mass accuracy for precursor ions and 0.6-Da mass accuracy for MS/MS data. The probability-based MASCOT scores are considered as significant when greater than the cutoff score that indicates either identity or homology ($p < 0.05$) for individual ions. One peptide hit sequence assignment is confirmed by de novo sequencing. All proteins are checked to meet the criteria set by SignalP predictions to increase the confidence in the assignment as secretory proteins and reduce the risk of contamination from cellular material. The SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP) is used to predict the presence and location of signal peptide cleavage sites in amino acid sequences of proteins. The S-score, C-score, Y-max, S-mean, and D-score for the signal peptide prediction, the hidden Markov model calculation for signal peptide probability, and the eukaryotic HMM model calculation for signal anchor probability are all evaluated for amino acid sequences (www.matrixscience.com). The proteins with five high scores and high signal peptide probability are considered as secreted proteins. Scaffold (www.proteomesoftware.com) is used to parse data from several runs, and either manual interpretation or software is used to quantify individual peptides. Tryptic peptides are used as internal standards. Their ratio must be 1:1. It is also recommended to use only doubly charged ions for the manual interpretation. Due to the overlap of the isotopes from triply charged ions, interpretation may otherwise lead to misinterpretation.

Evaluation

Determination of the complex secretory proteome of adipocytes and its metabolic changes induced by drug treatment such as insulin or rosiglitazone is possible with the advanced proteomics technologies described herein. To study the secreted proteins of adipocytes, a 2D liquid chromatography/mass spectrometry/mass spectrometry protocol has been established. With the use of reversed-phase high-performance liquid chromatography, intact proteins are separated in the first dimension into eight fractions and then digested with Lys-C and trypsin. Comparative differences after drug treatment are assessed using 18O proteolytic labeling strategies. With the advent of more and more sophisticated instrumentation and data analysis tools, protocols like this one will likely become standard tools for scientists in the research field of endocrinology, obesity, and diabetes. These protocols enable researchers to study the dynamic drug-induced changes in a comprehensive and systematic manner that was inconceivable just a few years ago.

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RNA Isolation and Real-Time Quantitative RT-PCR

Purpose and Rationale

Accumulating evidence suggests that white adipose tissue (WAT) plays a key role in the pathogenesis of obesity and its associated metabolic disorders via the production and release of a wide variety of hormones, cytokines, growth factors, and enzymes (Trayhurn and Beattie 2001; Kershaw and Flier 2004; Laclaustra et al. 2007; Frühbeck 2008). They are collectively known as adipokines, the majority of which are produced by adipocytes. The lipid-filled cells are the main constituents of WAT. Therefore, the study of gene expression patterns of adipokines in adipocytes and/or WAT under normal/basal and various treatments (e.g., drugs, hormones, and dietary manipulations) as well as pathological conditions (e.g., type II diabetes) has taken the center stage during the last decade.

Traditionally, levels of gene expression (i.e., of mRNA) in tissues/cells of interest have been determined with northern blot analysis, RNase protection assays, solution hybridization, and/or

in situ hybridization techniques. All of these assays are time-consuming and labor-intensive. Furthermore, with the exception of in situ hybridization, they all require relatively large quantities (at least 10 μg) of total or poly A+ RNA. With the advent of polymerase chain reaction (PCR) technology, semiquantitative reverse transcription PCR (RT-PCR) protocols have been developed to assess mRNA levels in samples as small as individual cells (Rappolee et al. 1989; Brady and Iscove 1993; Steuerwald et al. 1999). More recently, real-time PCR has revolutionized the way by which DNA and RNA levels are quantified, because of the ability to monitor the PCR product as it occurs in real time, rather than at the end of the PCR (i.e., conventional PCR)(Higuchi et al. 1993; Heid et al. 1996). There are numerous reviews and online resources that describe in detail the theories, the principles, and the applications of real-time PCR. The focus of this chapter deals with the practical aspects of real-time PCR as it applies to the detection of mRNAs, known as real-time quantitative RT-PCR (qRT-PCR), which is the most reliable and sensitive method for mRNA quantitation.

The next step in designing and conducting qRT-PCR is the choice of quantitation method. The quantitation of mRN can be achieved by a one-step or two-step RT-PCR. The major drawback of a one-step RT-PCR is the need to use fresh RNA sample and conduct RT-PCR for every target gene. As a consequence, a two-step RT-PCR protocol is presented in this chapter, exclusively. When calculating the results of quantitation assays, either absolute or relative quantitation may be used. The absolute quantitation assay is used to determine the absolute amount of the target mRNA by interpolating its quantity from a standard curve. However, the absolute quantities of the standards must first be known by independent means. In contrast, a relative quantitation assay is used to analyze changes in gene expression (i.e., mRNA levels) in a given sample relative to a reference sample (e.g., an untreated control sample). Given that it is often unnecessary to know the absolute amount of a target mRNA, relative quantitation assays are preferred and described here.

Total RNA Isolation and Purification from White Adipose Tissue and Primary Adipocytes

Purpose and Rationale

As its name implies, qRT-PCR involves two major steps: RT and real-time PCR. Similar to other mRNA detection methods, the success of qRT-PCR relies primarily on the quality (i.e., purity) of total RNA (Fleige and Pfaffl 2006), which must be free from genomic DNA contamination. Although there are numerous in-house protocols as well as commercial kits for isolation of total cellular RNA from tissues/cells of interest, the unique nature (i.e., extremely high lipid content) of WAT and adipocytes requires specially developed reagents, protocols, and kits. Here, a protocol is presented that makes use of conventional RNA extraction reagents and RNA isolation kits, which has given us consistently high yield and high purity total RNA (Guan et al. 2005). In addition, this protocol also eliminates the need to purchase additional reagents and kits that have been developed specifically for lipid-rich tissues/cells.

Procedures

White Adipose Tissue

Three hundred milligrams of fresh or frozen rat white adipose tissue (WAT) is supplemented with 1 ml of TRIzol® Reagent (Invitrogen, cat. no. 12183-555) in a sterile tube. Successful RNA isolation requires fast processing and careful handling of the tissues or cells before isolation. Endogenous RNases are released from cellular compartments immediately after harvesting the tissue. It is essential to inactivate these RNases as soon as possible to prevent RNA degradation. Therefore, the samples have either to be homogenized immediately after harvesting or to be flash-frozen in liquid nitrogen and stored at −80 °C. To prevent RNA degradation, it is important that the tissue is cut into small pieces to allow rapid and thorough freezing of the entire tissue. RNA isolation from WAT or mature adipocytes using TRIzol or TRIzol LS® reagents followed by purification with silica-based columns enables relatively high

yield of pure total RNA. Using this protocol, 60–80 μg of total RNA is typically obtained from 300 mg of rat WAT. The tissue is homogenized with a Polytron at medium speed for 15 s and then incubated at 22 °C for 5 min to allow complete dissociation of nucleoprotein complexes. The tissue homogenizer is cleaned off with large volumes of water before and after each use to avoid cross-contamination. The homogenate is centrifuged at 12,000× g for 10 min at 4 °C resulting in three layers, (i) top colorless layer, (ii) middle light pink-colored solution, and (iii) bottom layer and pellet containing extracellular membranes, cell debris, polysaccharides, and high-molecular-weight DNA, which should be discarded. Consequently, the top layer of excess lipid is removed and discarded. The light pink-colored solution containing the RNA is transferred into a fresh tube, leaving behind the pellet. After addition of 0.2 ml of chloroform and sealing of the tubes, they are vigorously shaken by hand for 15 s and then incubated at 22 °C for 3 min. Vortexing should be avoided in order to minimize DNA contamination of the RNA sample. Following centrifugation at 12,000× g for 15 min at 4 °C, the solution separates into a lower red phenol-chloroform phase, an interphase, and the colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase, whereas DNA and proteins are in the interphase and organic phase, respectively. The volume of the aqueous phase is about 60 % of the volume of the TRIzol reagent used for homogenization. The colorless upper aqueous phase containing the RNA is collected and transferred into a new tube. After addition of an equal volume of 70 % ethanol to the aqueous phase resulting in a final ethanol concentration of 35 %, the mixture is thoroughly mixed by vortexing and warmed to 22 °C. 700 μl of the mixture is transferred to a spin column supplied with the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma, cat. no. RTN-70) or other commercially available mini RNA isolation kits (e.g., Invitrogen or Qiagen). The spin column is centrifuged at 12,000× g for 15 s and the resulting flow through discarded. The same procedure is performed with the remaining RNA-containing mixture using the same spin column. Following addition of 250 μl of Wash Solution I to the column, centrifugation of the column at 12,000× g for 15 s, and removal of the flow through, the genomic DNA is degraded by addition of 80 μl of DNase I working solution (one part of DNase I stock solution, consisting of DNase I supplied with RNase-free DNase Set from Qiagen cat. no. 79254 and dissolved in 550 μl of RNase-free H_2O and stored at 4 °C for up to 6 weeks or at −20 °C in aliquots for up to 9 months without refreezing after thawing, to seven parts of buffer RDD supplied with the RNase-free DNase Set and mixed by gently inverting the tube without vortexing since DNase I is sensitive to physical denaturation) to each column. After incubation at 22 °C for 15 min, the column is washed once with 250 μl of Wash Solution 1 and then transferred to a new collection tube. Following two washes with 500 μl of Wash Solution 2 supplemented with ethanol according to the manufacturer's instructions and centrifugation at 12,000× g for 15 s, the flow through is discarded and the empty column re-centrifuged at 12,000× g for 2 min for removal of traces of ethanol, which must be eliminated since ethanol may inhibit downstream enzymatic reactions. The column is transferred to a new collection tube and then eluted for RNA by adding 80–100 μl of prewarmed elution solution (50–60 °C) to the center of the column membrane. To maximize RNA recovery, the tube containing the column is gently vortexed and incubated in a dry heating block at 50–60 °C for 3 min. Vortexing will spread the elution solution evenly on the membrane, and the incubation at 50–60 °C will significantly increase the RNA yield. If smaller amounts of fat tissues or adipocytes are used, the RNA elution volume has to be decreased to 40 μl or the RNA to be re-eluted using the same elution solution. After centrifugation at 12,000× g for 2 min, the eluted RNA is kept on ice, and the next steps are performed quickly before storing the RNA at −80 °C. For short-term storage, RNA can be stored at −20 °C; for long-term storage, it should be stored at −80 °C. Although RNA can be stored in H2Obidest or buffer, it is more stable in 70 % ethanol at −80 °C. It is recommended to store

RNA samples in aliquots to prevent damage to the RNA from successive freeze-thaw cycles and to reduce the risk of introducing RNases into the tube. The concentration and integrity of purified total RNA are assessed by UV absorbance spectrophotometry and standard agarose gel electrophoresis, respectively. A relatively pure RNA sample should yield an O.D. ratio of 1.80–2.0 at 260–280 nm. Samples diluted in H_2Obidest may give lower A260/A280 O.D. ratios. The use of 10-mM Tris/HCl (pH 7.4) is recommended for dilution of RNA samples for O.D. measurements. Before gene expression analysis, RNA integrity should be checked by standard agarose gel electrophoresis by running of 0.5–1 μg of total RNA on a 0.8 % agarose gel containing ethidium bromide. Intact total RNA will display two distinct and sharp bands representing 28S and 18S rRNA. The 28S rRNA band should be approximately twice as intense as the 18S rRNA. This 2:1 ratio of 28S/18S rRNA is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance and will lack the sharp rRNA bands or will have a lower 28S/18S rRNA ratio. Highly degraded RNA will appear as a very-low-molecular-weight smear.

White Primary Adipocytes

0.75 ml of TRIzol LS® reagent (Invitrogen, cat. no. 10296-010) is added to 0.25 ml of the sample containing freshly isolated or frozen mature primary white adipocytes from rats or mice. If the sample volume is less than 0.25 ml, the volume is adjusted to 0.25 ml with RNase-free H_2Obidest. If it is greater than 0.25 ml, the reagent is scaled up proportionally (the volume ratio of TRIzol LS reagent to the sample should always be 3:1). The cells are completely lysed by mixing several times with a pipette and incubating the lysate at 22 °C for 5 min to permit the complete dissociation of nucleoprotein complexes. Thereafter, the lysates are centrifuged at 12,000× g for 10 min at 4 °C. After removal of the top layer containing the excess lipid, the light pink-colored solution containing the RNA is transferred to a fresh tube, leaving behind the pellet. The subsequent steps are identical with those for RNA isolation from white adipose tissue pieces as described above.

Reverse Transcription

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat. no. 4368813) containing all components necessary for the quantitative conversion of up to 2 μg of total RNA in a 20-μl reaction volume to single-stranded cDNA is used. RT Master Mix (2×) (for 100 reactions) is prepared by mixing 420 μl of RNase-free H_2Obidest, 200 μl of 10× RT buffer, 200 μl of 10× random primers, 80 μl of 25 × 100-mM dNTP mix, and 100 μl of MultiScribe™ Reverse Transcriptase and stored in aliquots at −20 °C. Although oligo-dT primers can also be used for RT, the use of random primers is recommended, since this will allow the freedom of choosing whatever internal control one wishes to utilize in subsequent real-time PCR assays, including 18S rRNa and 28S rRNA, as well as other housekeeping genes, such as GAPDH, ß-actin, and α-tubulin. The reverse transcriptase is substituted with RNase-free H_2Obidest in preparing the negative control Master Mix. For every batch of RT reactions, one negative control (-RT) must be included. Up to 2 μg of total RNA is prepared in 10 μl of RNase-free H2Obidest in a sterile 0.5-ml thin-wall PCR tube. After addition of 10 μl of 2× RT Master Mix (a total of 20 μl of reaction volume) and gently mixing and briefly spinning, the tubes are placed in a thermal cycler for running of the RT reactions with the following program (based on the manufacturer's instructions) of 10 min at 25 °C, 2 h at 37 °C, and 5 s at 85 °C. The RT reactions are stored at −20 °C.

Real-Time PCR

TaqMan Assays

Optimal concentrations of primers and probe are determined following the guidelines developed for sequence detection systems by Applied Biosystems. The optimal concentrations of primers for housekeeping genes and genes of interest are between 50 and 150 nM and 200 and 300 nM, respectively. Consequently, the evaluation of the optimal concentrations of primers between 50 and 300 nM and 150 and 450 nM

for housekeeping genes and genes of interest, respectively, is recommended. When optimizing primers for SYBR Green I assays, it is also crucial to analyze the melting curve data for each primer concentration pair to ensure a single homogeneous product is being generated. If several primer combinations give very similar results, the primer combination with the lowest concentration is preferred to avoid primer dimer formation. Probe optimization is similar to primer optimization with selection of the probe and primer combination that results in the lowest CT value and the highest delta Rn value. Negative controls are set up with two different negative controls for each assay, one as no-template control (NTC) and the other as no-reverse transcriptase control (NRTC). At least two negative controls (NTC and NRTC) are used for quality control purposes. Ideally, signal amplification should not be observed in the NTC wells, and when observed, CT values should be at least five and preferably more than ten cycles from the CT values of the least concentrated samples. NRTC serves as an indicator of genomic DNA contamination, and their CT values should be at least five cycles more than those of the least concentrated samples. Another negative control to consider is NAC (i.e., no amplification control), which includes all the PCR components except for the DNA polymerase. This is useful if it is suspected that an increase in fluorescence may be observed in the reaction that is not due to actual amplification (e.g., in case the probe is degrading). Appropriate common reference or housekeeping genes are selected, such as GAPDH, ß-actin, α-tubulin, 28S rRNA, and 18S rRNA. Although using the same amount and quality of input RNA in each sample ensures that equivalent amounts of RNA are compared, it cannot compensate for variations in the efficacy of reverse transcription, which is required to produce cDNA for subsequent PCR. Therefore, it is imperative that researchers normalize expression levels of genes of interest to that of a reference gene (Thellin et al. 1999; Vandesompele et al. 2002). This step can remove inaccuracies due to variations in reverse transcription efficacy since RNA of the reference gene is reverse transcribed along with that of the gene of interest. Housekeeping genes, such as GAPDH, α-tubulin, β-tubulin, cyclophilin, 28S rRNA, and 18S rRNA, have often been used as reference genes for normalization (Suzuki et al. 2000), with the assumption that the expression of these genes is constitutively high and that a given treatment will have little effect on their expression. However, this assumption must be validated empirically, as the expression of housekeeping genes can vary under certain conditions (Zhong and Simons 1999; Schmittgen and Zakrajsek 2000; Murphy et al. 2003). In any case, it is crucial to select a reference or even multiple reference genes whose expression has been empirically tested to be constant across all experimental conditions in the study. Thereafter, standard curves have to be constructed using the known starting concentration of template from one of a variety of sources and performing a dilution series. The standard curve should consist of at least four dilutions. The samples for constructing standard curves are chosen based on their anticipated levels of mRNA. Ideal samples should contain higher levels of the mRNA. If it is not possible to make predictions, a small portion of cDNA from a few samples may be pooled or cDNA from untreated control may be used. Alternatively, a separate RT reaction may be set up specifically for standard curves. The dilution series should encompass a large range of concentrations, ideally covering the expected levels of target in experimental samples. To accomplish this objective, a three- to tenfold dilution series over several orders of magnitude should be generated. For instance, a typical serial dilution would consist of five points of a fivefold serial dilution, starting with 100 ng of total RNA per reaction (or the cDNA equivalent amount).

The PCR Master Mix (without cDNA templates; TaqMan Universal PCR Master Mix 2x; Applied Biosystems, cat. no. 4304437) is prepared for a 20-µl reaction volume per well in triplicate resulting in a total volume of 60 µl per sample for 384-well real-time PCR plates (384-well Clear Optical Reaction Plates; Applied Biosystems cat. no. 4309849) and optical adhesive covers (Applied Biosystems cat. no. 4311971). Assuming that 1.0 µl of RT

products will be used per well, the total volume of PCR Master Mix for each sample will be 57 µl. The amount of cDNA templates (i.e., RT products) to be used in real-time PCR should be determined according to their expected CT values, which can be estimated from those obtained during primer optimization. As a general rule, more RT should be used for lower-abundance mRNAs. However, the volume of RT must not exceed 10 % of the final PCR volume, since greater than 10 % RT will inhibit PCR. The following example assumes that 20 unknown samples are to be processed, one NTC, one NRTC, four standard curve samples, and one extra reaction volume to accommodate reagent losses during pipetting (for a total of 27 samples). The solutions are pipetted into a sterile 2.0-ml microfuge tube by mixing 21 µl × 27 = 567 µl of H2Obidest, 30 µl × 27 = 810 µl of TaqMan PCR Master Mix, 3 µl × 27 = 81 µl of TaqMan probe, and 3 µl × 27 = 81 µl of primer mix and subsequent gently mixing by vortexing and keeping on ice. After the optimal concentration of the probe has been determined, its concentrations have to be adjusted such that 1 µl of probe will be required per 20 µl of PCR. After the optimal concentrations of the primers have been determined, a ready-to-use primer mix is prepared containing both sense and antisense primers at a concentration (according to their predetermined optimal concentrations and diluted with appropriate volumes of sterile H2Obidest) such that 1 µl will be required per 20 ul of PCR. Primer Express 2.0 (Applied Biosystems) can be used to design probes and primers for both probe- and dye-based real-time PCRs. When designing primers for SYBR Green I assays (see below), all the default settings should be kept except for the amplicon requirements under which the minimum length should be increased from 50 to 100 and the maximum length from 150 to 200. The rationale is that a larger amplicon size (i.e., PCR product) makes subsequent sequencing and/or restriction enzyme digestion analysis easier. For first-time use, an Optical Adhesive Starter Kit should be ordered because it includes one compression pad and one applicator in addition to 20 optical adhesive covers. Although the compression pad is not needed with the real-time PCR machine, ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), the applicator is required regardless of the machine type. Thereafter, each PCR is prepared in a sterile 1.5-ml microfuge tube by adding 57 µl of the PCR Master Mix into the tube containing 3 µl of RT products and mixing well by vortexing and briefly spinning. 19 µl of each PCR is loaded into triplicate wells on a 384-well plate, which is done for all samples. The wells are immediately covered completely by placing one piece of the Optical Adhesive Cover onto the plate according to the manufacturer's instructions. Following centrifugation of the plate at 4,000× g for 2 min, the plate is run on an ABI PRISM 7900HT Sequence Detection System immediately or stored at −20 °C for up to 2 weeks. PCRs are run with a standard program encompassing incubation at 50 °C for 2 min, activation at 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension (detection) at 72 °C for 1 min. At the end of PCR, Analyze from the Analysis menu is selected, and the semilog view of the amplification plots is examined. Then, the default baseline setting is adjusted to accommodate the earliest amplification plot, and a threshold is selected above the noise close to the baseline, but still in the linear region of the semilog plot. Upon completion of the analysis, the data are exported to Excel for further analysis. The slope and Rsq values of the standard curve help to determine the sensitivity of a given assay. PCR cycles that generate a linear fit with a slope between about −3.1 and −3.6 are considered acceptable. The linearity is denoted by the R squared (Rsq) value, which should be very close to 1 (>0.985). Another quality indicator of your assay is that there should be a difference of approximately 3.3 in CT values between two standard curve points with a tenfold dilution.

SYBR Green I Assay

The procedures for SYBR Green I assays are identical to the TaqMan probe assay as described except that (i) a dye-based PCR Master Mix, SYBR Green I (Platinum SYBR Green qPCR SuperMix-UDG with ROX (2×); Invitrogen, cat. no. 11744; SYBR Green Master Mix from other

companies including Qiagen and Applied Biosystems are also appropriate), instead of TaqMan PCR Master Mix, is used; (ii) sterile H2Obidest is used to make up the volume difference when preparing the SYBR Green I PCR Master Mix instead of the TaqMan probe; and (iii) a melting (dissociation) curve and sequence (and/or restriction enzyme digestion) analyses are performed at the end of the PCR. Because SYBR Green I will bind to any double-stranded DNA, nonspecific amplifications in unknown wells will artificially increase the fluorescence signal and make it impossible to accurately quantitate these samples. In addition, to facilitate post-PCR analyses, such as sequencing and restriction enzyme digestions, a 5-min extension at 72 °C at the end of the PCR program is added. Most PCR products will melt somewhere in the range of 80–90 °C, although this melting point can vary with the size and sequence of the specific target. Ideally, the experimental samples should yield a single sharp peak within this temperature range, and the melting temperature should be the same in all the reactions. Furthermore, both NTC and NRTC should not generate significant fluorescent signals. If the dissociation curve reveals a series of peaks, it indicates that there is not enough discrimination between specific and nonspecific reaction products, which would render optimization of the qRT-PCR necessary. Finally, the validity of your SYBR Green I assay should be verified by sequence and/or restriction enzyme digestion analysis of the PCR product.

Calculation

For real-time PCR, both the probe (i.e., TaqMan probe)- and dye-based (i.e., SYBR Green I dye) PCR protocols are described. The primary advantage of the probe method is that the PCR specificity is guaranteed (i.e., there is no need to perform post-PCR analysis to verify that there are no nonspecific PCR products), because specific hybridization between probe and target is required to generate fluorescent signal. The major disadvantage is the requirement for the synthesis of different probes for different target sequences. However, the major advantage of the dye-base method is the reduced cost, especially when running multiple assays for multiple mRNAs, because no probes are required. However, the main disadvantage is that it may generate false-positive signals because the dye binds to any double-stranded DNA (e.g., both the target and nonspecific sequences). Consequently, post-PCR analysis (e.g., melting curve and sequencing of the PCR product) must be performed to validate the PCR specificity. In conclusion, both methods have distinct advantages and disadvantages, and it will be the decision of the researchers as to which one is best suited for their particular study.

There are two calculation methods used for relative quantitation: standard curve and comparative CT (threshold cycle number). Although these two methods give equivalent results, the standard curve method is routinely used since it requires the least amount of optimization and validation. The target and reference (i.e., housekeeping gene) amplifications are routinely run in separate wells using their respective standard curves for the following two reasons. (i) To use the comparative CT method, a validation experiment must be performed to show that the efficacies of the target and reference amplifications are roughly equal, a prerequisite that can be difficult, if not impossible, to meet. (ii) To amplify the target and gene reference genes in the same tube, limiting primer concentrations must be identified and shown not to affect the CT values. However, one caveat of using the standard curve method is the need to construct a standard curve for every assay and curve sample.

Evaluation

Adipose tissue has emerged as a major endocrine organ producing a wide spectrum of hormones and factors that play crucial roles in regulating cell turnover and function, not only locally within the adipose tissue but also in the brain and other key metabolic organ systems. It is known that gene activity is controlled at both transcriptional and posttranscriptional levels. Consequently, one of the most important means by which the activity of a gene is assessed is through the determination of levels of the corresponding mRNA. This process involves the isolation of total cellular RNA and subsequent analysis of the mRNA of interest.

Given the unique nature of adipose tissue and adipocytes (i.e., the high contents of lipid), special RNA isolation techniques that have been tested in both white adipose tissue and isolated mature adipocytes from rats and mice will be presented. Although several methods are available for mRNA quantitation, the real-time quantitative reverse transcription polymerase chain reaction protocol is the method of choice due to its superior sensitivity and reliability.

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Assays for Insulin and Insulin-Like Regulation of Gene and Protein Expression

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Contents

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Part of the blood glucose-lowering activity of insulin is based on massive and specific up- or downregulation of the gene expression for proteins/enzymes which directly regulate carbohydrate and lipid metabolism (e.g., GLUT4, PEPCK). Furthermore, changes in the intermediary metabolism provoked by insulin may secondarily lead to alterations in the gene/protein expression of other signaling/metabolic pathways. Consequently, it is important for the identification as well as characterization of compounds/drug candidates with insulin-like activity to analyze their effect on the expression of gene and proteins. For practical reasons, this analysis can be restricted to those genes/proteins which according to the present knowledge are relevant for mode of action of the compound/drug candidate. However, undoubtedly whole genome or proteome searches for changes in the expression levels have important advantages, in particular due to its unbiased nature. However, they require considerable experimental expenditure and time on basis of conventional technology. This may change completely with the successful introduction of protein chips and DNA microarrays for the study of protein and gene expression and further increase its attractiveness for routine application in characterization of novel compounds/drug candidates.

Protein Chips

General Considerations

Currently, mapping the proteome and understanding the roles of these proteins are still in the very early stages, but scientists are hopeful that individual proteins, as well as constellations or "fingerprints" made up of multiple proteins (even if those proteins' functions are unknown), will soon be used regularly for the characterization of compounds/drug candidates in drug discovery programs, in addition to application in the characterization of the probands for clinical trials regarding both desired and undesired effects of drug candidate envisioned. Indeed, the promise of these tests is so significant that many companies are already using such markers to select the most appropriate patients for clinical trials and to choose better drug candidates. Meanwhile, pharmaceutical and biotechnology companies have become acutely aware of the value of biomarkers for identifying those patients who may disproportionately benefit from a drug and those at highest risk of experiencing serious adverse effects. Biomarkers are regarded as a vital tool in streamlining drug discovery and development and accelerating approvals.

Proteins are the workhorses of the cell. Although genes represent the blueprint of life, proteins are the molecules that actually carry out the gene's instructions. In addition, because proteins are not confined to the cell that created them (as is mRNA) but float throughout the bloodstream, they are easier to find and measure. During the pre- and post-genome-sequencing euphoria of the late 1990s and early 2000, many researchers declared that the proteasome was the next frontier.

Challenges for Development

A major obstacle in protein chip development is the fact that proteins greatly outnumber genes. A single gene can code for multiple proteins, and each protein can undergo modifications that substantially change its activity. As a result, potentially several hundred thousand proteins can be required for the estimated 30,000 genes to do their work in the human body. This scenario helps explain the complexity of the human body despite the fact that it has relatively few genes compared with lower organisms. It also makes it much harder for scientists to completely map the proteasome. In addition, in contrast to the strand-like construction of mRNA and DNA, proteins have extremely complex structures. Within each protein's convoluted architecture, there are certain "active" sites that make the most difference in terms of the protein's activity. (It is in these sites that other molecules, including drugs, exert their activity.) When they are placed on a substrate, such as a chip, proteins may unfold, becoming inactive and useless for study.

In terms of the technology, the traditional method of using 2-D gels for measuring the protein expression in a clinical or experimental sample is tedious and cumbersome. Initially, there was great interest in finding ways to industrialize this approach by using mass spectrometry and automated systems. With the arrival of DNA microarrays and their success in the RNA measurement field, the dawn of "proteome-wide chips" seemed imminent. But the technological hurdles facing the field have not diminished substantially, and throughput in proteomics is still far behind what is achievable in areas such as gene sequencing and gene expression analysis.

As a result of the aforementioned challenges, many groups have taken a tip from the surging interest in gene-expression-profiling patterns. To develop such "signatures" of disease, researchers realized that they need only capture several proteins accurately and reproducibly. The protein marker signature does not need to be understandable. It just needs to be robust. Thus, interest in proteomics has now switched from being able to accurately quantify and identify all of the proteins in a particular sample to being able to distinguish "fingerprints" that are sufficient to separate one sample (e.g., progressive disease) from another (e.g., mild disease).

Technologies

Protein chips are mainly used to either identify protein-protein interactions or to find proteins in a sample. The former type of chip is primarily a drug discovery and development tool, while the latter type can be used either in drug discovery and development or in diagnostic development. The type of molecule used to capture the proteins is the fundamental difference between chips for research purposes, which can be used to find biomarkers, and those chips that will be used to diagnose disease or predict outcomes. When searching for protein biomarker signatures, one must be sure they are examining a wide range of proteins and accurately measuring the relative levels of all of these proteins. Once the markers have been identified, one can then concentrate on a particular subset of proteins and find the ideal binding molecules for use in a diagnostic system. Therefore, the key intermediate step is to locate the important marker proteins among the several hundred thousand proteins present in the body. A variety of molecules can be used to bind proteins, but antibodies are most often used. Primary antibodies are used to capture the protein (antigen) which is recognized by secondary antibodies (not sharing the antigenic determinant with the primary antibody but often labeled with biotin) and some type of readout (e.g., Cy5-labeled streptavidin) signal presence of the protein (antigen). To build such arrays, each antibody placed on the chip (often in the wells of a microtiter plate) must be generated and then thoroughly tested for its cross-reactivity and binding affinity. Because proteins can share domains, or certain common structures, cross-reactivity can cause serious problems. Determining that a protein is cross-reactive requires extensive screening against other proteins. In addition, it is very difficult to get antibodies that bind tightly to a particular protein. Haab (2001) showed that as few as 20 % of antibodies out of hundreds could provide specific and accurate measurements of matching proteins on a chip. Antibodies can be exquisitely specific, however, and researchers are only now beginning to learn some general rules on how to design good antibodies for proteins microarrays. A certain number of antibodies are already very well characterized. The pharmaceutical industry has been heavily reliant on these identified antibodies. The technology for making protein chips has also advanced, largely through the development of new methods to capture the proteins without degrading them.

New approaches to protein microarrays are also emerging. For example, mass spectrometry has recently been demonstrated to be useful for the selection of certain proteins from a mixture and then to "soft land" these proteins onto a microarray while retaining their shape and activity to a very high degree. Meanwhile, a bench-top system of arrays of silicon-nitride microcantilevers with customizable surfaces has been introduced to detect interactions between proteins, antigens, and DNA. This system does not require

labeling because the microcantilevers bend due to the interaction between two molecules. Furthermore, a digital proteome chip has been developed for a comprehensive protein expression analysis. The technology is predicted of being unusually rapid (a single sample can be processed in 1 h or less) and has unprecedented sensitivity, reproducibility, and accuracy. Very recently, isothermal rolling-circle amplification technology (RCAT) has been linked to antibodies as a means of signal amplification on traditional sandwich immunoassay-based protein chips. This detection method may be particularly useful because it remains localized and can detect protein analytes with zeptomole sensitivity and across a very broad dynamic range. Protein microarrays for interaction studies and other functional genomics studies will be most useful for drug discovery and development. By using a parallel procedure for protein production and purification technology, it is assumed to generate and purify thousands of different proteins for the generation of the most diverse "portfolio of functional protein content." A comparative evaluation of selected suppliers of protein chip technology including their applications reported so far has been provided by Branca (2004).

DNA Microarrays

Use for Drug Discovery

Purpose and Rationale

Changes and improvement in microarray technology have enhanced the ability to analyze transcription-factor-binding events genome-wide. Early versions of microarrays used for location analysis were manufactured by amplifying the promoter regions of genes from genomic DNA using PCR and spotting the amplicons on glass slides. In addition to being reasonable accessible and affordable, PCR-generated probes could cover the entire proximal promoter region of a gene with a single probe. But these arrays had limitations. Double-stranded PCR products are less effective hybridization probes than single-stranded oligomers. PCR-generated probes are more susceptible to variations in quality due to the processing required to generate them, and with only one probe reporting for each promoter, there is a relatively high rate of error associated with bad probes. Finally, the binding may occur anywhere within the probe sequence, which may be as long as 1 or 2 kB.

Procedure

Emerging microarray applications such as aCGH (array-based comparative genomic hybridization), SNP (single nucleotide polymorphism) analysis, location analysis (ChIP, on-chip-chromatin immunoprecipitation on a microarray), methylation analysis, splice variant analysis, and microRNA studies are all growing faster even than the already popular microarray application of gene expression. Inkjet-based array manufacturing processes provide the flexibility, long probe lengths, and density required by these applications. The use of ChIP and microarray technology has proved a powerful combination for analyzing transcriptional regulation (for a review, see Lee and Volkert 2006). One of the primary advantages of ChIP is that DNA-protein interactions are first fixed by the addition of a cross-linking agent to living cells, thus allowing for the identification of dynamic in vivo interactions between transcriptions factors and the genome. However, this approach has been relatively limited for exploring DNA-protein interactions across the genome as detection of the enrichment on this scale can be difficult. Enrichment is often detected by the use of PCR-based assays, which are easy to perform and highly informative, but detection is limited because it is difficult to perform these assays in a sufficiently high-throughput format for genome analysis. Alternative approaches, such as cloning and sequencing of enriched DNA or direct sequencing of enriched DNA, have also been used but are relatively labor intensive and depend on sequencing large numbers of fragments to approach complete genome coverage. Using microarrays for the identification of enriched DNA regions has circumvented some of the difficulties in detecting enrichment genome-wide. Immuno-enriched DNA can be labeled, hybridized to arrays, and

compared to control DNA that has been hybridized at the same time. As arrays can be designed to probe all known promoter regions (Boyer et al. 2005) or even the entire genome (Kim et al. 2005), this ensures complete coverage of the genome in a rapid and highly parallel fashion.

The current versions of arrays used for location analysis take advantage of newer printing technology. Features are now commonly composed of 60-mer oligonucleotides rather than long PCR amplicons. Several studies have shown that oligonucleotide probes are more sensitive than PCR probes in microarray analysis (Carter et al. 2003; Hughes et al. 2001; Li et al. 2002). Furthermore, it has been shown that results using longer oligonucleotides are more sensitive and reproducible than shorter ones. The 5070-base range seems to represent the "sweetspot" where hybridization efficiency, specificity, and sensitivity are co-optimized. These oligonucleotide arrays are manufactured by commercial vendors, which translates to an increase in both the quality and consistency of the arrays. For instance, there are currently three array designs for the study of transcription factor binding in the human genome available (for a review, see Lee and Volkert 2006).

Evaluation

Future studies will focus on expanding the transcriptional regulatory network for metabolic genes. It will be interesting to identify the targets of transcription factors that are themselves targets of metabolic transcriptions factors, thus expanding the transcriptional regulatory network responsible for the genetic program underlying the coordination of glucose and lipid metabolism. Transcription factors are responsible for recruiting additional components of the transcription apparatus, most notably chromatin-remodeling and chromatin-modifying complexes that affect transcription by regulating higher-order DNA structure. Consequently, one area of interest is identifying the targets of these various remodeling and modifying complexes and linking the network of transcriptions factors with the network of chromatin modifiers in liver, muscle, adipose, and pancreatic β-cells.

Application to Human Adipose Tissue/Cells

Purpose and Rationale

Adipose tissue plays a major role in obesity and metabolic disorders, as discussed in separate chapters of this book. The main functions of adipose tissue include (i) secretion of adipokines (a diverse set of bioactive molecules produced by adipose tissue), (ii) energy dissipation/thermogenesis, and (iii) energy storage. Different cell types of adipose tissue serve different functions. For instance, adipocytes store energy in the form of lipids (Large et al. 2004) as well as secrete adiponectin, an insulin-sensitizing hormone (Havel 2004), and leptin, a signal of energy balance (Havel 2004). However, non-adipose cells, which comprise the stromal vascular fraction, provide structural support (Aoki et al. 2004) and secrete angiogenesis-related cytokines (Aoki et al. 2004). Dysregulation of these cellular functions may be reflected in altered expression levels of the genes involved in pertinent pathways. Defining signature gene expression profiles of adipose tissue or its cellular constituents in disease states may also complement genetic approaches to identify disease susceptibility genes (Permana et al. 2004a). The process of isolating the cellular components of adipose tissue may alter some gene expression profiles. For instance, the transcription of many pro-inflammatory genes are increased after standard adipocyte isolation (Ruan et al. 2003). Regardless of this difficulty, the pursuit of better understanding of the different roles of each cellular component of adipose tissue plays is warranted.

The use of DNA microarrays to investigate normal and dysregulated adipose tissue gene activity has grown exponentially in the past decade. This technology provides comprehensive expression profiles of multitudes of genes simultaneously. Microarrays commonly use cDNA or oligonucleotide probes. cDNA microarrays involve spotting 3′-expressed sequence tags or known genes on glass slides (Duggan et al. 1999), whereas oligonucleotide microarrays contain combinatorially synthesized short oligonucleotides complementary to expressed genes as

well as expressed sequence tags and all identified exons (Lipshutz et al. 1999). The amount of labeled complementary deoxyribonucleic acid (cDNA) from experimental samples that hybridize to these probes correlates with the amount of messenger ribonucleic acid (mRNA) in the original samples. It is generally expected that the concentrations of the specific mRNAs in the samples reflect the concentrations and activities of the corresponding protein products of the genes, albeit this is not always the case (Cagney et al. 2005).

Here, the utility of DNA microarrays to study gene expression profiles of human adipose tissue, adipocytes, and stromal vascular cells (SVCs) is described. Although different kinds of microarrays, analytical methods, and data mining methods exist, sample preparation and hybridization methods for human oligonucleotide microarrays synthesized by Affymetrix and the resulting data analysis by Affymetrix software are specifically presented. The data can be further mined using the continually updated databases on genomic networks as described below. An application of these methods to investigate gene expression profiles in adipocytes and cultured SVCs of adipose tissue from obese compared to nonobese subjects has been reported previously (Lee et al. 2005; Nair et al. 2005).

Procedure

Adipose tissue (typically from abdominal subcutaneous or omental depot) can be obtained surgically (Kern et al. 2003) or using needle biopsy/aspiration (Permana et al. 2004b) with the help of reusable hypodermic needles with Luer-Lok (13 gauge × 2 in., short bevel, BD Biosciences) and several (up to five per incision site) 60 ml syringes, each containing about 10 ml of sterile 0.9 % NaCl solution, after the administration of local anesthesia. The latter procedure is only applicable to subcutaneous adipose tissue.

Surgery Samples

The tissue samples (about 1–3 g) are put in a sterile plastic tube containing Hank's Balanced Salt Solution (HBSS) or Medium 199 supplemented with 1 μg/ml of amphotericin B, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 2 mM GlutaMAX-1 (Invitrogen). Visceral adipose tissue samples are usually taken from patients undergoing abdominal surgery. If adipose tissue samples need to be transferred to another place for processing, they should be carried in tubes containing saline solution at 22 °C, not in a cold carrier or on ice, to prevent changes in gene expression profiles caused by exposure to cold temperature. The tissue sample is coarsely minced (into 25-mg pieces) using sterile scalpels and forceps in a laminar flow hood. The tissue pieces are cleaned from visible connective tissue, blood clots and blood vessels in HBSS supplemented with 5.5 mM glucose.

Needle Biopsy Samples

The adipose tissue is aspirated into a 60 ml syringe containing 0.9 % NaCl solution and then placed on tissue on a sterile nylon mesh and rinsed with more NaCl solution. If needle biopsy samples need to be transferred to another place for subsequent processing, they need to be diluted with more saline solution with gentle agitation to prevent blood clotting inside the syringes. The already fragmented tissue samples are cleaned from visible connective tissue and blood vessels in HBSS supplemented with 5.5 mM glucose in a laminar flow hood.

Subsequent Processing

For RNA extraction, the tissue samples are placed in 5–10 volumes of RNAlater (Ambion) solution to preserve for later extraction (stored at 4 °C overnight and then transferred to −80 °C for longer-term storage) or proceed with RNA extraction immediately. Tissue samples can also be frozen quickly in liquid nitrogen and stored at −80 °C for RNA extraction at a later time. For digestion, the tissue samples are transferred into a sterile 125 ml Erlenmeyer flask and processed further as below.

Adipose Tissue Digestion and Separation of Adipocytes and Stromal Vascular Cells

The tissue is digested in HBSS buffer containing 5.5 mM glucose, 5 % fatty-acid-free BSA, and

3.3 mg/ml type I collagenase for 30–60 min in a shaking 37 °C water bath at 120–150 strokes/min. The digestion mixture is passed through a sterile 230-um stainless tissue sieve into a sterile 50 ml tube. After allowing the adipocytes to float by buoyancy, the adipocyte layer is carefully pipetted into a separate tube. The adipocytes with M199 media containing 3 % FBS and 5 % fatty-acid-free BSA twice using the flotation method. After the final wash, the adipocytes can be lysed for RNA extraction. Thereafter, an equal volume of M199 is added to the infranatant containing SVCs. The mixture is centrifuged at 585× g for 5 min. The supernatant is carefully discarded, leaving the pellet in 1–2 ml of media. The S-V pellet is rinsed with M199 and subsequently with HBSS using the centrifugation method. At the end of the last rinse, discard as much of the supernatant as possible without disturbing the pellet. The SVCs can be lysed immediately or expanded in culture for RNA extraction, similar to the procedure for adipocytes. If the number of SVCs needs to be expanded to obtain adequate RNA, the cells can be cultured for several population doublings. SVCs are usually composed of a mixture of preadipocytes, macrophages, endothelial cells, and fibroblasts. Under 2-week culture conditions, preadipocytes will predominate in the culture and contamination from other cell types will be minimal (Nair et al. 2005; Permana et al. 2004b).

To culture the stromal vascular cells, the pellet is resuspended in 10 ml of M199 containing 10 % FBS. The cells are transferred to a culture flask/dish and then cultured in a 37 °C incubator for a day before washing the attached SVCs with PBS twice to get rid of red blood cells. The culturing of the cells is continued in fresh M199 media containing 10 % FBS until there are approximately 1.5 × 106 cells (e.g., the cells can be propagated in a 15-cm dish with media change every 2–3 days and harvested for RNA extraction when they are about 90 % confluent).

cDNA Preparation

The following procedures (recommended by Affymetrix) are used to prepare biotinylated cRNA target using 5 ug of purified total RNA as the starting sample, which will provide sufficient amount of cRNA target for hybridization of one or both subarrays of the HG-U133 set. High-quality (1.9–2.1, A260/A280) total RNA is required for DNA microarray analysis.

For first-strand cDNA synthesis (One-Cycle cDNA Synthesis Kit, Affymetrix), 5 μg of RNA sample is mixed with 2 μl of diluted poly-A RNA controls and 2 μl of T7-oligo(dT) primer and then supplemented with RNase-free water to a final volume of 12 μl in a 0.2 ml PCR tube. The tube is flicked for mixing and then centrifuged. After incubation for 10 min at 70 °C using a PCR machine, the tube is quickly chilled on ice and kept at 4 °C at least for 2 min. and then briefly centrifuged again. Sufficient First-Strand Master Mix is prepared by adding 4 μl of 5× first-strand reaction buffer, 2 μl of 0.1 M DTT, and 1 μl of 10 mM dNTP for each sample. The mixture is mixed well and centrifuged and a 7 μl portion of the First-Strand Master Mix transferred to each denatured sample tube. After incubation for 2 min at 42 °C, 1 μl of SuperScript II is added to each RNA sample, mixed by flicking, centrifuged, and then immediately incubated for 1 h at 42 °C. After cooling down to 4 °C for at least 2 min, the mixture is centrifuged and immediately used for the second-strand cDNA synthesis (One-Cycle cDNA Synthesis Kit).

For this, sufficient Second-Strand Master Mix is prepared in a separate tube with 91 μl of RNase-free water, 30 μl of 5× Second-Strand Reaction Mix, 3 μl of dNTP, 1 μl of E. coli DNA ligase, 4 μl of E. coli DNA polymerase I, and 1 μl of RNase H for each sample. After addition of 130 μl of Second-Strand Master Mix to each first-strand synthesis sample, the mixture is incubated for 2 h at 16 °C. After supplementation of 2 μl of T4 DNA polymerase, the incubation at 16 °C is continued for 5 min. Thereafter, the mixture is kept at 4 °C. Following the addition of 10 μl of 0.5 M EDTA, the double-stranded cDNA is cleaned up.

For cleaning-up (Sample Cleanup Module at room temperature), the second-strand cDNA mixture is transferred into a 1.5 ml microcentrifuge tube and supplemented with 600 μl of cDNA binding buffer. The tube is vortexed for 3 s and briefly centrifuged. The color of the mixture should be yellow. In case of orange or violet

color, 10 µl of 3 M sodium acetate (pH 5.0) is added, resulting in a color turn to yellow. 500 µl of the sample is pipetted into the cDNA Cleanup Spin Column in a 2 ml collection tube, which is then centrifuged for 1 min at 9,000× g. The flow-through is discarded. This step is repeated with the remainder of the sample. The collection tube with the flow-through is discarded. The column is transferred into a new 2 ml collection tube. 750 µl of the cDNA wash buffer is pipetted onto the spin column which is then centrifuged for 1 min at 9,000× g. The flow-through is discarded. After the opening of the cap of the column, the centrifugation is continued for 5 min at maximum speed. The collection tube with the flow-through is discarded. Thereafter, the column is transferred into a 1.5 ml collection tube. 14 µl of cDNA elution buffer is directly pipetted onto the membrane. After incubation for 1 min at 22 °C and centrifugation for 1 min at maximum speed, the average volume of eluate is 12 µl from 14 µl elution buffer.

For the synthesis of biotin-labeled cRNA by in vitro transcription reaction (IVT Labeling Kit, Affymetrix), all of the eluate from the cleanup procedure is transferred from the cleanup procedure into a 0.2 ml PCR tube and supplemented with 8 µl of RNase-free water, 4 µl of 10× IVT labeling buffer, 12 µl of NTP Mix, and 4 µl of enzyme mix; the tube is carefully tapped and then briefly centrifuged. The mixture is incubated for 16 h at 37 °C in a thermal cycler. The biotin-labeled cRNA can be stored at −20 °C or −70 °C, if not used for cleanup immediately.

For cleaning-up and quantification of biotin-labeled cRNA (Sample Cleanup Module at room temperature), an aliquot (0.5 µl) of the unpurified IVT product is analyzed for analysis by gel electrophoresis or Agilent 2100 Bioanalyzer to estimate the yield and size distribution of labeled transcripts. The cRNA sample is transferred to a 1.5 ml tube and supplemented with 60 µl of RNase-free water. After vortexing for 3 s, 350 µl of IVT cRNA binding buffer is added and the vortexing continued. If the IVT cRNA binding buffer forms a precipitate, it has to be redissolved in a 30 °C water bath and then kept at 22 °C. Before using for the first time, 24 ml and 20 ml of ethanol (96–100 %) is added to the cDNA wash buffer and IVT cRNA wash buffer, respectively. After the addition of 250 µl of ethanol (96–100 %) and mixing by pipetting, the sample is applied to the IVT cRNA Cleanup Spin Column in a 2 ml collection tube and then centrifuged for 15 s at 9,000× g. The column is transferred into a new 2 ml collection tube. After the addition of 500 µl of IVT cRNA wash buffer, the tube is centrifuged for 15 s at 9,000× g. The flow-through is discarded. Thereafter, 500 µl of 80 % ethanol is pipetted onto the column which is then centrifuged for 15 s at 9,000× g. The flow-through is discarded. After opening the cap of the column, it is centrifuged for 5 min at maximum speed. The collection tube with the flow-through is discarded. The column is transferred into a 1.5 ml collection tube. After pipetting 11 µl of RNase-free water directly onto the membrane and incubation for 1 min at 22 °C, the column is centrifuged for 1 min at maximum speed. After pipetting another 10 µl of RNase-free water onto the membrane and elution as above, the combined eluate (21 µl) can be stored at −20 °C or −70 °C if not used for quantification immediately.

For determination of the cRNA concentration and purity, the absorbance is checked at 260 nM and 280 nm of a 1:100 dilution of 1 µl of cRNA sample. Ratios between 1.9 and 2.1 of A260/A280 are acceptable. The adjusted cRNA yield is calculated to reflect carryover of unlabeled total RNA by subtracting 5 µg of starting total RNA from the cRNA yield. More than 50 µg of adjusted cRNA yield can be expected by starting amount of 5 µg of total RNA. The adjusted cRNA concentration is used for the fragmentation procedure. The quality of the unfragmented samples are checked by gel electrophoresis or Agilent 2100 Bioanalyzer.

For fragmentation of cRNA (Sample Cleanup Module), 40 µg of cRNA of adjusted concentration is transferred into a 0.2 ml PCR tube and supplemented with 16 µl of 5× fragmentation buffer and RNase-free water to a final volume of 80 ul for the preparation of fragmented cRNA for two subarrays of the HG-U133 set. After incubation at 94 °C for 35 min in a thermal cycler,

the reaction is put on ice. An 1 µl aliquot is saved for analysis on the bioanalyzer or gel electrophoresis to confirm the fragmentation. The undiluted fragmented cRNA is stored at −70 °C until hybridization is ready to be performed.

Microarray Hybridization and Scanning

Target cRNA hybridization step should be followed by washing, staining, and scanning without delay for the best results. For target hybridization, 600 µl of hybridization cocktail for two subarrays of the human genome HG-U133 set is prepared by mixing 30 µg of fragmented cRNA, 10 µl of control oligonucleotide b2, 30 µl of eukaryotic hybridization controls (GeneChip Eukaryotic Hybridization Control Kit, Affymetrix; the cRNA frozen stock has to be completely resuspended by heating to 65 °C for 5 min before aliquoting), 6 µl of herring sperm DNA (Promega), 6 µl of 50 mg/ml BSA (Invitrogen) solution, 300 µl of 2× hybridization buffer (200 mM MES/NaOH, 2 M NaCl, 40 mM EDTA, 0.02 % Tween-20; Affymetrix kit), 60 µl of DMSO, and nuclease-free water to a final volume of 600 µl. The hybridization cocktail is heated to 95 °C for 5 min, then incubated at 45 °C for 5 min in a heat block, and finally centrifuged for 5 min at maximum speed. The probe array is equilibrated to 22 °C, then filled with 1× hybridization buffer through one of the septa using a micropipettor with another tip for venting through the other septum, and subsequently incubated at 45 °C for 10 min. The buffer is removed from the array and cartridge, filled with 200 µl (for each array) of hybridization cocktail, and then hybridized for 16 h in a hybridization oven (Type 640) with 60 rpm at 45 °C.

For setting up of the fluidics station, the file location have to be defined if MAS is used by selecting Tools → Defaults → File → Locations from the menu bar and verify that all three file locations (files for probe information, fluidics protocols, and experimental data) are set correctly (not required if GCOS is used). After launching MAS 5.0 or GCOS (Affymetrix Microarray Suite 5.0 or GeneChip Operating Software) on PC-compatible workstation, MAS 5.0 software has been widely used but has recently been upgraded into GCOS by Affymetrix. Both software programs share basic similarities in data collection, management, and analysis. Product and technical support including manuals for both may be obtained at the following link: http://www.affymetrix.com/support/technical/byproduct.afx?cat=software. Also refer to the following site for the most recent Affymetrix software and manuals/technical sheets (http://www.affymetrix.com/products/software/index.affx), and when entering the experiment name, probe array type, and the additional information of sample name, sample type, and project if using GCOS, the Fluidics Station (Type 400 or 450/250) is turned on. Run → Fluidics is chosen from the GCOS menu bar. Subsequently, the intake buffer reservoir A is changed to wash buffer A (non-stringent wash buffer 6× SSPE, 0.01 % Tween-20) and intake buffer reservoir B to wash buffer B (stringent wash buffer 100 mM MES, 0.1 M of NaCl, 0.01 % Tween-20). Thereafter, the Fluidics Station is primed by selecting Protocol, Prime, and All Modules, and finally Run is clicked.

For probe array washing, staining, and scanning, 1,200 µl of fresh streptavidin R-phycoerythrin (SAPE, Invitrogen) stain solution is prepared for each array by mixing of 600 µl of 2× stain buffer, 48 µl of BSA solution, 12 µl of SAPE, and 540 µl of nuclease-free water and division into two aliquots of 600 µl each for the staining steps. Subsequently, 600 µl of fresh Antibody Solution Mix is prepared by mixing of 300 µl of 2× stain buffer (200 mM MES/NaOH, 2 M NaCl, 0.1 % Tween-20), 24 µl of BSA solution 6 µl of goat IgG stock (50 mg of goat IgG resuspended in 5 ml of 150 mM NaCl, stored at 4 °C, Sigma), 3.6 µl of biotinylated anti-streptavidin goat antibody (Vector Laboratories), and 266.4 µl of nuclease-free water. The hybridization cocktail is removed from the probe array immediately after 16 h hybridization and completely filled with about 250 µl of wash buffer A. This probe array can be stored at 4 °C for up to 3 h if washing and staining is not available immediately after hybridization. Washing and staining of the probe array is performed using one of either Fluidics Station 450/250 or 400 following the

automated protocol (EukGE-WS2v4) for the probe array format of HG-U133. The probe arrays are removed from the Fluidics Station at the end of washing and staining. The probe array glass window is checked for bubbles or air pockets. If there are large bubbles, fill the probe array without making large bubbles using Fluidics Station or manually. The probe array is kept in the dark at 4 °C until ready for scanning. Thereafter, the Affymetrix GeneChip Scanner 3000 is warmed up for at least 10 min or the Agilent GeneArray Scanner for at least 15 min before scanning. The excess fluid is cleaned from the probe array. One Tough-Spot is applied to each of the two septa with pressing to ensure that the spots remain flat. The glass window of probe arrays is cleaned. The cartridge is inserted into the scanner. The autofocus is tested to ensure that the Tough-Spots do not interfere. The Option button is clicked to check for the correct pixel value and wavelength of the laser beam in case GeneArray Scanner is used (pixel value 3 um; wavelength 570 nm). Run and Scanner are selected from the menu bar or click the Start Scan in the tool bar in MAS 5.0 or GCOS. The experiment name is selected and the Start button is clicked. The sample door on the scanner is opened, and the probe array is applied without forcing the probe array into the holder. Then the door is closed. OK is clicked in the Start Scanner dialog box. Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension.

Microarray Data Analysis

This is a simplified version of analysis options using Affymetrix microarray data analysis software. The technical manuals of the analysis software have extensive descriptions for choosing the various options, and the reader is referred to those websites. The general workflow outlining the sequence of steps involved in collecting, processing, analyzing, and interpreting microarray data is as follows:

> Register sample and define → Process probe array in fluidics station → Scan probe array and save image data (.dat file created) → Compute cell intensity data from the image data and save cell intensity data (.cel file created) → Analyze expression cell intensity data and save expression probe analysis data (.chp file created) → Generate expression analysis report (.rpt file created) → Statistical analyses (data saved as *.txt or *.xls files) → Biological interpretation (Data mining and biological pathways analyses).

For checking scanned images and converting fluorescence intensities to numerical values, double-click on each *.dat file to open it. Under the tab for "Image Settings," Autoscale and Pseudocolor are chosen and then "OK" is clicked. "Grid" tab is clicked on, and "G" and then View → Grid are selected from the menu bar to superimpose grid lines on the scanned image. The corners of the scanned image should fit within the grid. A picture of the image data is displayed in an image window when *.dat is opened. The software represents the fluorescence intensity value from each pixel on the array in a grayscale or pseudocolor mode and superimposes a grid on the image to delineate the probe cells (ref. technical manual). This is important because each grid cell represents a probe cell and the software calculates the average of intensities of the middle squares of each probe cell to generate a *.cel file during analyses. It is rare to have to adjust the grid. The grid is manually aligned by click-dragging the double arrows on the grid perimeters. Run → Analysis from the menu bar is selected. At the end of each analysis of a *.dat file (about 2 min), cell intensity data is computed and a *.cel file is created (single intensity value is computed for each probe cell). To analyze absolute expression values for an array, Tools → Analysis → settings → Expression is selected. In the Expression Analysis Settings dialog box, the "Use baseline comparison file" box must not be selected. Scaling tab is clicked on, "All probe sets" is selected, and default or user-defined scaling value is chosen. Absolute analysis results from several experiments (using the same type of probe arrays) can be directly compared if a user-specified target signal value is chosen consistently for all arrays using the "All probe sets" scaling option. Default values are chosen for normalization and mask, baseline, and parameter are probed. Probe array type is selected

(e.g., HG-U133A). Run → Analysis is selected from the menu bar. The cell intensity data is analyzed, and, in the Expression Analysis Window (EAW), the analysis output file (*.chp file) is displayed. For each transcript on the probe array, a probeset identifier, signal, detection call, detection p-value, and description are generated. The far left column is the unique Affymetrix probeset identifier, and the rightmost column provides a brief description of the sequence that the probeset represents. The "signal" denotes intensity. A "detection call" may be "Present", "Absent", or "Marginal" for each probeset, and the "detection p-value" provides an assessment of the statistical significance of each call. "Save results as" dialog box opens and the *.chp files can be saved at desired location with any name. Any *.chp file to open is double-clicked. In the EAW, Pivot tab is clicked and absolute analysis results are viewed in the pivot table. A *.rpt file (expression analysis report file that has quality control metrics) can be generated from each *.chp file by File → Report. There are several pivot table display options. The pivot table can be exported as a text file (*.txt) or Excel file (*.xls) by clicking on File → Save As. The *.chp files can also be published into public databases (e.g., GCOS server). Published data can be queried using data mining software as described below.

Statistical Analyses, Data Mining, and Pathway Analyses

Microarray data often are log-transformed to approximate normal distribution and to reduce the influence of single measurements. Standard statistical analyses such as the parametric Student's *t*-test and Mann-Whitney tests (when the data are not normally distributed) can be used to assess the significance of the data. Depending on the experiment or researcher's needs, other statistical tests such as multivariate Bonferroni correction or false discovery rate (Lee et al. 2005) can be applied. Biological interpretation is the next step after lists of genes with statistically significant expression patterns are generated. Databases with functional gene information, such as Ensembl (http://www.ensembl.org/index.html), Entrez, LocusLink, RefSeq, and the Gene Ontology database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi), may provide useful information. The following databases contain annotated biological information and serve as integrated single interfaces through which one can link to multiple databases to mine data about any given gene or sequence: (i) Affymetrix (NetAffx™ Analysis Center) (http://www.affymetrix.com/analysis/index.affx), (ii) UCSC Genomic Browser website (http://genome.ucsc.edu/), and (iii) NCBI Entrez website (http://www.ncbi.nlmnih.gov/gquery.fcgi).

To biologically interpret expression from sets of genes and identify relevant biological pathways with coregulated genes, one can utilize various biological pathway analyses, some of which are listed below. Most of these data mining software can upload *.chp or *.xls files after data analyses. These software incorporate graphics to summarize networks of biological interactions. One can place global gene expression datasets in the context of these pathway images to identify existing or unique pathways that may be significant in the tested hypothesis: (i) GeneSifter (http://www.genesifter.net/web/dataCenter.html), (ii) MetaCore (http://www.genego.com/metacore.php), (iii) Affymetrix portal (http://www.affymetrix.com/products/software/compatible/pathway.affx#GeneGo), and (iv) Ingenuity (http://www.ingenuity.com/).

Evaluation

Adipose tissue is increasingly recognized as a metabolically active endocrine organ with multiple functions beyond its lipid storage capability. Various constituents of the tissue, such as mature adipocytes and stromal vascular cells, have distinct functions. For example, they express and secrete different kinds of bioactive molecules collectively called adipokines. Altered adipokine secretion patterns characterize obesity and insulin resistance, which are major risk factors for T2D. The contribution of dysregulated adipokine expression to these diseases may be assembled from transcriptomic profiles of the tissue and/or its cellular constituents. These gene expression profiles may also complement genetic approaches to identify disease susceptibility genes.

The application of gene expression profiling using DNA microarrays to study human adipose tissue, adipocytes, and stromal vascular cells has been successfully demonstrated.

References and Further Reading

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Electrophoretic Mobility Shift Assay and Chromatin Immunoprecipitation

Purpose and Rationale

The continuing study of adipose tissue for the last two decades has completely transformed the perception that most investigators had of the organ. Nowadays, it is well known that adipose tissue plays a crucial role in the regulation of energy balance and acts as an active endocrine organ that mediates a number of physiological and

pathological processes (Kershaw and Flier 2004). The renewed interest in this tissue has warranted that the study of the molecular basis of the development of the adipose cell has become the main area of research for many investigators. The mechanisms driving adipocyte differentiation can be easily dissected using well-characterized cell lines, such as 3T3-L1 preadipocytes, as well as primary preadipocytes or adipocytes isolated from different sources. Adipogenesis involves not only a perfectly coordinated network of transcription factors but also a host of cofactors endowed with chromatin-modifying activities (Farmer 2006), including chromatin-remodeling complexes (Salma et al. 2004) as well as enzymes with histone-modifying activities (Fajas et al. 2002; Musri et al. 2006; Yoo et al. 2006), which are crucial for the establishment of the gene expression pattern characteristic of the mature adipocyte. These coregulators lack DNA binding specificity and are ubiquitously expressed; hence, their selectivity during adipogenesis is afforded by their interacting with sequence-specific transcription factors, which recruit them to the appropriate gene promoters at the proper time points (Farmer 2006; Hassan et al. 2001).

The study of transcriptional processes in higher eukaryotes has long been curtailed by a shortage of direct technical approaches. Until recent times, the study of transcription was mostly restricted to in vitro analysis using electrophoretic mobility shift assays (EMSA) and reporter gene assays. These methods have become increasingly inappropriate in light of the advancing knowledge of the role of chromatin as a major regulatory element in transcription. For this reason, the development of new techniques has been an important step forward in allowing the unraveling of the molecular basis of transcriptional regulation in a number of cell types, including adipocytes. The advent of the immunoprecipitation of chromatin (ChIP), in particular, afforded an opportunity to eavesdrop on what is happening inside the living cell at close quarters. In the last few years, ChIP has been established as a powerful method to examine the access of nuclear proteins to their target promoters in the natural chromatin environment, as well as to study the covalent modifications of the histones that constitute the nucleosomes spanning genomic regions of interest.

The ChIP procedure is based on the ability of formaldehyde to reversibly cross-link amino and imino groups of both amino acids and DNA that are found within a maximal distance of 2 Å from each other (Orlando 2000). This short range of action warrants that the cross-links generated this way represent direct interactions taking place in the cell at a determined time point (Katan-Khaykovich and Struhl 2002). By using a specific antibody directed against a particular transcription factor or a posttranslationally modified histone, it is possible to precipitate the protein of interest, pulling along with it the DNA sequences to which this protein is bound. To achieve this, the DNA should have been previously fragmented randomly into small pieces ensuring that the coprecipitated DNA actually represents the sequences found in the close vicinity of the selected protein. Once the formaldehyde-generated cross-links are reverted, the coprecipitated DNA can be analyzed by semi-quantitative PCR using primers designed to detect the presence or absence of a region of interest in the precipitate. The major limitations of the ChIP assay are the need for a not-always-available specific antibody and the inability to determine exactly where the binding is occurring, because it is difficult to shear the fixed chromatin into fragments of less than a few hundred base pairs. Moreover, this assay does not allow discernment of whether the binding of the transcription factor of interest to chromatin is direct or mediated by its interaction with other DNA-binding proteins. In this respect, EMSA remains the standard assay for determining which specific nucleotides within a region of DNA constitute a binding site for a particular factor. Although in later times it has been all put displaced by ChIP assays, EMSA is a relatively easy method that can be used to establish if the binding of a protein to DNA is direct and determine precisely where it is occurring.

The EMSA procedure is based on the observation that although an unbound DNA fragment migrates rapidly through a nondenaturing polyacrylamide gel, protein binding to the fragment

significantly slows its migration (Fried 1989; Revzin 1989). Because of the appearance of shifted or retarded bands as a consequence of protein binding, the assay is also often referred to as gel shift or gel retardation assay. Using an excess of an unlabeled oligonucleotide that competes with the labeled probe, the disappearance of the shifted band can be observed, allowing a quantitative calculation of the thermodynamic and kinetic parameters of the binding (Fried 1989; Fried and Crothers 1984). In contrast to the ChIP assay, the use of EMSA does not provide information about the actual occurrence of the observed binding in a specific cell line. Moreover, sites that require multiprotein complex formation to stabilize DNA-protein interactions, as well as the binding of non-sequence-specific coregulators, are difficult to study in vitro. Thus, by providing different kinds of information, ChIP and EMSA are actually complementary methods.

The purpose of this chapter is to provide guidelines to carry out ChIP and EMSA experiments using freshly isolated rodent preadipocytes or adipocytes as well as cell culture lines, such as 3T3-L1 at different times during the differentiation process. The general protocol is detailed with indications of some modifications that should be applied depending on the source of the cells used.

Application of ChIP to Adipose Cells

Procedure

Formaldehyde Cross-Linking and DNA Fragmentation

Cross-linking solution, prepared freshly by diluting 36.5 % formaldehyde (in 10 % methanol, Merck) in formaldehyde dilution buffer (50 mM HEPES/KOH, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, stored at 4 °C) to reach a final conc. of 11 %, is added to the cells maintained in cell culture medium to reach a final conc. of 1 % formaldehyde. (Formaldehyde 36 % may also be added directly to the cells to reach a final conc. of 1 %, but it has been shown that pH influences the final result of the cross-linking reaction; Jackson 1999. If fixation is allowed to proceed at physiological pH of 7.4, DNA-protein cross-links are favored in detriment of protein-protein cross-links. Tris-containing buffer should be avoided during fixation, since it provides a source of amine groups that will quench the reaction.) Thereafter, the cells are incubated for 10 min at 22 °C. (Fixation is one of the critical steps of the process. When analyzing nucleosomal proteins, such as histones, relative weak fixation conditions can be used, but longer fixation times may be required for transcription factors. Thus, a time-course experiment to assess the optimal extent of fixation is a sensible first step when testing, for the first time, an antibody in ChIP assays. Excess cross-linking can turn the samples refractory to sonication and result in reduced antigen availability, thus complicating the immunoprecipitation step.) Fixation is terminated by adding 1.25 M glycine (prepared in H2Obidest, stored at 4 °C) to attain a final concentration of 125 mM. Glycine quenches the fixation reaction by providing excess of amino groups. This step is optional and can be skipped if the samples are quickly processed.

In case of using adherent 3T3-L1 cells, the cell culture medium containing the formaldehyde is aspirated. Then the cells are washed twice with ice-cold PBS, subsequently scraped with a cell scraper into 1 ml of PBS, and finally centrifuged at 10,000× g for 1 min. After the removal of the supernatant, the cell pellet is resuspended in an appropriate volume of SDS lysis buffer. (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 1 % SDS, stored at 4 °C, equilibrated at 22 °C before use; the volume of SDS lysis buffer has to be adjusted according to the cell number. When using 3T3-L1 cells, about half confluent p100 dish for each immunoprecipitation has to be calculated under the consideration that a complete experiment includes at least two immunoprecipitations, e.g., the antibody of interest and a control immunoprecipitation performed with nonimmune serum or an unrelated antibody. Ideally, the correspondence 50–60 µl for each immunoprecipitation is used, i.e., 100–150 µl for each p100 dish. If the volume is too small to be sonicated, it has to be increased.)

In case of using freshly isolated primary preadipocytes, the suspended cells are centrifuged after fixation in 50 ml tubes at 4,000× g for 5 min. After removal of the supernatant, the pelleted cells are washed twice with 1 ml of ice-cold PBS and, after the last centrifugation step, resuspended in 7–10 volumes of SDS lysis buffer. (Typically, from the epididymal fat pads of two to three mice, 2–3 × 106 preadipocytes are obtained, which represent a pelleted volume of 30–40 µl, to be resuspended in 200–300 µl of SDS lysis buffer.)

In case of primary adipocytes, the cells are centrifuged after fixation in a 50 ml tube at 4,000× g for 5 min. Thereafter, the cells form a dense superior phase. Following aspiration of the infranatant with a 19-gauge syringe needle, the primary adipocytes are washed with 10 ml of ice-cold PBS and then resuspended in 10 volumes of adipocyte lysis buffer (5 mM Pipes/KOH, pH 7.9, 80 mM KCl, 1 % Igepal, stored at 4 °C). After incubation for 1 h at 4 °C on a rotating wheel resulting in rupturing of the cell membrane and release of the nuclei, the samples are centrifuged at 20,000× g for 10 min. The pelleted nuclei (typically representing a volume of 30–50 µl) are resuspended in 5–7 volumes of SDS lysis buffer.

All samples are incubated at 22 °C for 10 min on a rotating wheel and then sonicated on ice (for minimization of foaming and avoidance of overheating that may denature the chromatin) to generate 0.5- to 2-kb fragments. The high concentration of SDS results in breakage of the nuclear membrane, exposing the fixed chromatin and thus facilitating sonication. For this, the sonicator tip should be inserted to a distance of approximately 10 mm from the surface of the liquid if possible to reduce aerosoling and foaming. The walls of the tube with the probe should not be touched which will cause the energy to dissipate unproductively. Sonication (Branson Sonifier model 150 equipped with a microprobe) is performed at medium power for eight 15-s intervals at continuous setting, with waiting periods of several minutes in between pulses for foaming to subside. (Adequate DNA fragmentation is essential for successful ChIP assay. Fragment size can be checked by reverting the cross-link in an aliquot taken from each sample and running a 1 % agarose gel electrophoresis. This is an important control step when first performing ChIP experiments with a given cell line, but once the conditions have been optimized, it is no longer necessary to check the quality of sonication every time. An aliquot of the samples can still be reserved prior to immunoprecipitation to be used as the input control for PCR analysis later on. The samples can be stored at 4 °C while the DNA size is checked, if necessary. Fixed chromatin samples can be stored at 4 °C for at least 1 week or kept at −80 °C for long-term storage.) Finally, the samples are centrifuged in a microfuge at 20,000× g for 1 h at 4 °C to eliminate cellular debris and high-molecular-weight DNA-protein aggregates.

Immunoprecipitation

The supernatants are recovered and transferred to new tubes and then diluted tenfold using ChIP dilution buffer (16.7 mM Tris/HCl, pH 8.0, 0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 167 mM NaCl, stored at 4 °C and supplemented with 1 mM PMSF and protease inhibitors before use) to decrease the concentration of SDS in the samples. The samples are precleared by incubating every 1 ml with 60 µl of salmon sperm DNA/Protein A-agarose or Protein G-agarose (50 % slurry, prepared by swelling of 0.15 g of Protein A CL-4B from GE Healthcare with 2 ml of H2Obidest at 22 °C for 15 min on a rotating wheel, subsequent centrifugation of the beads at 1,000× g for 1 min, removal of the supernatant, two washing cycles with H2Obidest and one with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) of the pelleted beads and their resuspension in TE buffer, and final supplementation with 200 µg of sonicated salmon sperm DNA 500 µg of bovine serum albumin) at 4 °C for 60 min under gentle rotation (can be skipped if using anti-mouse Dynabeads) and then centrifuged. The supernatants are recovered and transferred in new microfuge tubes. For each individual immunoprecipitation, 2.5 µl (1–5 µg) of antibody (e.g., directed against dimethylated histone H3, Lys4; Upstate, Cat. No. 07-030) is added to 0.5 ml of supernatant. Pre-immune serum or nonimmune IgGs or an unrelated serum, preferable against a

nonnuclear protein, is added to one sample, which will be the negative control to check for specificity of the assay and to detect the DNA fragments that are nonspecifically precipitated. After incubation at 4 °C overnight on a rotating wheel, the samples are centrifuged at 11,000× g for 2 min to precipitate any aggregates that may have formed and that would otherwise be nonspecifically precipitated in the following steps. The supernatant is transferred into new tubes, supplemented with 30 µl of salmon DNA/Protein A or Protein G to each 0.5 ml sample and further incubated at 4 °C for 1–4 h to allow for the antibody-Protein A/Protein G complexes to form (for mouse monoclonal IgG1 antibodies, 30 µl of Protein G (Upstate) or 20 µl of washed sheep anti-mouse IgG Dynabeads (Dynal Biotech) have to be used instead because Protein A binds only weakly to mouse IgGs. When using Dynabeads, the centrifugation steps are substituted by placing the tubes for 2 min in magnet to concentrate the beads) and then centrifuged at 11,000× g for 2 min. The supernatant is discarded. (The supernatant of the control immunoprecipitation with nonimmune serum can be kept to be used later on as input DNA control for PCR analysis; alternatively, an aliquot may be taken prior to immunoprecipitation, but this saves material if limiting; however, this sample cannot be used to check DNA size, as it contains salmon sperm DNA which will mask the sample in agarose gel electrophoresis.) The pellet is washed with 1 ml of low salt immune complex wash buffer (20 mM Tris/HCl, pH 8.0, 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, stored at 4 °C) at 22 °C for 3–5 min on a rotating wheel and then centrifuged at 11,000× g for 1 min. After removal of the supernatant, the pellet is washed with 1 ml of high-salt immune complex wash buffer (20 mM Tris/HCl, pH 8.0, 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 500 mM NaCl, stored at 4 °C) at 22 °C for 3–5 min on a rotating wheel and then centrifuged at 11,000× g for 1 min. The washing steps of increasing stringency are intended to eliminate nonspecific protein-antibody interactions. After the removal of the supernatant, the pellet is washed with 1 ml of high-salt immune complex wash buffer (10 mM Tris/HCl, pH 8.0, 0.25 M LiCl, 1 % Igepal, 1 % sodium deoxycholate, 1 mM EDTA, stored at 4 °C) at 22 °C for 3–5 min on a rotating wheel and then centrifuged at 11,000× g for 1 min (if lower-stringency conditions are needed, this and the previous step can be omitted). The pellets are washed three times with 1 ml of TE buffer, each, to eliminate excess of LiCl in the samples and then centrifuged at 11,000× g for 1 min. After removal of the supernatants, 200 µl of elution buffer (0.1 M NaHCO3, 1 % SDS, freshly prepared before use) is added to each sample for elution. After incubation for 15 min at 22 °C on a rotating wheel, the samples are centrifuged at 11,000× g at 1 min. The supernatant is recovered and transferred into a new microfuge tube. With the pelleted beads, the elution step is repeated. The eluates (about 400 µl per sample) are combined.

Reversal of Cross-Linking and Sample Analysis

The eluted samples are supplemented with 16 µl of 5 M NaCl and input DNA (final concentration of 200 mM NaCl) and then incubated at 65 °C for 4 h to overnight to revert the cross-links. Formaldehyde cross-links are easily reverted by incubation at high temperature in the presence of salts and detergents. For digestion of the proteins, 8 µl of 0.5 M EDTA, 16 µl of 1 M Tris/HCl (pH 6.5), and 1.6 µl of 10 mg/ml Proteinase K (diluted in H2Obidest and stored at −20°) are added. After incubation at 45 °C for 1–2 h or at 37 °C overnight, DNA is recovered from the samples by phenol/chloroform-extraction (addition of one volume of phenol/chloroform/isoamylalcohol (25/24/1), vortexing, centrifugation at 12,000× g for 5 min, and recovery of the upper aqueous phase) and ethanol precipitation (addition of 1/10th volume of 3M sodium acetate, pH 5.5, 20-µg glycogen from 20 mg/ml stock solution as carrier and two volumes 100 % ice-cold ethanol, incubation at −20 °C for 1 h to overnight, and centrifugation at 12,000× g for 10 min at 4 °C). After removal of the supernatant, the DNA pellet is washed with 1 ml of 70 % ethanol. After centrifugation and removal of the supernatant, the pelleted DNA is dried on the air for several minutes and then resuspended in TE using the

same volume for all samples (the volume of resuspension depends on the initial cell number and should be as small as possible (e.g., 20–30 μl) for test multiplex semiquantitative PCR run on 12 % acrylamide gels (Mini-PROTEAN 3, Bio-Rad). Generally, input DNA needs to be diluted at least 50–100 times prior to PCR to be in the same working range as the samples. Multiplex primer pairs including a positive control (a promoter that is not modified by the experimental conditions tested) and a negative control for specificity (a promoter that is not expected to be precipitated) are preferable (Musri et al. 2006)). A series of dilutions of the input DNA is performed to work within the same range as experimental samples. Real-time PCR may also be used to analyze ChIP.

Application of EMSA to Adipose Cells

Procedure

Nuclear Extract Preparation

The method described by Schreiber et al. (1989) is an easily performed, two-step procedure that results in the purification of a crude nuclear fraction by first swelling the cells in a hypotonic buffer preserving nuclei intact and then extracting the nuclear pellet with a high-salt buffer.

In case of using 3T3-L1 cells, in a p100 dish, the cell culture medium is aspirated and the cells are washed twice with ice-cold PBS, then scraped into 1 ml of PBS, and transferred into a microfuge tube. After centrifugation at 8,000× g for 30 s at 4 °C, the pelleted cells are resuspended in 400 μl of Schreiber buffer A (10 mM HEPES/KOH, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, stored at 4 °C, supplemented with 1 mM PMSF, 1 mM DTT, and protease inhibitors immediately before use).

In case of using primary preadipocytes or mature adipocytes, the suspended cells are centrifuged at 4,000× g in a 50 ml tube, washed twice with 10 ml of ice-cold PBS, and finally resuspended in 1 ml of PBS and transferred into a microfuge tube. After centrifugation at 8,000× g for 30 s at 4 °C, the pelleted cells are resuspended in 400 μl of Schreiber buffer A (typically, epididymal fat pads of two to three mice or 2–4 × 106 preadipocytes or adipocytes corresponding to a packed cell volume of 70–100 μl are extracted for each nuclear extract preparation).

The suspended cells are incubated on ice for 15 min to allow swelling. After the addition of 25 μl of 1 % Igepal and vortexing for 15 s, the samples are centrifuged at 11,000× g for 30 s. Following the removal of the supernatant harboring the cytoplasmic fraction, 50 ul of Schreiber buffer C (20 mM HEPES/KOH, pH 8.0, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20 % glycerol (v/v), stored at 4 °C, supplemented with 1 mM PMSF, 1 mM DTT, and protease inhibitors immediately before use) is added to the pellets. After incubation at 4 °C for 15 min on a rotating wheel, the samples are centrifuged at 11,000× g for 5 min at 4 °C. The supernatant harboring the nuclear fraction is recovered, assayed for protein concentration using the Bradford method, and stored at −20 °C prior to use.

Probe Labeling

3′-End labeling of synthetic oligonucleotides containing the binding site of interest is performed with terminal deoxynucleotidyl transferase (TdT) and biotin-N4-CTP. (Enzyme, biotin-N4-CTP, and a 5× TdT reaction buffer can be purchased together as a 3′-End Biotin Labeling Kit from Pierce, cat. No. 89818. In addition, the kit encompasses a labeled control oligonucleotide, which can be used to assess the efficacy of the labeling. The probes to be used in EMSA assays may be generated in a number of ways, including digestion from DNA cloned in plasmids with appropriate enzymes to yield the region of interest, PCR amplification of an adequate template, or by synthesizing and annealing complementary oligonucleotides. The labeling of these probes can also be performed by a selection of techniques. Thus, radioactive labeling may be achieved by incubation of Klenow enzyme and deoxynucleotides including typically α32P-dCTP, with the 5′-overhang acting as template and equipped with at least an unpaired G residue. Blunt-ended probes or probes with a 3′-overhang

have to be labeled with polynucleotide kinase and γ32P-ATP. Alternatively, non-isotypic methods may be used for the labeling and detection of the probe.) Single-stranded oligonucleotides are the best substrate for TdT, although blunt-ended probes for 3′-overhang double-stranded oligonucleotides may also be used. Thus, when using synthetic oligonucleotides, it is convenient to label them separately and then anneal the labeled oligos.

The oligonucleotides are dissolved in TE buffer to reach a concentration of 10 μM for stock solutions, and these are diluted to 1 μM working concentration. The labeling reactions are prepared by adding the components in the following order, 25 μl of H2Obidest, 10 μl of 5× TdT reaction buffer, 5 μl of 1 uM single-stranded oligonucleotide, 5 μl of 5 μM biotin-N4-CTP, and 5 μl of 2U/μl of TdT and run by incubation at 37 °C for 30 min. After termination by the addition of 2.5 μl of 0.2 M EDTA, 50 μl of chloroform/isoamyl alcohol (24/1 by vol.) is added to each reaction mixture to extract TdT. Following brief vortexing and centrifugation at 11,000× g for 5 min for phase separation, the upper aqueous phase (50 μl) harboring the labeled oligonucleotides at 100 nM concentration is recovered. (To assess the efficacy of the biotinylation reaction, the detection of the labeled probe is compared to that of a series of dilutions of a control labeled oligonucleotide, for instance, that provided by the 3′-End Labeling Kit, by means of a dot blot assay.) The labeled oligonucleotides (final conc. 20 nM) are annealed by mixing 50 μl of forward oligonucleotide-biotin (100 nM), 50 μl of reverse oligonucleotide-biotin, and 150 μl of TE buffer together and subsequent incubation at 97 °C for 3 min and then at 37 °C for 2 h. The competitor unlabeled oligonucleotide is annealed by using the 10-μM stock solutions to obtain a final concentration of 2 μM (thus, the unlabeled double-stranded oligonucleotide represents a 100-fold concentration compared with the labeled probe) and mixing 50 μl of 10 μM forward oligonucleotide, 10 μM reverse oligonucleotide, and 150 μl of TE buffer together and subsequent incubation at 97 °C for 3 min and then at 37 °C for 2 h.

Hybridization and Electrophoresis

A native electrophoresis gel is prepared in 0.5× TBE with 7.3 ml of H2Obidest, 1.62 ml of 30 % acrylamide/bisacrylamide (37.5/1), 1 ml of 5× TBE (0.45 M of Tris base, 0.45 M of boric acid, 10 mM EDTA), 60 μl of 10 % ammonium peroxodisulfate, and 20 μl of TEMED (corresponding to adequate quantities for two Mini-PROTEAN 3 gels of 0.75-mm thickness). Once the gel is cast, a prerun is performed at 100 V for 30–60 min. (This step is intended to remove the ammonium peroxodisulfate in the gel and minimize temperature changes taking place during the actual electrophoresis of the samples, which may affect the stability of the bound complexes.) The hybridization mixes are prepared by mixing together 8 μl of H2Obidest, 2 μl of 100 mM Tris/HCl (pH 7.5–8.0), or 100 mM HEPES/KOH (pH 7.9) (may be used depending on the protein; the function of the buffer is to maintain the optimal pH to favor the DNA-protein interaction of interest); 2 μl of 10 mM DTT (100-mM stock, stored at −20 °C), 1 μl of 1 M KCl, or 1 M NaCl (the concentration of monovalent cations establishes the astringency of the conditions); and 1 μl of 10 mg/ml Poly(DI.DC) 1 mg/ml prepared in TE, a nonspecific competitor DNA used to minimize the binding of nonspecific proteins to the labeled probe by adsorbing proteins that will bind to an general DNA sequence (stored at −20 °C), 2 μl of 2 μM unlabeled oligonucleotide (4 pmols in H2Obidest), 2 μl of NE (5–10-μg protein in H2Obidest), and 2 μl of 20 nM labeled oligonucleotide (40 fmols in H2Obidest). (Depending on the transcription factor whose binding to DNA is to be tested, other components can be added to the hybridization mix. Some proteins may need the presence of divalent cations such as Mg2+ or Zn2+ to stabilize their binding to DNA. The astringency of the conditions can also be increased by adding Igepal to the mix, usually at a final conc. of 0.05 %. The time and temperature of the binding reaction also influence the final result.) The hybridization mixtures are incubated at 22 °C for 15 min, then supplemented with 2 μl of 10× loading buffer (250 mM Tris/HCl, pH 7.5, 40 % glycerol, 0.2 % bromophenol blue, 0.2 % xylene

cyanol, stored at −20 °C), and finally loaded onto the gel. (The ability of the DNA-protein complexes to withstand, as a whole, the short time required to migrate into the gel is a critical step on which the major part of the success of the procedure rests. The interactions are stabilized by the low ionic strength of the electrophoresis buffer used (0.5× TBE) and the DNA-protein complexes are quickly resolved upon entry on the gel.) The electrophoresis is run at 100 V until the bromophenol blue is three quarters down the length of the gel (which takes less than 1 h with a Mini-PROTEAN 3 vertical system). A nylon membrane (for nucleic acid hybridization, Pierce) is soaked with 0.5× TBE for at least 10 min. (In the case of radioactive labeling, the gel is placed on top of an 3MM Whatman filter paper, covered in Saran wrap, and dried in a vacuum gel drier at 80 °C for 1 h. The dried gel can then be exposed overnight to an X-ray film or analyzed using a phosphorimager.) The gel and nylon membrane are assembled in an electrophoretic transfer unit (e.g., Mini-PROTEAN 3 vertical transfer system equipped with a PowerPac Basic power supply, Bio-Rad), and the transfer in 0.5× TBE is running at 380 mA (and about 100 V) for 1–2 h. Subsequently, the DNA is cross-linked to the membrane at 120 mJ/cm^2 using a UV-light cross-linker (e.g., UVP cross-linker model CL-1000) equipped with 254-nm bulbs. At this point, the membrane can be stored dry at room temperature until the time of analysis. The membrane is blocked with 20 ml of blocking buffer (Pierce) for 15 min. Conjugation solution is prepared by adding 66.7 µl of stabilized streptavidin-horseradish peroxidase (HRP) conjugate to 20 ml of blocking buffer. Streptavidin binds to biotin and the HRP conjugated to it enables its detection by conventional chemiluminescence methods, e.g., using the chemiluminescence nucleic acid detection module from Pierce (cat. no. 89880). The blocking solution is decanted from the membrane and replaced with conjugate solution. After incubation at 22 °C for 15 min, the membrane is transferred to a new container and washed once with 1× wash buffer for 5 min. The buffer is decanted and the washing procedure is repeated four times. After transfer of the membrane to a new container, 30 ml of substrate equilibration buffer is added and the incubation continued for 5 min. The substrate working solution is prepared by mixing 50/50 luminol/enhancer solution with stable peroxide solution. After transfer of the membrane to a new container, the substrate working solution is added to the membrane and incubated at 22 °C for 5 min. The membrane is removed from the substrate working solution and liberated from excess of liquid by blotting with a filter paper, without drying to completeness. Finally, the membrane is exposed to an X-ray film for 1−5 min or measured for luminescence using a CCD camera (e.g., BAS3000 Lumi-Imager, Fuji Photo Film Co., Ltd.). Exposure and measurement times may be adjusted to obtain the desired signal.

Evaluation

Chromatin, long thought to be no more than a scaffold supporting DNA compaction inside the cell nucleus, has emerged in the last few years as a major regulatory element involved in the control of gene expression both acutely during interphase and programmatically throughout complex processes of development and differentiation. Adipogenesis is the result of an intertwined network of transcription factors and coregulators with chromatin-modifying activities and offers an excellent model for the study of transcriptional regulation. In this regard, electrophoretic mobility shift assay and immunoprecipitation of chromatin are complementary methods that can be used to study the binding of nuclear proteins to DNA and to characterize how these proteins interact with and modify chromatin to regulate gene expression and, more globally, cell differentiation. Some experimental strategies are presented on how to perform these two assays using 3T3-L1 cells and rodent primary preadipocytes and mature adipocytes.

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siRNA

During the past decade, a panel of methods has been developed for the validation of drug targets at the cellular level encompassing constitutive or regulated overexpression of the gene envisioned or its downregulation (antisense RNA, mouse embryonic fibroblasts from knockout mice and RNAi). RNAi displays a number of advantages regarding the expenditure in equipment, costs, and time required. Moreover, the incomplete efficacy of RNAi in downregulating gene expression (usually by up to 80 %) more closely reflects the pharmacological inhibition of transcription by small molecule compounds. In addition, there are considerable efforts to use RNAi as future therapeutics albeit many challenges have to be overcome.

RNA interference (RNAi) is an ancient mechanism of gene regulation, found in eukaryotes as diverse as yeasts and mammals, and probably plays a central role in controlling gene expression in all eukaryotes (Tijsterman et al. 2002). Using small-interfering RNA (siRNA) molecules, RNAi can selectively silence essentially any gene in the genome. Once in a cell, a short double-stranded RNA (dsRNA) molecule is cleaved by an RNase III, called "Dicer," into 21- to 23-nucleotide guide RNA duplexes with two-nucleotide 3′-overhangs, called siRNAs, that become bound to the RNA-induced silencing complex (RISC). Within the RISC, one of the two strands of the siRNA is chosen as the antisense strand via cleavage of the passenger strand (Gregory et al. 2005; Rand et al. 2005), so that they can target complementary sequences in messenger RNAs involved in a disease. An RISC-associated "slicer" RNase belonging to the Argonaute2 (Ago2) family then cleaves the target in the middle of the homology, which is

followed by further degradation mediated by an exosome complex (Liu et al. 2004; Rand et al. 2004), thereby interrupting the synthesis of the disease-causing protein. The RISC complex is naturally stable within the cell, enabling siRNA to cut multiple mRNA molecules consecutively and, therefore, suppressing protein synthesis in a potent and targeted way.

Design

The bulk of pharmaceutical applications of RNAi uses siRNA or its precursor. A completely foolproof algorithm of siRNA design for every target may remain an unattainable dream due to the inherent difficulty of predicting the various parameters that affect siRNA function (Heale et al. 2005; Amarzguioui et al. 2005; Gong and Ferrell 2004). However, some significant factors have been recognized. First, a successful application of RNAi demands that it be as specific to the target RNA as possible. Kim and coworkers (2004) demonstrated that 27-bp siRNAs are 10 to 100 times more potent and specific in vivo than their 21-bp counterparts. Second, Schwarz and coworkers (2002a) found that the formation of RISC is an asymmetric process in that only one strand of the siRNA is incorporated, not both. Third, it is now clear that the local secondary structure of the target RNA has dramatic influence on the accessibility for the siRNA (Vickers et al. 2003; Kretschmer-Kazemi and Sczakiel 2003; Luo and Chang 2004). Although siRNA designs have been proposed to incorporate this parameter (Heale et al. 2005; Amarzguioui and Prydz 2004; Gong and Ferrel 2004), the empirical nature of RNA structure prediction has left a room for uncertainty. Fourth, a number of studies have reported various degrees of positive and negative regulation of genes besides the intended target leading to "off-target" effects that are not easily explained by a fortuitous sequence homology of the siRNA to the off-target mRNAs (Sledz et al. 2003; Jackson et al. 2003; Bridge et al. 2003). In conclusion, one should follow all the guidelines suggested above but remember that the ultimate test is experimental.

Source

It is to be realized that RNAi-based target validation or therapy exploits the physiological machinery constitutively present in the normal cell and that dsRNA is the only entity needed to activate it. In other words, therapeutic RNAi is triggered by the simple introduction of target-specific siRNA. This is commonly achieved by one of the following methods. The 21-mer siRNA with the 3′-dinucleotide overhangs can be chemically synthesized and introduced into the cell. Alternately, relatively long sense and antisense strands are transcribed in vitro from recombinant DNA templates, annealed to produce the dsRNA, which is digested with Dicer to generate siRNA that is then transfected into the cells. Lastly, the dsRNA can be generated in vivo by transfection of the corresponding DNA clone, which is then processed by the intracellular Dicer to generate the siRNA.

Synthetic siRNA

The chemistry of RNA synthesis has significantly improved over the years with an additional boost from the recent demands of the RNAi market. Short RNAs containing a variety of modifications are now commercially available (Dorsett and Tuschl 2004; Manoharan 2004). Modifications are explored for a variety of goals, such as improved RNAi activity and lower IC50, enhanced stability, particularly against nucleases, and better pharmacokinetics and organ targeting. The effect of terminal and internal modifications of the siRNA on the silencing activity has been tested, and the consensus is summarized here. Additions at the 5′-end of the sense strand had little or no effect (Chiu and Rana 2003). A 5′-phosphate group on the antisense strand is essential for the incorporation into RISC. However, additions to the phosphate group are generally tolerated (Nykanen et al. 2001; Martinez et al. 2002). Tolerance at the 3′-end of the antisense depends on the derivative. While biotin, ddC, aminopropyl group, and puromycin do not inhibit siRNA activity, 2-hydroxyethyl phosphate, ethylene thymidine, and some fluorescent derivatives do (Schwarz et al. 2002b). Of all the

internal modifications, substitutions of the 2′-OH of ribose are the best studied and include -H, -OMe, -F, and -NH2. In general, all 2′-substitutions in the 3′-overhangs are tolerated (Elbashir et al. 2001; Amarzguioui et al. 2003). siRNAs with internal 2′-F are RNase resistant and may perform better than unmodified siRNA in cell culture (Capodici et al. 2002), but not necessarily so in animals (Layzer et al. 2004). Internally, 2′-O-methylated siRNAs also exhibit significantly greater stability in serum with sustained silencing activity, and siRNAs with alternate 2′-OMe and 2′-F substitutions have similar advantages as well. For reasons that are unclear, 2′-NH2 substitutions in either sense or antisense strand cause significant loss of silencing activity (Parrish et al. 2000). Replacement of the phosphodiester (P = O) backbone with phosphorothioates (P = S) or boranophosphonates (P = B) also offers nuclease resistance, but excessive substitution leads to loss of activity and/or increased toxicity (Manoharan 2004; Amarzguioui et al. 2003). Lastly, siRNA containing synthetic RNA-like nucleotide analog, known as "locked nucleic acid" (LNA), exhibits greatly improved biostability and enhanced inhibition of certain RNA targets (Braasch et al. 2003).

siRNA Transcribed In Vitro

Exogenous siRNA is also produced by the transcription of both strands of recombinant DNA templates, usually from a bacteriophage promoter, such as T7 or SP6 (Dudek and Picard 2004). As the resultant transcripts have 5′-triphosphates that may activate the undesirable IFN pathway, as mentioned before (Kim et al. 2005), the dsRNA product is either treated with phosphatase or trimmed with Dicer (Myers et al. 2003) in vitro before transfection.

Recombinant siRNA

The other common technique is to transfect target cells with DNA clones expressing shRNA, also known as DNA-directed RNAi or ddRNAi (Smith et al. 2000). The corresponding complementary DNA (cDNA) is cloned between two converging promoters, generating dsRNA in the recipient cells that is cleaved by cellular Dicer into siRNA. In an alternate strategy, a DNA segment containing a hyphenated inverted repeat produces an shRNA of 19 bp or longer upon transcription. The shRNA is processed essentially by the miRNA pathway using Drosha and Dicer as described previously to eventually generate the siRNA. Both strategies employ RNA polymerase III promoters, such as human and mouse U6, human H1, and human 7SK promoters. The commonly used vectors are of adenoviral, lentiviral, or retroviral origin. Recombinant expression opens the avenue for inducible and/or tissue-specific expression of the siRNA (Wiznerowicz and Tono 2003; Matsukura et al. 2003), which is not possible with synthetic siRNA. It also allows more sustained siRNA production over longer periods, critical in therapy for chronic diseases (see below). The potential hazards include viral infection, adverse immune reactions, and disruption or dysregulation of important genes via chromosomal integration of the viral genome (Bartosch and Cosset 2004). Furthermore, the parameters of shRNA design (such as the optimal length of the stem and the loop regions) are still ill defined and need further experimentation.

Delivery

There are two strategies for delivering siRNAs in vivo. One is to stably express siRNA precursors, such as short hairpin RNAs (shRNAs) from viral vector using gene therapy. The other is to deliver synthetic siRNAs by complexing or covalently linking the duplex RNA with lipids and/or delivery proteins. To solve the problem of cell penetration, most researchers have either complexed the RNA with a lipid or modified the RNA's phosphate backbone to minimize its charge. Despite the questions and unresolved problems, several companies are moving ahead to develop RNAi therapy for many diseases, including diabetes. The standard lipid reagents that were originally developed for DNA transfection, such as Oligofectamine (Invitrogen), continue to be the reagents of choice for cell culture studies (Spagnou et al. 2004). Recent

formulations claim to be specifically improved for RNA delivery and can be used in the presence of serum in cell culture and in live animals. Current options for the synthesis and design have been reviewed by Baric (2005).

Use for Metabolic Diseases

Numerous studies using highly sensitive microarray analyses have demonstrated that siRNAs can have off-target effects by silencing genes relevant for the regulation of carbohydrate and lipid metabolism in adipose, liver, muscle, and pancreatic β-cells. This makes siRNA an ideal tool for the cellular validation of molecular targets (e.g., enzymes, transcription factors) for the therapy of metabolic disorders, such as diabetes and obesity, as has been demonstrated elegantly by Zhou and coworkers (2002). In addition, RNAi-based therapeutics have potentially significant advantages over traditional approaches of treating diseases, including broad applicability, therapeutic precision, and selectivity avoiding side effects. This widespread applicability, coupled to relative ease of synthesis, and low cost of production make siRNAs an attractive new class of small-molecule drugs. RNAi-based drugs are designed to destroy the target RNA and therefore stop the associated undesirable protein production required for disease progression. The putative therapeutic potential for the use of siRNA, in general, and the cure of metabolic diseases, in particular, have been reviewed by Dallas and Vlassov (2006) and Rondinone (2006).

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Application to Adipose Cell Cultures

Purposes and Rationale

Suppression of gene expression in cells is one of the best strategies for analyzing the roles of a particular gene on cellular functions. Knockout mice have proven to be a powerful way of studying the specific role of a gene in vivo. However, this knockout strategy takes a long time and requires someone with great technical expertise. RNA interference (RNAi) is the process of using specific sequences of double-stranded RNA (dsRNA) to "knockdown" the expression of complementary genes (Meister and Tuschl 2004). This technique allows a powerful and easy reduction in the expression of a particular gene, often by 90 % or greater, allowing the analysis of the effect which that gene has on cellular functions (Meister and Tuschl 2004; Echeverri and Perrimon 2006; Elbashir et al. 2002; Pei and Tuschl 2006). Currently, a variety of commercial resources are available to start up RNAi experiments. Therefore, this technique is now widely used in vitro and even in vivo. For the best performance of this technique, investigators need to consider several steps, including the design of siRNA, an efficient delivery method (see above), and a means to monitor the biological effects of the siRNA introduced. Adipocytes have long been recognized as one of the most difficult cell types in which to perform gene delivery. There are many excellent recent reviews covering the mechanism and theoretical basis for the identification and selection of effective and specific siRNAs (Pei and Tuschl 2006; Sontheimer 2005; Tomari and Zamore 2005; Filipowicz et al. 2005; Valencia-Sanchez et al. 2006). Here, different transfection methods to obtain high efficacy of gene delivery and knockdown in adipocytes are described and compared.

Procedure

Three distinct transfection methods are compared with regard to efficacy and toxicity. The first method is performed with conventional liposome-type transfection reagents, such as

X-tremeGENE and Lipofectamine 2000. The second method is similar to the first one but distinct in the composition of transfection reagent. The third method is electroporation with Nucleofector.

Cell Culture

3T3-L1 preadipocytes are maintained in DMEM supplemented with 10 % calf serum. For induction of adipocyte differentiation, the cells are grown to confluence. The cells are then fed with differentiation medium (3:1 mixture of DMEM and Ham's F12 containing 10 % fetal bovine serum, 1.6 μM insulin, 0.0005 % transferrin, 180 μM adenine, and 20 pM triiodothyronine) supplied with 0.25 μM dexamethasone (250-μM stock solution in ethanol, stored in aliquots at −20 °C) and 500 μM isobutylmethylxanthine (500 mM stock solution in dimethyl sulfoxide, stored in aliquots at −20 °C). After 3 days, the cells are refed with fresh differentiation medium without dexamethasone and isobutylmethylxanthine and maintained over the following days. The transfection experiments are performed 8 days after the induction of differentiation. The amount of reagents described below refers to 1 well in a 12-well plate.

Transfection with Conventional Liposome-Based Transfection Reagents

The transfection reagents, X-tremeGENE (Roche Biochemicals) and Lipofectamine 2000 (Invitrogen) (stored at 4 °C), are gently mixed before use and then diluted with Opti-MEM 1 medium (Invitrogen) (1–10 μl of reagent in 49–40 μl of medium), again gently mixed and subsequently incubated for 5 min at room temperature. The siRNA (1–3 μl of 20-μM stock solution in RNase-free water, stored in aliquots at −20 °C) is diluted with Opti-MEM 1 medium, gently mixed and then incubated for 20 min at 22 °C to allow complex formation to occur. The solution may appear cloudy, but this will not inhibit the transfection. Subsequently, 400 μl of differentiation medium without antibiotics is added to the siRNA/reagent mix. After removal of the culture vessel from the incubator, the differentiation medium is replaced with the siRNA/reagent containing the medium. After 24–48 h of further culturing of the cells, total cellular RNA or protein is extracted.

Transfection with Polypeptide-Based Reagents

The siRNAs (20-μM stock solution in RNase-free water, stored in aliquots at −20 °C) and the DeliverX transfection reagent (stored at −20 °C) are thawed and stored on ice. The transfection reagent is sonicated (Bioruptor sonicator, Cosmo Bio) at a maximum speed and continuous power for 4 min to achieve a homogenous solution, then diluted (4.8 μl) with 45.2 μl of buffer 2, and again briefly mixed by vortexing and sonicated at maximum speed and continuous power for 4 min. The siRNA (3.2 μl of 20 μM stock solution in RNase-free water, stored in aliquots at −20 °C) is diluted with buffer 1. 50 μl of the siRNA/buffer 1 solution is mixed with 50 μl of the reagent/buffer 2 solution. After vortexing for 3 s or pipetting up and down several times, the mixture is incubated at 37 °C for 20 min and then supplemented with 400 μl of Opti-MEM 1 medium. After removal of the culture vessel from the incubator, the differentiation medium is replaced with the siRNA/DeliverX containing the medium. After 24–48 h of further culturing of the cells, total cellular RNA or protein is extracted.

Transfection by Electroporation

The adipocytes (cultured in 6-well plates) are washed with PBS and then treated with 0.25 % trypsin (Becton Dickinson) and 0.5 % collagenase (Sigma C6885), prepared in PBS containing 0.02 % EDTA for 2 min at 37 °C. After removal of trypsin and collagenase and tapping of the culture vessel several times, the cells are collected in proper amounts of PBS and then centrifuged at 1,000× g for 5 min. After the addition of 100 μl of solution V (Amaxa Inc.), the cells are completely suspended and supplemented with 5–10 μl of 20 μM siRNA stock solution. The mixture is transferred into the cuvette of the electroporation apparatus (Nucleofector device, Amaxa Inc.) and the program U-28 is chosen and started. Thereafter, the cells are resuspended into DMEM containing 10 % fetal bovine serum and seeded into a well of a 12-well plate. After culturing for

4–6 h, the medium is replaced with differentiation medium. After 24–48 h of further culturing of the cells, total cellular RNA or protein is extracted.

Evaluation

RNA interference is a powerful, quick, and easy technique to reduce the expression of a particular gene. However, investigators need to consider several steps for the experiments, including the design of the siRNA, an efficient delivery method, and a means for monitoring the biological effects of the siRNA introduced. Adipocytes have long been recognized as one of the most difficult cell types in which to perform gene delivery. Here three distinct transfection methods to obtain high efficacy of gene delivery and gene knockdown are described. The transfection efficacy of siRNA and shRNA is also compared.

In the first step of the RNAi experiments, the investigator has to decide whether to use siRNA or shRNA. Generally, the transfection efficacy of adipocytes with plasmid DNA is less than 10 %. In contrast, short dsRNA can be efficiently delivered into adipocytes. Also, siRNA is stable and significant amounts of siRNA exist in adipocytes even 10 days after transfection. Therefore, siRNA is the choice for mature adipocytes, unless investigators have a particular reason for using shRNA.

There are many companies providing "guaranteed to silence" or "validated" siRNAs. However, often Stealth siRNA from Invitrogen is adequate because of minimum induction of interferon responses. It is recommended to select a couple of sequences rather than a single one. It is likely that at least one of them knocks down the target gene. In case of preference to customize the relevant siRNA, several websites support the design of siRNA. If possible, at least two distinct sequences for the relevant target gene should be tested to exclude the possibility of off-target effects.

When dsRNA is delivered into cells, cellular responses called "interferon responses" occur. The induction of interferon responses depends on many factors, such as cell type, the length and sequence of the dsRNA, etc. (Meister and Tuschl 2004). Initially, it was thought that only longer dsRNA molecules (>30 nt) can trigger an interferon response and shorter (21 nt) siRNAs do not stimulate this cellular response. Recent extensive studies have revealed that even siRNA with the typical length of 21 nt can induce interferon responses. This holds also true for 3T3-L1 adipocytes transfected with commercial 21-nt length siRNA, which display induction of several interferon target genes. Interferon triggers the degradation of all messenger RNA and, eventually, cell death (Reynolds et al. 2006; Stark et al. 1998). These effects are unrelated to the reduction of target gene expression. Therefore, investigators are advised to use modified siRNA such as Stealth siRNA to reduce interferon responses or at least use control siRNA from the same company where the target siRNA is obtained. In any case, the effects resulting from the RNAi-mediated knockdown of target genes will have to be verified by other experiments.

Here, as conventional methods, two liposome-based reagents, X-tremeGENE and Lipofectamine 2000, are chosen. As a target gene, BMAL1 is selected. This gene is abundantly expressed in 3T3-L1 adipocytes and plays a role in the regulation of mature adipocyte functions (Aoyagi et al. 2005; Shimba et al. 2005). The siRNA used here almost completely knocks down the BMAL1 expression in proliferating cells. X-tremeGENE and DeliverX (polypeptide-based reagent) efficiently reduce the expression of the target gene. The comparison of X-tremeGENE and Lipofectamine 2000 indicates that the lipid composition of the reagent might influence transfection efficacy. Electroporation has long been used for transfection of genes into adipocytes. In fact, although efficacy of gene knockdown is slightly lower than other methods, electroporation can deliver a substantial amount of siRNA into adipocytes and knockdown the target gene. However, one of the biggest disadvantages of this method is that less than half of the cells can attach on the culture dish after 1 day of experiments. Therefore, this method is no longer the first choice of RNAi experiments with adipocytes. Both X-tremeGENE and DeliverX effectively reduce the expression of the target gene in specific siRNA-transfected cells. However, the absolute expression level in control cells transfected with

X-tremeGENE is less than those in DeliverX or Lipofectamine 2000 transfection cells. These results suggest that the X-tremeGENE may have efficient gene delivery activity but with high toxicity. Those who want to study metabolic changes by knockdown of a particular gene should carefully analyze the side effects of transfection reagents, especially liposome-based reagents. The lipids in the reagents may influence the metabolic pathway.

In conclusion, polypeptide-based transfection reagents, such as DeliverX, may be most effective and less toxic in RNAi experiments for adipocytes, although this method requires sonification of the reagent every time. If investigators require ease of use, then conventional reagents will suffice for the experiments. For evaluation of gene knockdown, detection of protein level and/or activity is established, and the mRNA level of the target gene should be determined by qRT-PCR.

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Effect on Peroxisome Proliferator-Activated Receptor

General Considerations

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear receptor superfamily (Issemann and Green 1990; Dreyer et al. 1992). They regulate glucose, lipid, and cholesterol metabolism in response to fatty acids and their derivatives, eicosanoids, and drugs used in the treatment of hyperlipidemia and diabetes. The PPAR subfamily contains three members known as PPARα, PPARβ/δ, and PPARγ. Each PPAR subtype shows a distinct tissue distribution (Keller and Wahli 1993; Green 1995; Devchand et al. 1996; Lemberger et al. 1996; Schoonjans et al. 1996a, b, c, 1997; Willson et al. 2000; Walczak and Tontonoz 2002) and ligand preference.

PPARα is expressed predominantly in metabolically active tissues, including the liver, kidney, skeletal muscle, and brown fat (Sher et al. 1993; Mukherjee et al. 1994), and its ligands include fatty acids, hypolipidemic drugs,

eicosanoids, and xenobiotics (Forman et al. 1995, 1997; Kliewer et al. 1997; Devchand et al. 1996; Lehmann et al. 1995).

PPARγ is highly expressed in adipocytes (Elbrecht et al. 1996; Mukherjee et al. 1997), is involved in control of lipid storage, and plays a critical role in the regulation of adipocyte gene expression and the induction of adipogenesis and adipocyte differentiation (Tortonoz et al. 1994, 1995; Brun et al. 1996; Wu et al. 1998; Lowell 1999). Thiazolidinedione derivatives which are antidiabetic agents are potent and selective activators of PPARγ (Young et al. 1995; Henke et al. 1998; Murakami et al. 1998; Reginato et al. 1998; Ribon et al. 1998; Vázquez et al. 2002). The meanwhile widely used antidiabetic drugs, pioglitazone and rosiglitazone, preferentially bind to the γ-isoform and modulate the expression of a number of genes in predominantly adipose tissue but presumably also in muscle and liver tissues, ultimately leading to substantial increase in insulin sensitivity (Lehmann et al. 1995). Berger and coworkers (1996) found a correlation of antidiabetic actions of thiazolidinediones in db/db mice with the conformational change in PPARγ. Su and coworkers (1999) described the use of a PPARγ-specific monoclonal antibody to demonstrate thiazolidinediones induced PPARγ receptor expression in vitro.

Murphy and Holder (2000) suggested a therapeutic potential of PPARγ agonists in the treatment of inflammatory diseases and certain cancers. Steppan and coworkers (2001) and Berger (2001) showed that adipocytes secrete a signaling molecule which they called resistin (for resistance to insulin). Circulating resistin levels in mice are decreased by thiazolidinediones and are increased in diet-induced and genetic forms of obesity.

Although the function of PPARδ, which is expressed ubiquitously, is less well known, this nuclear receptor may be involved in the regulation of cholesterol and lipid metabolism (Oliver et al. 2001; Shi et al. 2001). Fatty acids and cyclooxygenase 2-derived prostacyclin (PGI2) are natural ligands for PPARδ (Gupta et al. 2000). Several highly selective and potent synthetic ligands (e.g., L-165041, GW501516) activate PPARδ at nanomolar concentrations (Oliver et al. 2001; Glinghammar et al. 2003).

All subtypes share a common structural organization with a highly conserved N-terminal DNA binding domain (DBD) and a moderately conserved C-terminal ligand-binding domain (LBD) that contains a carboxyterminal activation function motif (AF2). All of the PPAR subfamily members heterodimerize with the receptor for 9-cis retinoic acid (RXR) and bind to target gene peroxisome proliferator elements (PPREs), a direct repeat of the sequence AGGTCA separated by one nucleotide (DR-1) (Dreyer et al. 1992; Kliewer et al. 1992).

Unliganded nuclear receptors, such as retinoic acid receptors (RARα) and T3Rα, are able to repress basal transcription through recruitment of corepressors (Chen and Evans 1995). Among the three PPARs, PPARδ distinguishes itself by displaying remarkably potent transcriptional repression activity. In contrast, unliganded PPARα and PPARγ do not repress PPRE-mediated transcription, presumably due to their inability to bind nuclear receptor corepressors SMRT and NCoR on the PPRE DNA element (Shi et al. 2001). Agonist binding to nuclear receptor LBD provokes a conformational change of the AF-2 motif that produces a suitable binding surface for recruitment of coactivators (Xu et al. 1999).

Recombinant Cell Lines

Purpose and Rationale

Because of the involvement of PPARs in many critical physiological and pathological functions, in particular with regard to metabolic diseases, the identification of high-affinity ligands would create useful tools for studying the PPAR role in the normal physiological and disease states as well as for drug discovery, in particular for the therapy of metabolic diseases, such as type II diabetes. To characterize PPAR specificity of synthetic ligands, stable HeLa-derived reporter cell lines in which PPARα, PPARβ, and PPARγ agonists induce luciferase activity have been constructed.

These cell lines stably express a chimeric protein containing the yeast transactivator GAL4 DBD fused to LBD regions of PPARs (GAL4-PPARs) and contain a luciferase reporter gene driven by a pentamer of the GAL4 recognition sequence in front of the β-globin promoter. The GAL4-PPAR chimeric receptors have been chosen because this assay format eliminates background activity from endogenous receptors and allowed quantitation of relative activity across the three PPAR subtypes with the same reporter gene. Additional assays for the characterization of PPAR-modulating compounds and drug candidates are given in ▶ Measurement of Blood Glucose-Lowering and Antidiabetic Activity.

Lipogenesis Assay

The lipogenic activity of PPARγ agonists can be assessed by determining the uptake of radioactive glucose uptake and its incorporation into the total lipids of cells (see ▶ Assays for Insulin and Insulin-Like Activity Based on Adipocytes). The amount of glucose incorporation into lipids in these cells provides a measure of cell differentiation, since preadipocytes are less efficient in de novo lipogenesis compared with mature adipocytes on the basis of their different expression levels of lipogenic genes, such as GLUT4 and GPAT. Moreover, the analyses of the expression of adipocyte marker genes (aP2, LPL, leptin) and of the accumulation of TAG in the cytoplasm confirmed by oil red O staining are other methods to evaluate the lipogenic activity of PPARγ agonists and thereby indirectly their binding affinity to PPARγ.

Protease Digestion Assay

Procedure

The protease digestion assay can be performed according to the method of Allan and coworkers (1992) with minor modifications. The plasmid pSG5-hPPARγ 1 is used to synthesize 35S-radiolabeled PPARγ 1 in a coupled transcription/translation system. The transcription/translation reactions are subsequently divided into portions of 22.5 µl volume. 2.5 µl PBS with or without a thiazolidinethione are added. The mixtures are incubated for 20 min at 25 °C, separated into 4.5 µl aliquots, and 0.5 µl distilled water and distilled water-solubilized trypsin are added. The protein digestions are allowed to proceed for 10 min at 25 °C and then terminated by the addition of 20 µl denaturing gel loading buffer and boiling for 5 min. The products of the digestion are separated by electrophoresis through a 1.5-mm 12 % polyacrylamide-SDS gel. After electrophoresis, the gels are fixed in 10 % acetic acid/40 % methanol for 30 min, treated in EN3HANCE for an additional 30 min. and dried under vacuum for 2 h at 80 °C. Autoradiography is then performed to visualize the radiolabeled digestion products.

Evaluation

For evaluation of the protease digestion assay, the partially protease-resistant conformation product of PPARγ is visualized by autoradiography on SDS-PAGE after incubation with the thiazolidinedione and increasing concentrations of trypsin.

Living Cell Luciferase Assay

The luciferase assays were used by Seimandi and coworkers (2005) to investigate the differential responses of PPARα, PPARδ, and PPARγ reporter cell lines to selective PPAR synthetic ligands. For this, cells are seeded at a density of 40,000 cells per well in 96-well white opaque tissue culture plates. Tested compounds are added 8 h later, and cells are incubated for 16 h. Experiments are performed in quadruplicate. At the end of incubation, culture medium is replaced by medium containing 3×10–4 M luciferin. Luciferase activity is measured for 2 s in intact living cells using a Wallac MicroBeta luminometer (PerkinElmer).

Lysed Cell Luciferase Assay

Procedure

Cells are seeded at a density of 200,000 cells per well in 24-well test plates. Test compounds/drug

candidates are added 8 h later, and cells are then incubated for 16 h. Cell extract preparation is done essentially as recommended by Promega. Compound-containing medium is removed, and cells are washed with PBS. Cells are lysed in 25 mM Tris/phosphate (pH 7.8), 2 mM EDTA, 1 % Triton X-100, 2 mM DTT, and 10 % glycerol. Protein concentration is estimated with the Bradford assay kit and BSA as standard.

Cell protein extracts (100 µl) are loaded onto 96-well white opaque tissue plates, and luciferase activity is measured after injection of luciferase detection buffer (20 mM tricine, pH 7.8, 1.1 mM (MgCO3), 4.0 mM Mg (OH)2•5H2O, 2.7 mM MgSO4, 0.1 mM EDTA, 33 mM DDT) and luciferase assay substrate (0.5 mM luciferin, 0.5 mM ATP, 0.3 mM coenzyme A) using a luminometer (e.g., Berthold Technologies). Luminescence is measured for 2 s.

Evaluation

In the transactivation assay, each compound is tested at various concentrations in at least two independent experiments. For each experiment, tests are performed in duplicate or triplicate for each concentration, and data are analyzed as means and standard deviations. Individual agonist concentration-response curves, in the absence and presence of antagonist, are fitted using the sigmoidal dose-response function of a graphics and statistics software program (e.g., GraphPad Prism, version 4.0). EC50 (effective concentration for half-maximal luciferase activity) and IC50 (half-maximal inhibitory concentration) values are calculated from equations used to fit the data in this graphic software. Transactivation data are presented as EC50 and IC50 values for each compound tested. To analyze the significance of PPAR isotype activation, PPAR agonist treatment is compared with controls using one-way analysis of variance (ANOVA) with the help of GraphPad Prism software.

In this assay system, the expression of PPAR isotypes differentially modulates the reporter gene basal activity and thus provides interesting information on the recruitment of cell type-specific coregulators by PPARs. Using specific PPAR agonists and antagonists, these cell lines permit an easy, rapid, and specific identification of subtype-selective PPAR ligands in a standardized microtiter plate assay. Furthermore, since HG5LN cells are putative host cells for other GAL4 nuclear receptor chimeras, RAR, thyroid hormone receptors, and steroid receptors, the assay system may be helpful to characterize the nuclear receptor selectivity of their nuclear receptor ligands which could also play important roles in the pathogenesis and therapy of metabolic diseases.

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Effect on Proliferation

Purpose and Rationale

Both insulin and IGF-1 stimulate the growth and proliferation of cells which express the corresponding receptors at adequate number. However, they differ considerably in their relative potency with IGF-1 exerting at least 100-fold higher/lower mitogenic/metabolic activities compared to those of insulin. The in vivo metabolic action of IGF-1 can be compared with insulin in adult rats using the following methods (DeMeyts 1994; Ulrich and Schlessinger 1990; Schlessinger and Ulrich 1992; Simpson et al. 1998): (i) dose dependence and time dependence of blood sugar decrease after intravenous injection, (ii) the antilipolytic effect (decrease of nonesterified fatty acids) after i.v. injection, and (iii) stimulation of glucose disposal during euglycemic clamping after intravenous infusion. Furthermore, all other assays described for insulin can be applied. Gazzano-Santoro and coworkers (1998) described a cell-based potency assay for IGF-1.

Procedure

Cell Culture

Cells from the human cell line HU-3, established from the bone marrow of a patient with acute megakaryoblastic leukemia, are adapted to grow in the presence of human thrombopoietin. Removal of thrombopoietin results in decreased proliferation and rapid loss of viability. Cells are cultured in RPMI-1640 medium with 2 % heat-inactivated human serum, 2 mM glutamine, 10 mM HEPES (pH 7.2), and 5 ng/ml thrombopoietin in culture flasks. They are grown in suspension at 37 °C in a humidified 5 % CO^2 incubator and are routinely subcultured every 2 or 3 days when densities reach 0.8–1.5 × 106 cells/ml.

Alternatively, the proliferative potency of insulin analogs can be determined by measuring the proliferation of human mammary epithelial cells (HMEC, Clonetics, USA) in culture. HMEC obtained at passage 7 and expanded and frozen at passage 8 are used at passage 10 for all experiments. Cells are cultured in growth medium (fully supplemented MEGM, BioWhittaker, 10 ng/ml hEGF, 5-µg/ml insulin, 0.5-µg/ml hydrocortisone, 50-µg/ml gentamycin, 50-ng/ml amphotericin-B, 13-mg/ml bovine pituitary extract) which is changed every day. For a growth experiment, the medium is changed to assay medium lacking insulin but containing 0.1 % BSA.

Assay

The HU-3 cell growth assay is performed under serum-free conditions in assay medium consisting of RPMI-1640 supplemented with 0.1 % BSA, 10 mM HEPES (pH 7.2), and 50-µg/ml gentamycin. HU-3 cells are washed twice in the assay medium and resuspended at a density of 0.25 × 106 cells/ml. In a typical assay, 100 µl of

a cell suspension (25,000 cells/well) and 100 µl of IGF-1 at varying concentrations are added to flat bottomed 96-well tissue culture plates at 37 °C and 5 % CO2 and cultured for 2 days. 40 µl of alamarBlue™ (undiluted) is then added and the incubation continued for 7–24 h. The plates are allowed to cool to room temperature for 10 min on a shaker, and the fluorescence is read using a 96-well plate fluorometer with excitation at 530 nm and emission at 590 nm.

The HMEC cell growth assay is performed in 96-well Cytostar-T scintillation microplates (Amersham Pharmacia). On day 1, HMEC cells are seeded at a density of 4,000 cells/well in 100 µl of assay medium. Insulin in the growth medium is replaced with graded doses of recombinant human insulin or insulin analog/compounds from 0 to 1 µM final concentration. After 4-h incubation, 0.1 µCi of 14C-thymidine in 10 µl of assay medium is added to each well and plates are read at 48 h and/or 72 h in a microtiter plate scintillation counter.

Evaluation

Recombinant human insulin and IGF-1 are controls used in each assay run, and recombinant human insulin is on each assay plate. Results expressed as relative fluorescence units are plotted against IGF-1/compound concentrations using a four-parameter curve-fitting program. Test compounds are compared with the standard. Typically, the maximal growth response is between three and fourfold stimulation over basal. Response data are normalized to between 0 % and 100 % responses equal to 100 X (response at concentration X – response at concentration zero) divided by (response at maximal concentration – response at zero concentration). Concentration-response data are fit by nonlinear regression employing appropriate software. The analysis of the proliferative capacity of novel recombinant insulin analogs with improved kinetic profile in comparison with IGF-1 and insulin is of tremendous importance.

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Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes

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General Consideration

After having established insulin-like activity of compounds/drug candidates in primary or cultured adipose, muscle, and liver cells or tissues with one or several of the metabolic assays described above (see chapters "▶ Assays for Insulin and Insulin-Like Activity Based on Adipocytes" and "▶ Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms"), it is often useful to elucidate the molecular mode of action of these compounds/drug candidates for further characterization and optimization, in particular regarding selectivity and potency. For this, detailed knowledge in the molecular mechanisms of the insulin signal transduction cascade as well as of cross-talking insulin-like signaling pathways as well as the availability of appropriate reliable and robust cell-free and cell-based assays reflecting these events is required. The following view results from the current experimental findings but, due to limitations in space and rapid progress still made in this area, has to be considered as simplified and temporary, only.

Upon binding of insulin to the IR (insulin receptor) in the major insulin-responsive target cells (adipocytes, myocytes, hepatocytes), the insulin signal transduction machinery, a complex network of protein–protein interactions and protein (serine/threonine/tyrosine) phosphorylation and dephosphorylation cascades between a multitude of cellular signaling components (e.g., enzymes, adaptor proteins, structural proteins), is activated and transmits the insulin signal to a variety of metabolic (predominantly) and mitogenic (to a minor degree) effector systems (e.g., glucose transport, GLUT4 translocation, glycogen synthesis, gene expression, cell differentiation, DNA synthesis) (Biddinger and Kahn 2006; Myers and White 1995; Saltiel 1996; Yenush and White 1997; White 1998; Watson and Pessin 2006). For compounds/drug candidates with insulin-like/sensitizing activity, it is important to differentiate between metabolic and mitogenic signaling in cells of both high (e.g., adipocytes) and low insulin responsiveness (e.g., fibroblasts).

Two major pathways within the insulin signaling cascade have been dissected and are thought to mediate the different biological functions of the hormone: (i) Activation of phosphatidylinositol-3′-kinase (PI3-K) plays a pivotal role for the regulation of glucose transport and cellular trafficking as well as glycogen synthesis and lipolysis by insulin. (ii) Formation of the Shc–Grb2 complex leads to activation of the Ras pathway which has been linked to insulin regulation of both cell growth and gene expression, although this has been questioned recently. Many of the proteins involved in these two cascades have been identified at the molecular level (White 1997, 2002; Holman and Kasuga 1997). The most upstream located one, the insulin receptor (IR), is a transmembrane tyrosine kinase which, when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates a number of intracellular substrates, among them one or more 50- to 60-kDa proteins, including Shc, a 15-kDa fatty acid-binding protein, and several so-called insulin receptor substrate proteins, IRS-1/IRS-2/IRS-3/IRS-4, and Gab, which act as adaptor proteins lacking any enzymatic activity. Following tyrosine phosphorylation, the phosphotyrosine residues of the IRS polypeptides act as docking sites for several Src homology 2 (SH2) domain-containing adaptor molecules and enzymes, including PI3-K, Grb2, SHP2, Nck, and Fyn. The interaction between the IRS proteins and PI3-K occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit.

PI3-K is essential and may even be sufficient for many of the insulin-regulated metabolic processes, including stimulation of glucose transport and glycogen synthesis and inhibition of lipolysis (Yeh et al. 1997; Alessi and Downes 1998; Shepherd et al. 1998; Shepherd 2005; Cheatham et al. 1994; Herbst et al. 1995). PI3-K activation results in the production of phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4)-bisphosphate which leads to binding and concomitant activation of the membrane-associated serine/threonine protein kinases, PDK1/2 (the nature of PDK2 remaining unclear so far). The binding of the pleckstrin homology domain of PKB to the PI3-K-generated phosphoinositides both recruits PKB to the plasma membrane and via phosphorylation by PDK1/2 stimulates its kinase activity. Activation of Akt requires that it undergoes phosphorylation at two sites (Welsh et al. 2005). PDK1 phosphorylates Akt at Thr308, a residue located in its kinase-domain activation loop. In addition, Ser473 in the C-terminal hydrophobic motif of Akt has long been known to undergo phosphorylation, but the identity of the kinase responsible has been controversial. Recently, however, an enzyme complex consisting of mTOR (mammalian target of rapamycin) and RICTOR (rapamycin insensitive companion of mTOR) has been shown to phosphorylate Akt at Ser473 in response to insulin (Hresko and Mueckler 2006; Sarbassov et al. 2005).

Thus the current knowledge favors the view that most metabolic insulin signals emerge from insulin-dependent tyrosine phosphorylation of the IRS proteins (and additional adaptor proteins located in lipid rafts, see section "Insulin-Like Signal Transduction via Plasma Membrane Microdomains (Caveolae and Lipid Rafts)"), whereas the mitogenic insulin action is apparently coupled to tyrosine phosphorylation of the Shc proteins. The latter serve as docking sites for the Grb–SOS complex which possesses GDP–GTP exchange activity for the small G-protein Ras and is activated by binding to Shc. In turn GTP-loaded Ras interacts with and activates the Raf serine kinase which phosphorylates the dual specificity kinase, mitogen/extracellular signal-activated kinase MEK (= MAPKK). Activated MEK phosphorylates the mitogen-activated protein kinases MAPK, ERK1 (p44), and ERK2 (p42), which in turn phosphorylate and activate a number of transcription factors (e.g., c-jun, c-fos) and structural proteins ultimately leading to increased gene and protein expression as well as DNA synthesis in insulin-like fashion (Gustafson et al. 1998).

In addition to the identification of the signal transduction pathways directly leading from the IR to downstream targets, several cross talks have been delineated between signal transmission by insulin and other hormones/growth factors

(e.g., EGF, angiotensin) or diverse exogenous stimuli (e.g., H2O2, phosphoinositolglycans, see section "Glycosyl-Phosphatidylinositol-Specific Phospholipase (GPI-PL) and Insulin-Like Signaling") which either mimic to a certain degree (insulin-like activity) or modulate in a positive or negative fashion (insulin-sensitizing or insulin-desensitizing activity) metabolic and/or mitogenic insulin action in various cellular systems. Since none of these ligands activate the IR kinase directly, their signaling pathways may converge with that of insulin at a more distal signaling step (Argetsinger et al. 1995; Huppertz et al. 1996; Kowalski-Chauvel et al. 1996; Velloso et al. 1996; Verdier et al. 1997; Baron et al. 1998). Soluble phosphoinositolglycan molecules (PIG) which have been shown to exert partial insulin-like effects in diverse cellular and subcellular systems (Müller et al. 1997, 2005; Kessler et al. 1998; Frick et al. 1998; Leon and Varela-Nieto 2004) can also be classified into the latter category. Interestingly, the second-generation sulfonylureas, glibenclamide and, in particular, glimepiride, have been demonstrated to stimulate glucose transport and non-oxidative metabolism in adipose and muscle cells in vitro by causing IR-independent tyrosine phosphorylation of IRS-1/IRS-2 and stimulating the downstream located insulin signaling events (Müller et al. 1994; Müller and Geisen 1996; Müller 2005). This insulin-like signaling in cells of peripheral tissues may explain the insulin-independent blood glucose-lowering activity of glimepiride/glibenclamide as has been reported in a number of animal studies (Geisen 1988; Müller et al. 1995; Müller 2000, 2005), which supplements the potent blood glucose decrease via insulin release provoked by these widely used antidiabetics.

Insulin Receptor (IR) Activation

General Considerations

Insulin exerts its biological effects through a plasma membrane receptor that possesses a tyrosine kinase activity. Binding of insulin to the α-subunits of the IR induces autophosphorylation in trans on tyrosine residues of its β-subunits and thereby stimulates the intrinsic tyrosine kinase activity toward intracellular substrates (such as IRS proteins and Shc) that play crucial roles in the transmission of the signal (see "▶ Assays for Insulin and Insulin-Like Regulation of Energy Metabolism"). Alterations in tyrosine phosphorylation of the IR have been described in insulin-resistant states, such as diabetes and obesity (Combettes-Souverain and Isaad 1998). The discovery of pharmacological agents that specifically activate the tyrosine kinase activity of the IR in cell-free and cell-based assay systems has recently been reported (Zhang et al. 1999) and apparently formed the starting point for a number of drug discovery efforts for future insulin-like or sensitizing drugs for the treatment of insulin-resistant patients.

The first critical node in the insulin signaling network is, by definition, IR and the associated IRS. The IR is a tetrameric protein that consists of two extracellular α-subunits and two intracellular β-subunits. It belongs to a subfamily of receptor tyrosine kinases (RTKs) which also includes the insulin-like growth factor-1 receptor (IGF1R) and an orphan receptor, known as the IR-related receptor (IRR). Each of these receptors is the product of a separate gene, in which the two subunits are derived from a single-chain precursor or proreceptor that is processed by a furin-like enzyme to give single α-β dimers linked with disulfide bonds to form the tetrameric α2β2 holoreceptor. This configuration allows for the creation of a hybrid IR–IGF1R complex, which serves as an additional receptor isoform modulated by insulin-like/insulin-sensitizing stimuli in differential fashion. The activity of the IR is tightly regulated, as unchecked activation or inactivity would lead to profound metabolic (and presumably proliferative) consequences. There are two splice isoforms of the IR, and each has a different affinity for insulin and IGF-1 (DeMeyts et al. 1976).

Functionally, the IR behaves like a classical allosteric enzyme in which the α-subunit inhibits the tyrosine kinase activity that is intrinsic to the β-subunit. Insulin binding to the

α-subunit, or removal of the α-subunit by proteolysis or genetic deletion, leads to partial derepression, i.e., activation of the kinase activity in the β-subunit by stabilizing the open/relaxed rather than the closed/tense conformation of the activation loops of the two receptor halves. Following this initial activation, transphosphorylation of the β-subunits at tyrosines 1158, 1162, and 1163 leads to an additional stabilization of this conformational change even in the absence of ongoing insulin binding (Ellis et al. 1986). The determination of the crystal structure of the tyrosine kinase domain of the human IR has provided a better understanding of the molecular mechanism involved in the stimulation of its kinase activity. In the unphosphorylated state, Tyr-1162 is located in the active site of the enzyme and plays an autoinhibitory role by competing with the binding of protein substrates. This tyrosine remains in the unphosphorylated form in the basal state, because other residues in the activation loop also impair ATP binding. The crystallization of the trisphosphorylated form of the kinase domain has shown that autophosphorylation of these three tyrosines results in a dramatic change in the conformation of the activation loop (Hubbard 1997). This conformation change permits unrestricted access to the binding sites for ATP and protein substrates. It has been postulated that conformational changes induced by ligand binding move the kinase domain of the two β-subunits of the IR nearer to each other, thereby allowing frans-phosphorylation of tyrosine 1162 and adjacent tyrosines in the activation loop.

It is important to note that unlike other RTKs that bind directly to the cytoplasmic tails of downstream effectors, the IR and IGF-1R engage specific proteins that are known as the IRS proteins and mediate the binding of intracellular effectors (see above). In addition, the IR and the IRS proteins share common mechanisms of regulation: They are negatively regulated by dephosphorylation by protein tyrosine phosphatases (PTPs), phosphorylation by serine/threonine kinases which interfere with IR/IRS function by sterically blocking the interaction of IR and IRS or modifying IR kinase activity or downstream signaling of IRS, binding of inhibitory adaptor and signaling proteins (e.g., suppressor of cytokine signaling SOCS1/3, plasma membrane glycoprotein PC-1), and ligand-induced downregulation of IRS at the translational and transcriptional level. Furthermore, the IR is also downregulated at the protein level by ligand-induced internalization and degradation. Some of these regulatory processes may be dysregulated during the pathogenesis of insulin-resistant, hyperinsulinemic states, including obesity, type II diabetes, and metabolic syndrome. The following methods may be helpful for the identification and characterization of insulin-like and insulin-sensitizing compounds/drug candidates as well as of insulin analogs with favorable kinetic (short- or long-lasting) action profiles using cell-free and cell-based assay systems.

Insulin Binding

Purpose and Rationale

IR binding studies have been performed with various animal tissues and primary and cultured cells of rodent and human origin. Human adipocytes can be used to study simultaneously IR binding and metabolic effects of insulin (Hjøllund 1991). The binding tests are of value to characterize newly synthesized insulin analogs (Schwartz et al. 1987; Ribel et al. 1990; Vølund et al. 1991; Robertson et al. 1992).

Preparation of 125I-Insulin

Most investigators use the "chloramine-T procedure" to iodinate insulin. The reaction is carried out in a 20-ml glass vial in an ice bath with continuous magnetic stirring. To 2.5 ml 0.05 M phosphate buffer (pH 7.5), 2.0 mCi Na125I and 15 μl of a 1 mg/ml insulin solution are added. Then, 0.5 ml of a chloramine-T (50 mg/ml) solution is added dropwise over the course of 1 min. After 10 min, 0.7 ml of a freshly prepared sodium

metabisulfite solution (50 mg/ml in 0.05 M phosphate buffer, pH 7.5) is added. One ml of this reaction mixture is transferred to 10 ml 2 % BSA for determination of specific activity. In order to absorb unreacted 125I and damaged products, 2.0 g 20–50 mesh AG 1X-8 resin (Bio-Rad) is added (equilibrated in 1 ml 0.05 M phosphate buffer, pH 7.5, containing 0.1 mg/ml thiomerosal and 20 mg/ml crystalline BSA). The reaction mixture is stirred for 10 min, decanted from the resin, and diluted to a concentration of less than 25 pC/ml in a solution of 0.8 M glycine, 0.2 M NaCl, 0.05 M phosphate (pH 7.5), and 2.5 mg/ml crystalline BSA. The final solution is stored in multiple aliquots at −70 °C.

Binding to Purified Insulin Receptor

IRs are partially purified by affinity chromatography on WGA–Sepharose according to the procedure introduced by Ellis and coworkers (1986). Confluent flasks of cells are solubilized with 50 mM HEPES/KOH (pH 7.6), 1 mM PMSF, and 1 mg/ml bacitracin. The lysate is then centrifuged (100,000 × g, 1 h, 4 °C) and loaded on a 2-ml WGA–Sepharose column for 2 h at 4 °C. After washing of the column with buffer C (50 mM HEPES/KOH, pH 7.6, 150 mM NaCl, 0.1 % TX-100), bound proteins are eluted with 0.3 M N-acetyl-D-glucosamine in buffer C. The IR is further affinity purified batchwise by adsorption to monoclonal anti-hIR antibodies which are subsequently bound to anti-mouse IgG coupled to Sepharose beads. After several washing cycles with buffer C (10,000 × g, 2 min, 4 °C), portions of the hIR-mAb-anti-IgG beads are suspended in 50 µl of 20 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 20 mg/ml BSA, and 0.05 % TX-100 and then incubated with 125I-labeled insulin (300 mCi/mg, 0.2 µCi, 5 µM) in the presence of increasing concentrations of unlabeled insulin for 1 h at 4 °C. Subsequently, the hIR-mAb-anti-IgG beads are collected by centrifugation (10,000 × g, 2 min, 4 °C), rapidly washed with 500 µl of buffer C containing 20 mg/ml BSA, and finally counted in a gamma counter.

Binding to Cultured Cells

Chinese hamster ovary (CHO) cells expressing the wild-type human IR (CHO-hIR) are maintained in Ham's F12 medium supplemented with 10 % FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml) (F12 medium). Cells (~106 cells/100-mm plate) are cotransfected with plasmid DNA (10 µg of expression plasmid plus 2 µg of pSV2NEO) in the form of a calcium phosphate precipitate with the addition of a glycerol shock (20 % glycerol in F12 medium) after 4 h. After 36 h, the cells are split 1:20 and allowed to grow for 24 h prior to addition of G418 (400 µg/ml). Ten to fourteen days later, colonies from five plates (~250–500 colonies) are harvested with trypsin/EDTA (0.05 % trypsin, 0.02 % EDTA) in Ca2+-free and Mg2+-free Hank's basic salt solution and are replated in 24-well culture plates in F12 medium. Two days later, semiconfluent cells (~4 × 104 cells/well) are washed twice with buffer B (100 mM HEPES/KOH, pH 7.8, 120 mM NaCl, 1.2 mM MgSO4, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose, 1 % BSA) and are then incubated with 16 h at 4 °C with 0.5 ml of buffer B containing 125I-labeled insulin (30,000 cpm, 120 Ci/g) plus increasing concentrations of unlabeled insulin. Cells are washed twice, solubilized with 0.5 ml of 0.03 % SDS, and counted in a γ-counter.

Binding to Human Primary Adipocytes

Procedure

Subcutaneous adipose tissue (~4–5 g) is obtained from the abdomen of patients undergoing gastroenterological surgery. Patients suffering from any endocrine or metabolic disorder or taking drugs known to affect metabolism should be excluded. Other exclusion criteria are impaired glucose tolerance measured by determination of fasting blood glucose and the 2-h value after a 75-g oral glucose load. The adipose tissue is finely

chopped and incubated for 90 min at 37 °C in 25 mM HEPES/KOH (pH 7.4), containing HSA (25 g/l) and collagenase (0.5 g/l). The isolated adipocytes are subsequently washed five times in HEPES/KOH (see above) containing 50 g/l HSA.

IR binding studies with isolated human adipocytes are performed in a 300-µl cell suspension containing about 1 × 105 cells/ml in 10 mM HEPES (pH 7.4), 50 g/l HSA at 37 °C. The iodine-labeled ligand ([125I]TyrA14-monoiodinated insulin, specific activity about 350 mCi/mg) in a final concentration of 20 µM is incubated with increasing amounts of unlabeled human insulin and the insulin analog/compound/drug candidate to be tested. The reaction is stopped by adding 10 ml of chilled 0.150 M NaCl and subsequent centrifugation through silicone oil (Pedersen et al. 1981, 1982; Zeuzem et al. 1984). Nonspecific binding is measured by incubating tracer in the presence of a large excess of unlabeled insulin.

For kinetic analysis of binding, the association is studied by incubation of the [125I]TyrA14-monoiodinated insulin for various times (1–240 min), and the reaction is terminated as described above. At each time point, the nonspecific binding is measured and subsequently subtracted from the corresponding data for total binding. Dissociation rates are determined by first incubating isolated human adipocytes at 37 °C with either [125I]TyrA14-insulin or the test compound labeled in the same position for 90 min to achieve steady-state binding conditions. Each incubation mixture is then centrifuged for 60 s. The adipocytes are rapidly washed twice by diluting with buffer to the original volume at 4 °C, and the centrifugations and aspirations are repeated. After the third aspiration, the cells are diluted to the original volume with buffer alone or native insulin or the insulin analog or compound to be tested at a final concentration of 0.2 µM at 22 °C. At this hormone concentration a maximal effect of 125I-insulin dissociation is reported (Podlecki et al. 1984; DeMeyts et al. 1976). The reaction is stopped at various times between 10 and 180 min, and cell-associated radioactivity is determined.

IR Conformational Change Using BRET and FRET

Purpose and Rationale

BRET is a natural phenomenon in which resonance energy transfer occurs between luminescent donor and fluorescent acceptor proteins (Xu et al. 1999; Angers et al. 2000). Resonance energy transfer occurs when part of the energy of an excited donor is transferred to an acceptor fluorophore, which re-emits light at another wavelength. Resonance energy transfer only takes place if the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule overlap sufficiently and if the donor and the acceptor are in proper distance (10–100 Å) and relative orientation (Tsien 1993; Wu and Brand 1994). In the BRET methodology, the first protein partner is fused to Renilla luciferase (Rluc), whereas the second protein partner is fused to a fluorescent protein (e.g., yellow fluorescent protein YFP). If the two partners do not interact, only one signal, emitted by the luciferase, can be detected after addition of its substrate cœlenterazine. If the two partners interact, resonance energy transfer occurs between Rluc and the YFP, and an additional signal, emitted by the YFP, can be detected. The strict dependence of BRET on the molecular proximity between energy donors and acceptors makes it a system of choice to study changes in the interaction between two proteins, which usually (in the unstimulated state of the target cell) exist as separate entities or are already assembled in a preformed complex susceptible to changes in their relative neighborhood or orientation in response to appropriate stimuli.

Binding of insulin to the IR induces a conformational change that brings the two β-subunits of the IR into close proximity, allowing transphosphorylation of one IR β-subunit by the other subunit (Hubbard 1997; Combettes-Souverain and Isaad 1998). This autophosphorylation stimulates the tyrosine kinase activity of the IR toward intracellular substrates, such as IRS-1 and initiated downstream signaling to the terminal

metabolic insulin effector systems, such as GLUT4 translocation, which are impaired in insulin-resistant and diabetic states. Therefore, methods that allow monitoring of the activity of the IR constitute important tools for the search for insulin-like compounds and putative antidiabetic drugs.

Biazzo-Ashnault and coworkers (2001) developed a FRET-based assay for monitoring IR kinase activity. In this assay, the IR has to be immobilized on a microtiter plate with anti-IR antibody. A kinase reaction is then performed using a phosphorylation cocktail containing a biotinylated substrate. After incubation with a fluorescent donor, a streptavidin-labeled fluorescent acceptor that to the biotinylated substrate is added. Energy transfer only occurs if the fluorescent donor coupled to anti-phosphotyrosine antibody is in close proximity to the streptavidin-labeled fluorescent acceptor, i.e., if the peptide substrate is phosphorylated on tyrosine residues. Obviously, this is still a heterogeneous assay involving time-consuming incubations and washing steps.

By contrast, Boute and coworkers (2001) developed a completely homogeneous test, based on the BRET methodology, to follow IR kinase activity. A chimeric human IR, in which one IR β-subunit is fused to Renilla luciferase (Rluc) and the other IR β-subunit is fused to yellow fluorescent protein (YFP), is produced in HEK cells and partially purified by wheat-germ lectin chromatography. Insulin-induced conformational change could be detected as an increase in energy transfer (BRET signal) between Rluc and YFP. This BRET signal corresponds to the ligand-induced conformational changes of the IR before any phosphorylation event and faithfully reflects the activation state of the IR (Boute et al. 2001).

Procedure

Cell-Based Assay

HEK-293 cells maintained in DMEM supplemented with 4.5 g/l glucose and 10 % FBS (Invitrogen) are seeded at a density of 1.2 × 106 cells per 100-mm dish. Transient transfection is performed 1 day later using FuGene 6 (Roche Diagnostics) with either 0.3 μg of IR-Rluc cDNA and 0.3 μg of empty vector or with 0.3 μg of IR-Rluc and 0.3 μg of IR-YFP cDNAs per 100-mm dish. BRET measurements on intact cells are performed essentially as described by Angers and coworkers (2000). Two days after transfection, HEK-293 cells are detached with Trypsin-EDTA and resuspended in PBS. Approximately 60,000 cells per well are distributed in a 96-well microplate. Cells are incubated for 2–10 min in the absence or presence of 100-nM insulin. Coelenterazine is added at a final concentration of 5 μM, and light-emission acquisition is started immediately. For use of adherent cells, cells are transfected exactly as described above, but 1 day after transfection, cells are transferred into 96-well microplates (white CulturPlate-96, Packard) at a density of 30,000 cells per well.

In Vitro Assay on Partially Purified Fusion IR

BRET measurements are performed using partially purified fusion IR. Two days after transfection, cells are extracted in buffer containing 1 % Triton X-100, 20 mM MOPS, 2.5 mM benzamidine, 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 1 μg/ml each of aprotinin, pepstatin, antipain, and leupeptin. Fusion receptors are partially purified by chromatography on wheat-germ lectin Sepharose by Tavare and Denton (1988). Partially purified fusion IR are aliquoted and stored at −80 °C for subsequent use. In vitro measurement of BRET signal is performed using 4.5 μl of wheat-germ lectin (WGL) eluate (approximately 2 μg of proteins) preincubated in 96-well microplates for 45 min at 20 °C in a total volume of 60 μl containing 30 mM MOPS, 1 mM Na3 VO4, and different concentrations of ligands. Coelenterazine (7 μl, 2.6 μM final concentration, Molecular Probes, USA) is added to the preparation, and light-emission acquisition at 485 nm (filter window 20 nm) and 530 nm (filter window 25 nm) is started immediately using a microplate analyzer (e.g., Fusion, Packard).

Evaluation

The BRET ratio has been defined previously (Angers et al. 2000) as [(emission at 530 nm) − (emission at 485 nm) × Cf]/(emission at 485 nm), where Cf corresponds to (emission at 530 nm)/(emission at 485 nm) for the Rluc fusion protein expressed alone in the same experimental conditions (i.e., IR-Rluc transfected alone).

The BRET signal detected in this assay closely reflects the activation state of the IR independently of any phosphorylation reaction. It is a completely homogeneous procedure, requiring no washing step. Thus, the procedure allows for very rapid determination of the activation state of the IR and can be easily used in throughput screening tests for the search of novel compounds/drug candidates with insulin-like activity. Indeed, partially purified fusion IR can be prepared on a large scale by WGL chromatography and stored at −80 °C for subsequent use. This preparation can be distributed in an automated way in 96-well microplates, and the effect of molecules on IR activity can be measured within a few minutes using the BRET method. Therefore, the same preparation can be used for multiple screening rounds. Similar procedures should be applicable to any receptor in which ligand binding induces dimerization or conformational changes detectable by BRET. Thus, this assay could be a valuable tool for the search of compounds/drug candidates with antidiabetic properties.

For screening purposes it turned out that the in vitro rather than the cell-based assay has to be used (Boute et al. 2001). The chimeric IR is correctly expressed and functional in HEK293 cells, but an unexpected high basal BRET signal was observed. Obviously, the choice between an in vivo and in vitro assay has to be done on a case-by-case basis. However, it must be kept in mind that in an intact cell assay, cells have to be distributed and cultured in microtiter plates for each screening round, which is highly time-consuming.

Comparison with FRET Technology

Before the development of BRET in 1999, fluorescence resonance energy transfer (FRET) has been used widely to study protein–protein interactions or to monitor conformational changes within a given protein. In the FRET methodology, luciferase is replaced by a fluorophore that is excited using monochromatic light at the appropriate wavelength (Cubitt 1995; Hovius 2000; Zacharias 2000). A major advantage of FRET is to permit, under microscopic observation, visualization in a single living cell of protein–protein interactions at the subcellular level (Wouters 2001). This is difficult to perform with the present version of the BRET methodology because of the low intensity of light emission by luciferase.

However, FRET presents several disadvantages that make BRET a technology of choice in many cases. In FRET, the donor fluorophore must be excited using monochromatic light. Because of the overlapping absorption and emission spectra of the existing mutants of GFP, direct excitation of the acceptor fluorophore by the light used to excite the donor often complicates the interpretation of the results. This obviously does not occur in BRET because excitation of the donor partner does not require exogenous illumination. Therefore, the quantification of resonance energy transfer is much easier in BRET than in FRET (Tsien 1998). Another related advantage of BRET is that the relative levels of expression of donor and acceptor partners can be quantified independently by measuring the luminescence of the donor and the fluorescence of the acceptor (Xu 2002). Moreover, in FRET, excitation of the donor by illumination of the sample can induce photobleaching of the donor fluorophore, which results in loss of signal with time (Tsien et al. 1993; Cubitt 1995). In addition, in FRET, illumination of the sample often induces autofluorescence that is emitted by endogenous cell components. The resulting background can easily be dealt with when FRET is assessed in a single cell with imaging techniques, by using an unlabeled region of the cell as an internal reference for the autofluorescence background (Tsien 1998). However, this background might become a serious problem when interactions are measured on populations of cells or molecules using non-imaging methods, such as fluorometry in microtiter plates (as would be desirable for throughput screening assays).

Recent studies indicate that a tenfold improvement in sensitivity could be attained by using BRET rather than FRET, in an otherwise identical assay (Arai 2000, 2001). Finally, it must be taken into account that the instrumentation requirements for BRET are simpler than those for FRET, which needs an excitation light source. A comparative evaluation of BRET vs. FRET technologies with emphasis in their putative application in high-throughput screening has been provided by Boute and coworkers (2002). A chimeric human insulin receptor, in which one receptor β-subunit was fused to Renilla luciferase and the other receptor β-subunit was fused to yellow fluorescent protein, was produced in HEK cells and partially purified by wheat-germ lectin chromatography. Insulin-induced conformation change could be detected as an increase in energy transfer (BRET signal) between Renilla luciferase and the yellow fluorescent protein.

IR Tyrosine Phosphorylation

Phosphorylation with Purified IR

Procedure

Preparation of Purified IR

IR is extracted and purified from human placenta as described by Yamaguchi and coworkers (1983) with modifications from Ozawa and coworkers (1998). After preparation of placental membranes from one piece of fresh normal human placenta by differential centrifugation, a suspension containing the membrane proteins is mixed with the same volume of a 50 mM Tris/HCl buffer (pH 7.4) containing 2 % Triton X-100 and protein inhibitors (2 mM BAEE, 1 mg/ml pepstatin, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.1 mM PMSF) for 45 min at 4 °C with stirring. A clear supernatant is obtained by centrifugation (100,000 × g, 90 min, 4 °C) and is transferred to 40 ml of a WGA–Sepharose affinity column. The fractions including the protein are pooled and applied to 6.3 ml of an insulin–Sepharose column. After thorough washing of the column with 50 mM Tris/HCl (pH 7.4) containing 1.0 M NaCl, 0.1 % Triton X-100, and 0.1 mM PMSF, the IR is eluted with 50 mM acetate buffer (pH 5.0) containing 1.0 M NaCl and 0.1 % Triton X-100. The active fractions assessed by [7] I-insulin binding activity are collected and concentrated by pressured dialysis using a Diaflo ultrafiltration membrane PM-30 (Amicon Inc.). The purified IR is stored at −80 °C. The purity of the IR is assessed by SDS-PAGE and silver staining. The purified IR results in two bands of 135 and 90 kDa, which correspond to α- and β-subunits of the IR, respectively. The stability of the purified IR is determined by the kinase activity of IR at various time intervals after elution of the insulin–Sepharose column. There is no apparent change for the kinase activity up to 1 month, which is evaluated from the magnitude of phosphorylated artificial substrate, poly (Glu80Tyr20), incubated with insulin and its receptor. The IR prepared by the present method is thus of sufficient stability. Kinase activities of purified IR are typically found to change from one extraction to another. In order to control this kinase activity so that it would to be the same throughout the experiment, the concentration of the purified IR has to be adjusted by dilution before the IR is applied for measurements in the present method.

Radioactive Assay

The direct effect of insulin/compounds/drug candidates on the purified IR is assessed by using random copolymers of (Glu80Tyr20)n serving as a substrate for the IR kinase according to Braun and coworkers (1984). The purified IR is preincubated with 0.48 μM insulin at 22 °C for 1 h in 630 μl of a solution (solution A) containing 25 μM ATP and 5.0 mM MnCl2. The random copolymer of (Glu80Tyr20)n is dissolved in a buffer solution (solution B) containing 0.1 % Triton X-100, 5.0 mM MnCl2, 10 mM MgCl2, 50 μCi [γ-32P]ATP, and 50 mM HEPES/KOH (pH 7.4) to its final concentration of 0.5 mg/ml. A 30-μl portion of solution B is taken into a microtube. Phosphorylation of (Glu80Tyr20)n is initiated by adding 15 μl of the preincubated IR solution into the microtube, and

the solution is incubated for 2–120 min. After each incubation time, the sample is applied to filter paper (Whatman 3 MM). The filter paper is washed six times with 10 % TCA and then with acetone and finally air-dried. Radioactivity is counted with a liquid scintillation counter. The magnitude of phosphorylated random copolymers incubated with maximally effective concentrations of insulin is about four to six times higher than that without insulin at each time, demonstrating the activity of the IR preparation by the present purification method. The insulin-like activity of compounds/drug candidates is calculated as percentage of the maximal insulin effect.

Phosphorylation with Intact Cells

Detection by Immunoblotting

IR is immunoprecipitated from cell lysates (9 × 106 cells) by incubating with rabbit anti-IR antibody (3 μg) in buffer A (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 % Nonidet-P40, 1 % sodium deoxycholate, 0.1 % SDS, 1 mM sodium pyrophosphate, 100 μM pervanadate, complete protease inhibitor mix tablets) overnight at 4 °C with rotating. Prewashed protein G-sepharose beads (50 μl of packed beads) are added, and the solution is mixed in a rotator for 2 h at 4 °C. The beads are washed three times in buffer A, and protein is eluted using SDS sample buffer (65 mM Tris/HCl, pH 6.8, 1.3 % SDS, 13 % glycerol, and bromophenol blue) with heating at 95 °C for 5 min. SDS-PAGE is carried out by the method of Laemmli on 10 % polyacrylamide gels using a mini apparatus (e.g., Novex) in 25 mM Tris base, pH 8.3, 192 mM glycine, 0.1 % SDS at 140-V constant voltage. Electrophoretic transfer from SDS-polyacrylamide gels to polyvinylidene fluoride (PVDF) (anti-phosphotyrosine probing) or nitrocellulose (anti-IR probing) is carried out in a mini-blot apparatus (e.g., Novex). The blotting conditions are 40 V for 2 h in 12 mM Tris base, pH 8.3, 96 mM glycine, and 20 % methanol. Membranes are blocked in 1 % polyvinylpyrrolidone (PVP-40, PVDF membranes) or 5 % nonfat milk (nitrocellulose) and washed in 25 mM Tris (pH 7.2), 150 mM NaCl, and 0.1 % Tween 20. Phosphotyrosine-containing proteins are detected using anti-phosphotyrosine (4G10) antibody-HRP-conjugated diluted 1:2,000 in 1 % PVP-40. The β-subunit of the IR is detected using rabbit anti-IR diluted 1:1,000 in 5 % milk followed by 1:2,000 dilution of anti-rabbit-HRP. Reactive proteins are visualized using chemiluminescent detection and a LumiImager.

Detection by ELISA

Purpose and Rationale

A new assay method has been introduced by Ozawa and coworkers (1998) for the identification and evaluation of compounds/drug candidates with insulin-like activity and interfering with the insulin signaling pathway. It is based on the on/off switching mechanism of the IR-mediated insulin signaling. The Y939 substrate peptide, a synthetic peptide of 12 amino acid residues, consists of a tyrosine-phosphorylated domain of IRS-1 and of a binding domain of IRS-1 to PI-3 K. The Y939 peptide is immobilized on an avidin-coated 96-well microtiter plate. Upon binding of insulin to the IR prepared in total or immunoprecipitated from lysates of treated cells, insulin stimulates its receptor kinase activity in a sample solution and phosphorylates the tyrosine residue of the Y939 peptide immobilized on the well surface. The sample solution is washed out, and a selective binding protein for the phosphorylated tyrosine, monoclonal anti-phosphotyrosine antibody labeled with horseradish peroxidase, is added and forms a phosphorylated peptide–antibody complex. The amount of complex thus formed is detected by enzymatic amplification with horseradish peroxidase that can produce a green-colored product with an absorbance at 727 nm capable of being detected by colorimetry. The amount of phosphorylated Y939 peptide thus measured is expected to be a selective and sensitive measure of the extent of the insulin signaling induced by compounds/drug candidates.

Procedure

Preparation of Biotin-Y939 Substrate Peptide

The Y939 substrate peptide is biotinylated by reaction of a maleimide group of biotin-PEAC5-maleimide with a thiol group of cysteine introduced at the carboxy-terminal end of the Y939 peptide. 1.0 mg of Y939 peptide (7.2 × 10–7 mol) is dissolved in 160 µl of PBS buffer (150 mM NaCl, 3.0 mM KCl, 10 mM phosphate buffer, pH 7.2), and 2.5 mg of biotin-PEAC5-maleimide (3.6 × 10–6 mol) dissolved in 40 µl of DMSO is added dropwise to the Y939 peptide solution with gentle stirring. The reaction mixture is left for 12 h at 25 °C. The reactants are purified by reverse phase HPLC performed on a 801-SC system (Japan Spectroscopic Co., Japan), equipped with a Kaseisorb LC ODS-300–5 column and a UV detector, using a linear gradient of 0–80 % acetonitrile in 0.05 % trifluoroacetic acid at a constant flow rate of 1.0 ml/min over 60 min. The eluents are monitored by UV absorbance at 220 nm. Ten milliliters of an eluent containing biotinylated peptide is collected and lyophilized. To evaluate the ratio of covalently immobilized biotin molecules to the Y939 peptide, the amounts of the Y939-immobilized biotin and the Y939 peptide in the eluent are determined as follows. After lyophilization, the biotin-Y939 conjugate is dissolved in 1.0 ml of a PBS buffer, and its absorbance at 280 nm is measured. Assuming that the observed absorbance is due to a tyrosine residue in a Y939 peptide, total amount of Y939 peptide is obtained from the absorbance and molar absorptivity for tyrosine of 1.34 × 103 M^{-1} cm^{-1}. The amount of Y939-conjugated D-biotin is evaluated by a spectrophotometric assay method.

Immobilization of the Y939 Peptide on a 96-Well Microtiter Plate

All 96 wells of a microtiter plate are incubated with 150 µl each of avidin solution (0.1 mg/ml avidin in PBS buffer) over 18 h at 4 °C. After the immobilization of avidin on the well, each well of the plate is washed with a PBS-T buffer solution (0.05 % Tween 20 in PBS buffer). To fill up space between the immobilized avidin molecules with BSA molecules, a 200-µl portion of a BSA solution (1.0 % BSA in PBS buffer) is added to each well, and the plate is left for 4 h at 4 °C. The BSA solution is discarded, and each well is washed four times with the PBS-T solution. After washing with the PBS-T solution, 150 µl of biotin-Y939 conjugates (1.0 µg/ml in the PBS-T solution) is added to each well, and biotin–avidin complexation is carried out for 1 h at 4 °C. An excess of the conjugates is discarded, and the well is washed four times with the PBS-T solution.

Kinase Reaction

A sample solution is prepared in a microtube, consisting of 0.05 % Triton X-100, 50 µM ATP, 1.7 mM MnCl2, a given concentration of IR, 50 mM HEPES/NaOH (pH 7.4), and each concentration of insulin. The sample solution is incubated in a microtube at 25 °C for 10 min, and 150-µl portion of the sample solution thus prepared is introduced in a given well. For obtaining a background absorbance, the sample solution without insulin is also introduced in one of the wells. The plate is shaken on a mixer for 1 h at 37 °C to react the IR immune complex with the Y939 peptide immobilized on the well surface. The plate is washed three times with the PBS-T solution and then two times with a TBS solution (20 mM NaCl, 3.0 mM KCl, and 20 mM Tris/HCl, pH7.4).

Enzymatic Reaction and Absorptiometric Assay

After dilution of monoclonal anti-phosphotyrosine antibody labeled with horseradish peroxidase in the TBS solution to its final concentration of 0.5 µg/ml, 150 µl of the solution is added into each well. After standing for 2 h at room temperature without shaking, the plate is washed six times with a TBS-T (0.05 % Tween 20 in the TBS buffer) solution. To evaluate the amount of the antibody specifically bound to the phosphorylated Y939 peptide, 175 µl of a DA-64 solution (0.4 mg/mL of DA-64 in 2.0 % H2O2, 50 mM citric acid, and 100 mM Na2HPO4) is added to each well and is incubated with shaking for 1 h at 37 °C in the dark. After the enzymatic reaction, the DA-64 solution is subjected to

absorbance measurement. A 150-µl portion of the DA-64 solution is diluted with 1.0 ml of Milli-Q water, and the absorbance of the solution in a 1.0-ml cuvette is measured at 727 nm against water with a spectrophotometer.

Evaluation

For obtaining net insulin-dependent signals (absorbance), a background absorbance obtained by introducing a buffer solution without insulin is subtracted from the observed absorbance in the presence of a given concentration of insulin. Changes in absolute value of this net absorbance are not negligible from one microtiter plate to another, seemingly because of unexpected alteration in the experimental conditions such as concentration of antibody and/or a decrease in activities for IR kinase. In order for the net absorbances obtained from one microtiter plate to be comparable to those obtained from another, the measured net absorbance for a given concentration of insulin is normalized against absorbance for 1.0 µM insulin in terms of B/Bmax × 100, where B is the absorbance for each concentration of insulin and Bmax that for 1.0 µM insulin.

Detection by TRF

Purpose and Rationale

A time-resolved fluorescent assay using Wallac's DELFIA system (DELFIA assay) has been developed to monitor changes in IR tyrosine phosphorylation. Usually phosphorylation levels are analyzed by measuring the incorporation of radioactive phosphate (Tornqvist and Avruch 1988), by immunoblotting, or by ELISA (Krutzfeldt et al. 1999) using phosphotyrosine antibodies (see section "IR Tyrosine Phosphorylation"). These methods suffer from various drawbacks including the use of radioisotopes, tedious and limited sample analysis with respect to immunoblotting, and low dynamic range, in particular in colorimetric assays using ELISA. In contrast, the DELFIA assay using time-resolved fluorometry is a highly sensitive, nonradioactive assay performed in microtiter plates amenable to automation. A formate similar to that of the DELFIA assay has been used by Okada and coworkers (1998) to measure the effect of sodium orthovanadate on IR autophosphorylation of erythrocytes from normal and diabetic patients.

Procedure

Cell Culture

Chinese hamster ovary (CHO) cells overexpressing the hIR (CHO-hIR) are maintained in DMEM, 10 % (by vol.) FBS, and 1 % (by vol.) penicillin–streptomycin at 37 °C under 5 % CO2. Before treatment with insulin/compounds/drug candidates, cells are serum starved for at least 14 h by incubation in DMEM, 0.5 % (w/v) BSA, and 1 % penicillin–streptomycin.

In Vitro Phosphorylation of the IR

Wheat germ agglutinin-purified hIR is prepared from total membranes of CHO-hIR as described (sections "Binding to Purified Insulin Receptor" and "Phosphorylation with Purified IR") and then phosphorylated by incubating 700 µg of hIR with 50 mM HEPES (pH 7.4), 10 mM MnCl2, 0.1 % (by vol.) Triton X-100, 1 µM insulin, 0.5 mM ATP, and 100 µM sodium orthovanadate for 1 h at room temperature. The phosphorylated hIR is purified by passing the solution over a PD-10 column (Pharmacia) in 50 mM HEPES (pH 7.4), 0.1 % Triton X-100, and 100 µM sodium orthovanadate and concentrated by centrifugation in a 2-ml Amicon concentrator (30MWCO).

Biotinylation of Anti-IR Antibody

Anti-IR antibody Ab-3 (800 µl of 1.3 mg/ml, Upstate Biotechnology) is biotinylated by incubating overnight at 4 °C with 60 µM of freshly prepared Sulf-NHS-LC-biotin (Pierce) dissolved in 4 mM bicarbonate (pH 7.8), 35 mM NaCl, and 1 % (by vol.) glycerol (final vol. 1 ml). Buffer exchange (PBS) and excess biotin removal are facilitated by diluting and concentrating in centricon units (30MWCO). The level of biotin incorporation is quantitated by comparing to a

standard curve constructed using the biotin-induced displacement of avidin from the dye 2-(4-hydroxybenzene) azo benzoic acid (HABA; Pierce; absorption of HABA-avidin 500 nm) according to the manufacturer's instructions. The typical yield is between 4 and 10 mol of biotin per mole of protein.

Preparation of Phosphorylated IR from Cells

Cells are grown in 6-well plates until ~60 % confluency and serum starved overnight. The cells are washed with DMEM, incubated in 2 ml of the same medium, and treated with various concentrations of insulin/compounds/drug candidates for 5–10 min. Following the treatments the medium is removed, and the cells are immediately frozen in liquid nitrogen and then stored at −80 °C until cell lysis. Cells are lysed in 200 µl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 mM pyrophosphate, 100 µM pervanadate, "Complete" protease inhibitor tablet). The cells are gently scraped from the dishes and centrifuged (14,000 rpm, 15 min, 4 °C, Eppendorf centrifuge). The supernatants are used in the DELFIA assay.

Quantitation of Receptor Phosphorylation

All incubations of the DELFIA assay are carried out at room temperature with agitation in a final volume of 200 µl of DELFIA assay buffer unless otherwise stated. Streptavidin-coated plates are washed with DELFIA wash buffer for 15 min. After removal of wash buffer, anti-IR-biotin or anti-EGFR-biotin (100 ng/well) is added for 30 min. The plate is washed three times with wash buffer, and cell lysates are added, and the plate is incubated for 1 h. The plate is again washed three times with wash buffer, europium-labeled anti-phosphotyrosine antibody (100 ng/well) is added, and the plate is incubated for 1 h. Following extensive washing (at least five times), 180 µl of enhancement solution is added. The plate is shaken vigorously for 30 min and read in a microtiter plate reader using a europium protocol (excitation at 340 nm, emission at 615 nm, delay 400 ps, 1,000 ps/cycle).

Optimization of Conditions

This time-resolved fluorescent (TRF) assay for measuring tyrosine-phosphorylated proteins in cells has been optimized by Waddleton and coworkers (2002). For this, biotinylated anti-IR antibody is used to capture both phosphorylated and non-phosphorylated IR from cells and for immobilization to the streptavidin-coated plate. Europium-labeled anti-phosphotyrosine antibody is used to bind only the phosphorylated IR. Time-resolved fluorometry of europium is then used to measure the level of phosphorylated IR. To establish conditions for the DELFIA assay, in vitro phosphorylated IR partially purified from CHO-hIR cells is used. The optimum concentration of both the capture antibody (anti-IR antibody) and the anti-phosphotyrosine antibody is typically ~100 ng. Using in vitro phosphorylated partially purified CHO-hIR (0.13–0.52 µg/ml), the Eu signal typically reaches three orders of magnitude and remains linear to up to ~800,000 Eu counts. The minimal amount of phosphorylated IR necessary for a reliable signal is ~0.1 pmole (in 200 µl). The signal is specific for the phosphorylated form of IR since non-phosphorylated IR tested at similar concentrations does not produce a significant increase in background signal. Establishing the optimal conditions for the DELFIA assay, it has been demonstrated that the phosphorylation status of the IR does not affect the ability of the capture antibody to immunoprecipitate IR from cell lysates.

Evaluation

Stimulation of the cells with insulin followed by measurement of IR phosphorylation by DELFIA resulted in an EC50 of ~25 nM. This is similar to that reported by Biazzo-Ashnault and coworkers (2001) who used a FRET assay to measure IR tyrosine kinase activity by following phosphorylation of a synthetic peptide. Using the nonspecific protein tyrosine phosphatase (PTP) inhibitor, pervanadate, a tenfold increase in basal IR phosphorylation is typically detected by the DELFIA assay, whereas analysis of the same samples by immunoblotting reveals a threefold

increase, only. This difference is likely due to enhanced sensitivity of the DELFIA assay, particularly in the lower range where quantitation of scanned immunoblots is less accurate. Pervanadate results in a 50 % increase in insulin-induced IR phosphorylation compared to treatment with ligand alone (10 nM), as measured by both DELFIA and immunoblotting. Comparing the well-established technique of immunoblotting with anti-phosphotyrosine antibodies to the DELFIA assay, similar results are usually obtained. In addition, Waddleton and coworkers (2002) demonstrated that this assay can be used to monitor IR phosphorylation in a normal (i.e., IR non-overexpressing) cell line and in response to phosphatase inhibitors. They also show the rapid and easy adaptation of the Delphia format assay to monitor changes in the phosphorylation status of other cellular proteins. These findings provide strong evidence that changes in the phosphorylation status of the IR in response to PTP inhibitors and most likely insulin-like compounds/drug candidates can be monitored using the DELFIA assay.

Yeast-Based Method

General Considerations

A number of mammalian cell-based assays have been developed in order to analyze IR kinase activity in response to compounds/candidate drugs in a cellular environment that most closely resembles the natural physiological conditions (see sections "Phosphorylation with Intact Cells" and "IR Conformational Change Using BRET and FRET"; Sims and Allbritton 2003; Sato et al. 2002). However, mammalian cell-based assays are expensive and time-consuming. Moreover, the effects of redundant processes on the measured output can be difficult to control and to distinguish from the effects that are specific for the IR. The yeast *Saccharomyces cerevisiae* represents an inexpensive and rapid alternative for measuring the activity of the IR in a heterologous, yet eukaryotic environment. The fact that yeast does not have any endogenous RTKs and no bonafide non-receptor tyrosine kinases offers the advantage of a null background for the expression of the mammalian IR and for the measurement of the effects of compounds/drug candidates on the specific target (Barberis 2002; Gunde 2004; Hughes 2002). A yeast growth selection system for monitoring mammalian RTK activities and for detecting inhibition by specific inhibitors, which is based on the Ras recruitment system (RRS) developed by Aronheim and collaborators, has been introduced recently (Broder et al. 1998; Aronheim et al. 1994, 1998) and further modified and improved by Gunde and Barberis (2005). In particular, it may be useful for the throughput screening for insulin-like compounds/drug candidates acting directly at the IR.

Purpose and Rationale

Cdc25p, the yeast homologue of mammalian son of sevenless (Sos), is a Ras guanyl nucleotide exchange factor (Ras-GEF) functioning upstream of the Ras proteins in *S. cerevisiae*. The yeast temperature-sensitive cdc25–2 strain cannot grow at 37 °C, because the mutant Cdc25 protein carried by this strain is unable to activate the endogenous Ras protein at this non-permissive temperature. Aronheim and coworkers (1994, 1998) have shown that membrane targeting of the mammalian Sos is sufficient to activate Ras leading to complementation of the cdc25–2 temperature-sensitive allele. The yeast growth selection system for monitoring human IR is based on the RRS.

Procedure

Yeast Strains and Yeast Spotting Assay

Cells from the yeast strain cdc25–2 (Matα, ade5, cdc25–2, his7, lys2, met10, trp1, ura3–52, leu2) transformed with the hIR are plated on 2 % glucose agar plates. Four different colonies of each transformation are picked and spotted onto two sets of galactose plates (3 % galactose, 2 % raffinose, and 2 % glycerol) that are incubated at 25 °C or 37 °C. For this spotting assay, the yeast

suspensions are not adjusted to the same concentration prior to spotting, whereas for the serial dilutions, the yeast cultures are adjusted to the same concentration. Plates incubated at 25 °C are scanned after 4 days, whereas plates incubated at 37 °C are scanned after 7 days. For serial dilutions, exponentially growing cultures are serially diluted in sterile water starting with a concentration of 4 × 107 cells/ml. Five microliters of each dilution is spotted onto two sets of galactose plates, corresponding to about 100,000, 20,000, 4,000, 800, 160, and 32 cells per spot. Plates are incubated at 25 °C and 37 °C and scanned after 4 and 11 days, respectively.

Preparation of Yeast Extracts and Immunoblot Analysis

Exponentially growing *S. cerevisiae* cultures expressing the hIR are diluted to a final absorbance at 600 nm (A600) of 0.5 in 4 ml of Ura/-Leu/-Trp dropout medium containing 2 % glucose and are grown for 4 h. Cells are harvested by centrifugation, and whole-cell extracts are prepared as described (Kaiser et al. 1994). Samples of whole-cell extracts are subjected to SDS-PAGE and blotted on nitrocellulose membranes following standard protocols.

Growth

Exponentially growing cultures expressing hIR are washed once with water and diluted to a final A600 of 0.1 ml of Ura/-Leu/-Trp dropout medium containing 3 % galactose, 2 % raffinose, and 2 % glycerol. As a control, cells expressing M-Grb2-Ras (Q61L)ÄF are similarly grown, washed, and diluted to a final A600 of 0.03. One hundred fifty microliters of the diluted cells is added to 96-well plates containing 1.5 µl of the compounds/drug candidates at the desired concentrations (1 % final DMSO concentration). The plates are incubated at 37 °C, and growth at the indicated time points is quantified by measuring A595 with a microtiter plate reader.

Evaluation

As alternatives to this yeast Sos recruitment system (RRS) to monitor protein–protein interactions at the plasma membrane, the reverse RRS for the identification of protein–protein interactions with integral membrane proteins (Kohler and Müller 2003), the split-ubiquitin system (Hubsman et al. 2001), and the SCINEX-P system (Stagljar et al. 1998) have been developed during the last decade. The split-ubiquitin system has been used to detect novel proteins that are associated with the mammalian ErbB3 RTK (Thaminy et al. 2003). Ehrhard and coworkers (2000) developed a method that uses the yeast G-protein signaling pathway as readout to select for protein–protein interactions. This assay has been validated with known binding partners, such as the RTK fibroblast growth factor receptor 3 (FGFR3) and SNT-1, that constitutively bind to the FGFR3 intracellular domain. The latter two methods are feasible for detection of interactions between an integral membrane protein and a soluble cytoplasmic protein, but, in contrast to the direct RTK assays, the binding of cytoplasmic proteins to the RTKs is independent of receptor activity. Furthermore, Kohler and Müller (2003) recently applied an adaptation of the RRS to the analysis of interactions between two membrane-associated proteins, namely, the myristylated and phosphorylated EGFR intracellular domain and a myristylated Grb2–Ras fusion protein. Due to the fact that yeast cells do not have endogenous mammalian-type tyrosine kinases, the yeast-based growth selection system offers the advantage of a null background eukaryotic organism for expression of these mammalian membrane-bound kinases, such as the IR. This assay may be of value in throughput screenings for the identification of cell-active activators (and inhibitors) of RTKs.

Phosphorylation of Signaling Components by Insulin and Insulin-Like Stimuli

General Considerations

Protein kinases (PK) and phosphatases (PP) have been implicated in a variety of cellular processes such as proliferation, differentiation, metabolism, and apoptosis. Over one-third of the proteins in

the human proteome are phosphoproteins, and the family of PK and PP represents up to 5 % of the human genome. These enzymes may cause an increase in or suppression of the activity of other enzymes, receptors, regulatory proteins, or transcription factors, mark proteins for destruction, allow proteins to move from one subcellular compartment to another, or enhance or impede protein–protein interactions. In many cases these components and processes are embedded in various signaling pathways, such as the insulin signal transduction cascades. Thus, any change in the level, activity, or localization of PK and PP has a major impact on the regulation of these processes. Not surprisingly, the dysregulation of protein phosphorylation has been associated with diabetes.

There are estimated to be over 500 different PK in the human proteome that catalyze the transfer of phosphate from ATP to serine, threonine, and tyrosine. PK can be divided into two broad classes, serine/threonine and tyrosine kinases, although a few PK are able to phosphorylate both types of amino acid residues (dual specificity kinases). Because of the key role that PK play in cellular functions, they have become one of the most important drug targets. As such, protein phosphorylation is in the focus of much interest for the development of compounds to selectively regulate the activity of diabetes-related PK. Thus, there is a need for assay systems that monitor the activity of these enzymes under a variety of experimental conditions and for the development of selective inhibitors or activators of these enzymes for a variety of therapeutic applications, among them metabolic diseases, such as diabetes.

Purpose and Rationale

One strategy is based on the identification of selective novel inhibitors of PK, which have been implicated with interference of insulin signal transduction via serine phosphorylation of key signaling components, such as IRS-1 (see "► Assays for Insulin and Insulin-Like Regulation of Energy Metabolism" and section "Expression, Phosphorylation, Activity and Interaction of Insulin Signaling Components"), thereby blocking activation of downstream components, such as PI3-K. These compounds/drug candidates should act as insulin sensitizers bypassing the insulin resistance in muscle and liver tissues of type II diabetic patients. The other strategy relies on the identification of activators of PK, which function as positive elements in the insulin signal transduction cascade, such as the IR. These compounds/drug candidates may induce insulin-like signals in muscle and liver tissues of type II diabetic patients. In any case, the effect of large libraries of compounds on the phosphorylation of specific insulin signaling components has to be analyzed in a rapid, reliable, and sensitive fashion.

Procedure

Numerous methods have been designed to measure PK activity. Early methods require physical separation of substrate protein or peptide from its phosphorylated product. One method is to use radiolabeled ATP that allows the incorporated phosphate to be detected after the capture of the protein/peptide substrate by binding to phosphocellulose membranes (Witt and Roskoski 1975). This type of PK assay requires multiple steps and the use of radioactivity with its accompanying hazards and costs. Previously, a very selective and highly sensitive assay for various protein kinases was developed that is based on the use of biotinylated peptide substrates and [γ-32P]ATP. This assay is fast, quantitative, and does not require any alteration of the peptide consensus sequence of the optimal PK substrate, which is routinely done with phosphocellulose membrane-based kinase assays. Although this assay system offers many advantages such as specificity, sensitivity, and convenience, it suffers from the fact that it is not homogeneous, i.e., it requires transfer of reaction products to a filter plate, and it is radioactive. Others have developed assays for measuring the enzyme activity of PK that are homogeneous and increase throughput for measuring the PK activity of many samples. Some of these are radioactivity-based assays, such as the scintillation proximity assays (SPA) or fluorescence-based assays, such as time-resolved

fluorescence resonance energy transfer (FRET) and fluorescence polarization (FP). The SPA and flashplate assay have been used as a high-throughput homogeneous assay of PK activity that does not require an antibody (Braunwalder et al. 1996; Antonsson et al. 1999), but still has some disadvantages. Although the signal-to-noise ratio (S/N) is high for the SPA, it uses radioactivity ([γ-33P]ATP), and its efficiency is dependent on the energy of the isotope used ([γ-33P]ATP is preferred over [α-32P]ATP). It also requires centrifugation or washing steps. Homogeneous nonradioactive assay formats measuring PK activity, which include FP and FRET, have been developed to eliminate the disadvantages of radioactivity and washing steps (Seethala and Menzel 1997; Park et al. 1999). FP is a ratiometric technique that reflects the rotational motion of a fluorescent molecule in solution with the rotational velocity dependent on the apparent molecular size (Pope et al. 1999). Although FRET and FP are nonradioactive, their signal-to-noise ratios are low, and they rely on the availability of very high-affinity and selective antibodies. This represents a major drawback of FRET, its reliability on the dual-label format in which an anti-phosphopeptide antibody is labeled with a fluorescence donor and is brought into close proximity to the peptide conjugated to a fluorescence acceptor, and only when these two molecules are within close proximity does transfer occur. FP on the other hand uses only one labeled fluorescent molecule, and it is ratiometric and thus minimizes interference by sample quenching. The disadvantages, however, are the very low S/N ratio that in some cases is less than 1.5, depending on the assay. The optimization of the assay may be difficult to attain because one cannot easily predict the appropriate location and spatial configuration for the fluorescent label. A major disadvantage of both FP and FRET assays is that they are dependent on the availability of a phospho-specific antibody. Another drawback to the use of antibodies is that their high specificity for a given phosphopeptide often prevents the same antibody from being used with different substrates. Anti-phosphotyrosine-specific antibodies are broadly reactive since they are relatively insensitive to the surrounding amino acid context. In contrast, anti-phosphoserine-/anti-phosphothreonine-specific antibodies are highly dependent on the amino acid sequence surrounding the phosphorylated residue and thus tend to only bind a limited diversity of phosphorylated sites (Wu et al. 2000). In general, a new antibody must be generated for each different substrate, which can be both time-consuming and expensive. There are also ELISA-based assays for PK activity, but these also require multiple steps in addition to being dependent on availability of a phospho-specific antibody to capture product (Angeles et al. 1996).

Several antibody-free nonradioactive assays for PK activity have been reported. One of these is based on the differential electrophoretic mobility of the fluorescent peptide substrate and its phosphorylated product using capillary electrophoresis and is compatible with throughput formats. This type of assay requires specialized high-throughput capillary electrophoresis setup and equipment. Another assay method replaces ATP in the reaction with ATPγ S leading to the formation of a thiophosphorylated peptide product (Jeong and Nikiforov 1999). This product is then chemically biotinylated by a sulfur-specific biotinylation reagent. Streptavidin is then added to the fluorescently tagged peptide, and an FP measurement is taken to measure the amount of product formed. The potential disadvantages of this assay include long incubation times and altered kinetics for some PK due to the ATPγ S. Another antibody-free FP assay of PK activity that has been reported uses polyarginine to discriminate fluorescently labeled phosphorylated and non-phosphorylated peptides based on charge interaction of the phosphate group and the positively charged high molecular weight polyarginine (see section "Fluorescence-Based Assay"; Coffin et al. 2000; Simeonov et al. 2002). Another potential antibody-free assay of PK activity is the so-called IMAP assay that employs trivalent metal ions immobilized on nanoparticles to bind phosphate groups on molecules and thus increase their FP values (Huang et al. 2002). This approach is similar in concept to immobilized metal affinity chromatography (IMAC) used to specifically isolate

phosphopeptides from mixtures containing both phosphorylated and non-phosphorylated peptides (Andersson and Porath 1986). Finally, the development of microchip instruments has led to an additional approach for measuring the PK activity (Cohen et al. 1999). In the microfluidic assay of PK activity, fluorophore-labeled peptide substrate and product are separated based on a difference in their charge-to-mass ratio. Separation of product and substrate is achieved by electrophoresis through an electric field, followed by quantitation of the individual fluorescent peaks. The currently most important and widely used PK assay technologies as well as novel ones with interesting future applications are described briefly.

Scintillation Proximity Assay (SPA)

For the scintillation proximity assay (SPA), the final assay conditions are 50 mM Tris (pH 8.0), 10 mM MgCl2, 0.01 % Triton X-100, 1 mM DTT, 1 or 3.75 μM ATP, 0.375 μM biotinylated peptide substrate, 160 ng/reaction kinase, 10 μM compound (in 10 mM DMSO, diluted 1:100 at least), and 0.125 μCi/reaction of [33P]ATP. The 40-μl reactions are incubated at room temperature for 4 h. A stop reagent (40 μl per well) containing 0.2 mg streptavidin-coated SPA beads (Amersham), 40 mM EDTA, and 74 % CsCl is added to the reaction. After sealing the plates and incubating for 2 h at room temperature, the plates are read on a microtiter plate TopCount scintillation counter.

Fluorescence Polarization (FP) Assay

Purpose and Rationale

FP is a solution-based nonradioactive homogeneous assay that requires no separation of components for the analysis and in many but not all cases (see below) depends on the availability of appropriate phosphosite-specific antibodies. FP, first described in 1926, depends on the differing rotational properties of small (<10,000 Da) vs. large (>100,000 Da) molecules. Fluorescent molecules excited by a plane of polarized light emit light in that same plane if the molecules do not move or rotate during the course of the excited state (1–5 ns). If the fluorescent molecules change position during their existence in the excited state, the polarized light will be emitted in a plane different with respect to the excitation plane. Large molecules such as proteins (or small molecules bound to proteins) do not rotate or move significantly during the lifetime of the excited state. Therefore, they do not become depolarized during the course of an FP experiment. Small molecules, such as peptides, tumble more rapidly and thus become depolarized during the course of the FP experiment. This change in polarization can be detected by monitoring the amount of fluorescence in the vertical vs. the horizontal plane following excitation in the vertical plane. Mathematically this can be expressed as polarization value (P) = (IV − IH)/(IV + IH), where IV and IH are the intensities of the vertical and horizontal components of the emission light, respectively. FP is a dimensionless entity and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. This is a fundamental advantage of FP, in that each sample has an internal calibration that thus limits variations in intensity due to source fluctuation, fluorescence quenching by contaminants or inhibitors, or scattering caused by turbidity. The major limitation of FP lies in the limited sensitivity and signal-to-noise ratio, requiring specialized fluorimeters.

Procedure

Most commercially available FP-based assays of PK activity require an anti-phospho-specific antibody. There are several disadvantages to the use of antibodies in FP assays. Although anti-phosphotyrosine-specific antibodies are common and relatively amino acid context independent, they are expensive. Anti-phosphoserine- or phosphothreonine-specific antibodies can be difficult and costly to produce and are sensitive to amino acid sequence surrounding the phosphorylation site and hence cannot be used for all serine/threonine peptide substrates (see sections

"Phosphorylation of Signaling Components by Insulin and Insulin-Like Stimuli" and "Generic Assay for Protein Kinases (PK) and Phosphatases (PP) Based on Phosphate Release"). Unlike most other homogeneous fluorescent assays of PK activity, reagents required for the zinc FP assay are simply a fluorescein-labeled peptide and commonly used, inexpensive laboratory reagents, with no antibody required.

Modifications of the method have been established which are based on the use of zinc (Scott and Carpenter 2003), polyarginine (Coffin et al. 2000), and competitive mode of assaying (Seethala and Menzel 1998; Turek et al. 2001; Kristjansdottir and Rudolph 2003).

Fluorescence-Based Assay

Purpose and Rationale

To address the various drawbacks of existing assays, a novel, robust system to measure the activity of several PK has been developed. This assay is rapid, can be completed in less than 2 h, and can be carried out in multiwell plate formats such as 96- or 384-well plates. The signal-to-noise ratio is very high, and unlike FRET or FP, it uses a simple measure of fluorescence intensity. The assay also does not rely on the availability of high-affinity and selective antibodies, as is the case with other existing technologies. Finally, the assay is easily adapted to robotic systems for drug discovery programs.

Procedure

It has been reported that phosphorylation of peptides on serine (Murray et al. 1996) or tyrosine (Dass and Mahalakshmi 1996) decreases the proteolytic activity of proteases. Thus, phosphorylation of peptides by PK can be monitored by the increase in resistance to protease cleavage of the phosphorylated product. The difference in cleavage rate of the peptide in the absence and presence of phosphorylation can be used as a measure of the PK activity. It is noteworthy that the activity of PP can also be determined using a similar approach. The cleavage rate can be monitored by HPLC separation of the peptides before and after phosphorylation. However, this approach is tedious and time-consuming, requires large amounts of substrates and PK/PP, and is not practical for a large number of samples. To develop a PK assay based on this principle that is sensitive, simple, and scalable to high-throughput formats, Kupcho and coworkers (2003) introduced a fluorescence technology based on the use of rhodamine 110 (R110)-modified peptide substrates.

Free R110 is a fluorescent molecule, but when it is covalently linked via both its amino groups in a bisamide form, both its visible absorption and its fluorescence are suppressed. Upon enzymatic cleavage, the non-fluorescent bisamide substrate is converted in a two-step process, first to the fluorescent monoamide and then to the fluorescent monoamide and then to the even more fluorescent free R110. Both of these hydrolysis products exhibit spectral properties similar to those of fluorescein, with peak excitation and emission wavelengths of 496 and 520 nm, respectively. When the peptide substrate is not phosphorylated, it can be digested by aminopeptidase, which releases free R110 and thus enhances fluorescence at 520 nm. Phosphorylation of the peptide slows down the proteolytic cleavage of the peptide, and thus minimal change is observed in the fluorescence. Using the appropriate consensus sequence for the peptide substrates, which have to be coupled to R110 in a bisamide form according to the protocol from Kupcho and coworkers (2003), the activity of a multitude of different PK can be monitored.

Capillary Electrophoresis-Based Assay

Purpose and Rationale

Separation of non-phosphorylated substrate from the phosphorylated product can be accomplished by capillary zone electrophoresis. Dawson and coworkers (1994). Although the separations are very efficient, each separation run takes about 10–15 min, making this approach too slow for

the screening of large chemical libraries. The microchip is uniquely suited for the development of PK assays based on separation of substrate and product. Using computer control, it is possible to dispense nanoliter volumes of reagent solutions into the channel network. Enzymatic phosphorylation of the substrate takes place, while the mixture is flowing through a reaction channel. An aliquot of the mixture is then injected into a separation channel where the substrates and product are electrophoretically separated. The separation requires only a fraction of the time needed in traditional capillary electrophoresis. Cohen and coworkers (1999) presented the successful development of a microchip-based assay for a model PK, PKA, activity. This assay format may be applied to a wide range of different PK, for which fluorogenic substrates do not exist but where the outcome of the enzymatic reaction can be assessed by the separation of substrate from product.

Procedure

The assay buffer for on-chip experiments using PKA is 100 mM HEPES/KOH (pH 7.5), 5 mM MgCl2, 100 μM ATP, 50 μM cAMP, and 1 M NDSB-195 (dimethylethylammonium propane sulfate) and is filtered through a 0.22-pm disposable Millipore syringe filter. The design and fabrication of the chips is given in the published report (Cohen et al. 1999). Prior to use, the chips are thoroughly cleaned with deionized water, 2-propanol, a filtered solution of 1 N NaOH for 15–30 min, rinsed with water and finally filled with assay buffer. To run experiments, the microchips are filled with the required reagents and placed in a specially constructed holder on the stage of an epifluorescent inverted microscope (480/40-nm excitation filter; 535/40-nm emission filter). A lid containing Pt electrodes is placed over the chip. Upon lid closure, the electrodes are inserted into the wells of the chip. These electrodes are connected to the high-voltage controller. Currents and voltages are applied to platinum wire electrodes immersed in reagent wells on the microchip.

Evaluation

The capillary electrophoresis-based assay format may replace other methods of analysis of PK, namely, radiolabel assays and other more cumbersome heterogeneous formats, with the true appeal of the microchip being its potential for application to high-throughput screening. Significant reductions in assay time and reagent consumption will offset the expense by the need for instrumentation and software development. Optimization of channel dimension for the direct introduction of compounds/drug candidates from a microtiter plate into the on-chip reagent stream of substrate and enzyme as well as of reagent concentrations as methods to manipulate on-chip incubation times for enzymes of varying turnover rates has meanwhile been achieved.

Dephosphorylation of Insulin and Insulin-Like Signaling Components

General Considerations

The PTPs (protein tyrosine phosphatases) are a structurally diverse family of both receptor-like and nontransmembrane hydrolases that have been implicated in the control of numerous physiological processes, including growth, differentiation, and metabolism (Johnson et al. 2002). Approximately 75 PTPs have been identified to date. These enzymes are characterized by the presence of a conserved catalytic domain of 240 residues which contains the unique signature motif, C(X5)R, that defines this enzyme superfamily (Andersen et al. 2001; Alonso et al. 2004). The structural diversity is manifested by the variability in noncatalytic sequences fused to the amino or carboxy terminus of the catalytic domain. These noncatalytic segments frequently serve a regulatory function, including ligand binding for receptor PTPs and targeting of cytoplasmic PTPs to defined subcellular locations. Despite variations in primary structure and differences in substrate specificity, key structural features in the catalytic site and mechanism are conserved

among all members of the PTP superfamily (Barford et al. 1994, 1998; Zhang 2003). Although great progress has been made in illustrating the, somewhat unexpected, structural diversity within this large enzyme family, relatively little is known of the physiological function of individual PTP. Presumably this structural diversity reflects a broad range of functions for the PTP family in vivo. However, it is clear on the basis of the role of protein tyrosine phosporylation in insulin signal transduction that PTPs play important roles in the downregulation of the insulin signal either for the physiological termination of insulin action after removal of insulin from the circulation or during the pathogenesis of type II diabetes in course of the development of insulin resistance in peripheral tissues. In particular, the potential role of PTP1B as a negative regulator of insulin action, causally related to the pathogenesis of insulin resistance and type II diabetes, has been suggested by a number of studies using knockout animals and cultured cells with ectopic overexpression and inhibition by small molecules or antisense oligonucleotides (Johnson et al. 2002; Elchebly et al. 1999). Consequently, PTP, in general, and PTP1B, in particular, have been recognized as attractive targets for the identification of insulin-like/insulin-sensitizing drugs for future antidiabetic therapy.

Purpose and Rationale

Considerable work has been directed at identifying PTPs that dephosphorylate IR as possible targets for insulin-like compounds/drug candidates. Several receptor PTPs (e.g., PTP-α, LAR) and non-receptor PTP (PTEN, PTP1B) have been considered candidates based on studies of their overexpression, substrate trapping mutants, mouse knockout models, and other approaches (Cheng et al. 2002; Tonks 2003; Bourdeau et al. 2005). Since the inhibition of PTP1B causes prolonged and increased IR phosphorylation, it is a potential therapeutic target in the treatment of type II diabetes and insulin resistance.

As interest in understanding PTP1B and its role in the regulation of insulin signal transduction and pathogenesis of type II diabetes grows, the accurate measurement of tyrosine phosphorylation on proteins becomes increasingly important. Therefore to aid in the identification of selective, potent PTP1B inhibitors, novel assays have recently been designed to follow changes in tyrosine phosphorylation of the IR and of additional so far unknown substrate proteins as well as for the identification of novel PTP1B substrate proteins in normal and insulin-resistant muscle, adipose, and hepatoma cell lines which all express functional PTP1B and IR.

In addition to PP, lipid phosphatases have also been implicated as negative regulators of insulin signaling and action, in general, and insulin-stimulated GLUT4 translocation, in particular. In addition to positive regulators of GLUT4 translocation, negative regulators have also been studied intensely because they might provide opportunities for pharmacological interventions. For example, the endogenous attenuation of PI3-K signaling occurs through the dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3,]. Two phosphoinositide phosphatases, SHIP2 (type II SH2-domain-containing inositol 5′-phosphatase) and PTEN (phosphatase and tensin homologue deleted on chromosome ten), have been implicated in negatively regulating insulin signaling (Jiang and Zhang 2002). SHIP2 removes the 5′ phosphate from PtdIns(3,4,5)P3 to generate phosphatidylinositol-3,4-bisphosphate [PtdIns (3,4)P2]. Although the Ship2 KO mice die shortly after birth and are severely hypoglycemic, heterozygous animals show improved glucose tolerance and enhanced insulin sensitivity (Clement et al. 2001).

In contrast to SHIP2, PTEN is a 3′-specific PtdIns(3,4,5)P3 phosphatase that generates PtdIns(4,5)P2. Overexpression of PTEN prevents the accumulation of PtdIns(3,4,5)P3 and also inhibits insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (Nakashima et al. 2000). Moreover, the genetic ablation of PTEN in adipose tissue of mice has recently been shown to enhance insulin sensitivity and resistance to pharmacologically induced diabetes (Kurlawalla-Martinez et al. 2005).

Protein Tyrosine Phosphatase (PTP) Activity Measurement Using DIFMUP

Purpose and Rationale

A variety of substrates have been developed for measurement of the hydrolytic activity of PTP with the rather unspecific para-nitrophenylphosphate (PNPP) and specific phosphotyrosine-containing peptides being most widely used. These assays have significantly improved our understanding of substrate specificity and catalytic mechanism of PTP. However, their use for routine inhibitor screening is rather limited due to a variety of reasons, in particular, the requirement for synthesis of a phosphopeptide, low sensitivity in detection, susceptibility to color interference, and discontinuous nature (i.e., termination of the reaction by addition of acidic malachite green reagent or alkaline solution; Zhang and Dixon 1994; Zhang et al. 1993). A number of fluorogenic compounds used for the analysis and characterization of PTP have been described. Recently, Huang and coworkers (1999) and Wang and coworkers (1999) described the development of fluorescein monosulfate monophosphate as a dual fluorogenic substrate for both phosphoserine/threonine phosphatases and PTP. The similarity in their reaction mechanisms seems to be the reason that phosphatases from the different families can use these small non-peptidic substrates, albeit with pronounced differences in their catalytic efficiencies. Wang and coworkers (1999) described the development of fluorescein monosulfate monophosphate and their use as sensitive fluorogenic substrates for several PTPs. The demonstrated catalytic efficiencies which are comparable to those of phosphopeptide substrates favor their use in alternatively more sensitive assay designs. Recently, another fluorogenic substrate, 6,8-difluoro-4-methylumbiliferyl phosphate (DIFMUP), was found to be appropriate for measurement of PTP activity (Welte et al. 2003). This substrate has initially been developed for phosphoserine/threonine phosphatases (Gee et al. 1999) and subsequently optimized for use in the high-throughput assay format (Welte et al. 2005). The DIFMUP-based assay may be helpful for the kinetic analysis and characterization of small-molecule inhibitors for PTP, which have been implicated as negative regulators in insulin signaling (e.g., PTPα, LAR, CD45, TC-PTP, YOP, SHP2), in general, and PTP1B, in particular.

Procedure

All reactions are carried out at 37 °C in black polystyrol 96-well plates with flat bottoms. For measurement of PTP1B, PTPα, LAR, CD45, TC-PTP, YOP, and SHP2, the reaction buffer contains 50 mM HEPES (pH 6.9), 150 mM NaCl, 1 mM EDTA, and 2 mM DTT, and for VHR, the reaction buffer contains 50 mM sodium acetate (pH 5.6), 100 mM NaCl, 1 mM EDTA, and 2 mM DTT. The substrate stock solution is prepared in DMSO at a final concentration of 10 mM and stored at −20 °C. For measurement, DIFMUP is diluted tenfold in the desired reaction buffer, yielding a final concentration of 1 mM. Fluorescence excitation of hydrolyzed DIFMUP (Molecular Probes) and the fluorescent standard DIFMU is measured at 358 nm, and emission is detected at 455 nm in a fluorescence plate reader (e.g., Spectramax, Molecular Devices).

For kinetic analysis, the DIFMUP stock solution is pre-diluted in reaction buffer and added to the reaction mixture to yield final concentrations of 0–300 μM. The assay is initiated by adding 10 μl of substrate to 90 μl of reaction buffer containing the pre-diluted PTP (final concentrations: PTP1B, 100 ng/ml; PTPα, 500 ng/ml; LAR, 125 ng/ml; CD45, 100 ng/ml; TC-PTP, 100 ng/ml; SHP2, 2 μg/ml; YOP, 50 ng/ml).

Compounds/drug candidates are pre-diluted in reaction buffer containing 100 ng PTP1B to the indicated final concentrations ranging from 0.8 to 80 μM in a final volume of 90 pl. The reaction is started by adding 10 μl of substrate buffer containing 1 mM DIFMUP, and the intensity of released DIFMU is monitored continuously for 5 min. Malachite green and PNPP assays are performed as described (Zhang and Dixon 1994;

Zhang et al. 1993). Initial velocities of the reactions are used to calculate kinetic constants using Sigma Plot software. The initial velocities are plotted against the inhibitor concentrations, and the IC50 is calculated.

Detailed analysis of the binding mode of compounds/candidate drugs is performed as follows. For analysis of the time dependence of inhibition, different inhibitors are incubated at their IC50 concentrations with PTP1B for 0, 10, and 15 min. The reaction is started by adding 10 μl of substrate buffer containing 1 mM DIFMUP, and the reaction is followed for 10 min. For analysis of the reversibility of inhibition, compounds/drug candidates are incubated for 10 min with PTP1B. After addition of DIFMUP to a final concentration of 10 μM, the reactions are followed continuously until the fluorescence emission does not increase further. The reactions are restarted by adding DIFMUP to a final concentration of 100 μM, and the release of DIFMU is monitored for an additional 10 min.

Evaluation

The fluorogenic substrate, DIFMUP, was developed as a substrate for phosphoserine/threonine phosphatases to enable continuous measurement of activity (Gee et al. 1999). Its hydrolysis by a phosphatase results in the release of fluorescent DIFMU, which can be easily followed in continuous mode by a fluorescence reader. A great advantage of this substrate is its low pKa value of 4.7 compatible with pH 7.0 during the reaction without prior alkalization (Sun et al. 1998). Furthermore, the substrate yields a higher fluorescence quantum than other frequently used fluorogenic substrates, such as 4-methylumbelliferyl phosphate. This substrate is accepted a recombinant fragment of human PTP1B (Welte et al. 2003). This finding has meanwhile been extended by a detailed kinetic and comparative analysis of PTP reactions using DIFMUP and other substrates (Welte et al. 2005). Compared to PNPP, DIFMUP is a better substrate for PTP1B as reflected in both its lower Km and higher turnover number. However, dephosphorylation of a phosphotyrosine-containing peptide derived from the IR is even more efficient, indicating the requirement of amino acids adjacent to the phosphoamino acids.

DIFMUP is accepted by other PTPs from different families. These include CD45, PTPα, SHP2, YOP, LAR, and TC-PTP. For these PTPs a clear relationship between the relative fluorescence signal and the enzyme concentration is detected, which, however, with regard to the protein amount required differed by two orders of magnitude between LAR, TC-PTP, CD45, YOP (50–500 μg) and PTPα, SHP2 (0.5–2 μg) with PTP1B, and the closely related TC-PTP showing the highest affinity for DIFMUP (Welte et al. 2005). These findings hint at subtle differences in the catalytic sites between these PTP for hydrolysis of optimally bound DIFMUP. Taken together, differences in the substrate recognition and catalytic sites of the various PTPs are presumably monitored in the course of DIFMUP binding and cleavage. In contrast, no major kinetic differences between a number of phosphatases were reported for the hydrolysis of 3,6-fluorescein diphosphate and other fluorogenic substrates (Huang et al. 1999; Wang et al. 1999), arguing for similar and more flexible binding modes to the substrate recognition and catalytic sites of these apparently more unspecific substrates.

The search for inhibitors of PTPs requires a robust test system to assay a large number of compounds for a multitude of different enzymes. A novel non-peptidic inhibitor, a benzooxathiazoldioxide derivative (Petry et al. 2002; Liu et al. 2002) exhibits in different PTP1B assays using either DIFMUP, PNPP, or the IR phosphopeptide as substrate an IC50 of 3.8, 5.1, and 3.9 μM, respectively. DMSO used as solvent for the inhibitors did not affect cleavage of DIFMUP to up to 4 % final concentration during the assay, reflecting the robustness of the assay.

PTPs all share a nucleophilic cysteine residue located in the catalytic cleft, which is sensitive to oxidation or covalent modification (Zhang 2003; Johnson et al. 2002). Therefore, characterization of the nature of their inhibition by small-molecule inhibitors is important, which requires detailed kinetic studies. The possibility of continuous

measurement of DIFMUP hydrolysis allows rapid characterization of the reversible nature of inhibition. For this, the enzyme is incubated in the absence or presence of inhibitor in the presence of 10 μM DIFMUP. After incubation for 10 min, when hydrolysis of DIFMUP stops due to substrate depletion (as revealed by approaching a plateau level of fluorescent DIFMU generated), a tenfold excess of fresh DIFMUP is added to the reaction. This leads to complete reversion of inhibition of DIFMUP hydrolysis as reflected in the almost identical initial velocities of the reactions containing or lacking (control) the inhibitor after fresh DIFMUP has been added. The degree of inhibition is often found to depend strictly on the incubation time of the inhibitor with PTP1B prior to DIFMUP addition.

In summary, the high sensitivity and compatibility with a broad pH range of the fluorogenic DIFMUP assay provide advantages with regard to saving enzyme and miniaturization. Consequently, the DIFMUP assay will be useful for the development of throughput screening assays for a variety of physiologically important PTPs as has been already been demonstrated for the receptor PTP and LAR (Pastula et al. 2003). In addition to its technical advantages, DIFMUP seems to reflect the binding mode of phosphotyrosine-containing peptide substrates more closely than the widely used non-peptidic substrates, PNPP and 3,6-fluorescein diphosphate. These characteristics may facilitate the future search for competitive and reversible PTP inhibitors as antidiabetic drugs.

PTP Identification Using Substrate Trapping

Purpose and Rationale

To understand fully the role of PTP in cellular function, in general, and in insulin signal transduction, in particular, it will be necessary to identify multitude of the physiological substrates of the individual members of the PTP family. It has shown in numerous studies that alteration of the nucleophilic Cys to Ser or Ala allows some PTPs to be isolated in a complex with their target substrates. Thus it appears that the Cys → Ser/Ala mutants may be utilized to isolate all combinations of PTP1B and its substrates. Additional alternative "substrate-trapping" PTP mutants have been identified by systematic mutational analysis of the invariant residues that were predicted from the crystal structure to be important in catalysis. In one such mutant form of PTP1B Asp-181, the residue that functions as a general acid to facilitate cleavage of the scissile P–O bond in the substrate has been altered to Ala. Clearly, a definition of the spectrum of phosphotyrosyl proteins dephosphorylated by PTP1B in vivo will be a prerequisite for the elucidation of the physiological function of that enzyme. Such substrate identification or trapping may be achieved by the following method.

Procedure

Immunoprecipitation, Immunofluorescence, and Activity Assay

Cells transformed or transfected with vectors coding for PP are lysed 44–48 h after transfection in 50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 5 mM iodoacetic acid, 10 mM sodium pyrophosphate, 10 mM NaF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM benzamidine (1 ml per 10-cm dish, 0.5 ml per 6-cm dish). PTP1B is immunoprecipitated from lysates with a monoclonal antibody (mAb). GST-PTP1B fusion proteins are precipitated with glutathione–Sepharose. Precipitates are collected by centrifugation (5,000 × g, 15 s, 4 °C), washed four times with 0.7 ml of ice-cold lysis buffer, and finally heated at 95 °C for 5 min in 60 μl of 2 × Laemmli sample buffer. mAb to phosphotyrosine are typically used for immunoblotting at the following concentrations: 4G10 (Upstate Biotechnology) 2 μg/ml and RC20b (Transduction Laboratories) 0.5 μg/ml. The levels of expression of wild type, C215A, and mutant PTP1B should always be equivalent. For immunofluorescence experiments, cells are seeded onto glass coverslips at 2.5–3 × 105 per 6-cm dish, transfected

with 10 μg of PTP1B cDNA, wild type or mutant, and processed for immunofluorescence staining at 36 h after transfection as described by Lorenzen and coworkers (1995).

Evaluation

Considering that the substrate-trapping mutant, PTP, has the ability, through interaction with phosphotyrosine residues, to increase the basal phosphorylation of their target substrates, one might expect them to promote ligand-independent signaling. However because these mutants form stable complexes with target substrates, they may also interfere with signaling in vivo in a manner analogous to the wild-type phosphatase. Thus, if the site of tyrosine phosphorylation on the substrate is critical for a protein–protein interaction required for signaling or if the substrate is an enzyme and the phosphorylation site is located close to the active site, steric hindrance resulting from binding of the mutant PTP may exert an effect that is functionally equivalent to dephosphorylation.

The successful application of this technique to identify additional substrates of PTP1B, and substrates of other PTP, will require expression of the Asp → Ala mutant PTP in cells and selection of an appropriate stimulus to trigger tyrosine phosphorylation of the substrate and accumulation of a complex with the mutant PTP. For PTP1B, IR is naturally abundant in myocytes, hepatocytes, and adipocytes, and its basal rate of autophosphorylation generates enough tyrosine-phosphorylated protein to be trapped by PTP1B-D181A and to be detected by anti-phosphotyrosine immunoblotting. Its high molecular weight, characteristic of growth factor receptors, facilitates identification. Of the less-phosphorylated substrates of PTP1B, the finding that phosphorylation of p70 is enhanced upon cotransfection with v-Src and D181A may enable enough of this substrate to be isolated to allow its identification by peptide sequencing. In addition to the use of trapping mutants to identify novel PTP substrates, it also may be possible to identify the site within the cell at which a PTP acts on a particular target, through colocalization of the substrate and the Asp → Ala mutant PTP.

These findings indicate that PTP1B can display substrate specificity in vivo and bolster the hypothesis that the subcellular targeting is an important mechanism by which such specificity is achieved. The substrate-trapping mutant introduced by Flint and coworkers (1997) is altered in a residue that is an invariant catalytic acid in all members of the PTP family. Therefore, the use of this mutation should be generally applicable to any PTP and may represent a powerful tool with which to delineate the physiological substrate specificity of other members of the family, thereby revealing important insights into their function in vivo and their potential as drug targets.

Lipid Phosphatase Activity Measurement

Purpose and Rationale

The methods currently available for the assay of lipid metabolizing enzymes, in general, and lipid phosphatases, in particular, rely on the release of radioactive isotopes with some form of substrate/product separation step before quantitation. Estimation of cellular mass of phosphoinositides usually requires labeling of cells with [3H]inositol or [32P]orthophosphate followed by deacylation and HPLC analysis. Estimation of phosphoinositide mass in tissues is at present restricted to PtdIns(3,4,5)P3 and requires extraction of the lipids and removal of the phosphoinositol head groups followed by a radiometric displacement assay which also requires synthesis of radiolabeled inositol(1,3,4,5)tetrakisphosphate. These assays also suffer from the drawback that the labeled substrates or precursors required are expensive and samples require extensive processing before analysis.

A novel assay for quantification of phosphoinositides is based on their ability to bind specifically to certain pleckstrin homology (PH) domains (Gray et al. 2003). PH domains are the major intracellular targets of PtdIns(3,4,5)P3, PtdIns(3,4)P2, and several other

phosphoinositides. Recently the number of characterized PH domains binding inositol lipids with a broad range of affinity and specificity has increased (Lemmon and Ferguson 2001). Other recently discovered phosphoinositide-binding domains, like the phox homology domain (Xu et al. 2001; Bravo et al. 2001) and FYVE domains (Gillooly et al. 2001), could potentially also be used in the assay formats described herein. Other critical components of the assay concept are biologically active short acyl chain phosphoinositides that recently have become commercially available. Important structural features of these include short acyl side chains (diC4 to diC8), rendering them water soluble, and the addition of biotin to the terminus of the sn-1 acyl chain while still allowing selective recognition of the inositol head group. Finally, sensitive nonradioactive detection of lipid/PH domain complexes relies on either TR-FRET using Lance reagents (e.g., Perkin–Elmer Wallac) or the recently introduced AlphaScreen technology (BioSignal Packard). In both cases, PtdIns(3,4)P2 or PtdIns(3,4,5)P3 present in samples are detected in competition assays by its ability to dissociate signal generating complexes between PH domain, biotinylated lipid, and detection reagents. The assay systems presented here allow the assay of lipid phosphatases in a homogeneous format compatible with throughput screening and, in the case of the TR-FRET format, are capable of real-time kinetic measurements.

Procedure

Detection of Biotinylated Phosphoinositide/PH Domain Complexes

The GRP1 PH domain (amino acids 263–380) is PCR cloned from a mouse brain cDNA library as described by Gray and coworkers (1999). The protein is expressed from the pGEX 4T1 vector (Amersham Pharmacia) in *Escherichia coli* and affinity purified on glutathione–agarose using the manufacturer's standard protocols. The purified PH domains are labeled with Lance chelate according to the manufacturer's protocols.

AlphaScreen detection is performed in 384-well microtiter plates in 50 mM HEPES/KOH (pH 7.4), 50 mM NaCl, and 0.1 % BSA. Biotinylated, short-chain (diC6) phosphoinositides and GRP1 PH–GST are added at 15 and 3.75 nM, respectively. Donor and acceptor AlphaScreen beads (Perkin–Elmer) are added at 5 µg/ml to a final volume of 50 µl. Plates are incubated in the dark for 5 h to ensure binding is complete and then read in an AlphaQuest AD instrument (Perkin–Elmer) using standard settings. The TR-FRET sensor complex consists of 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM DTT, and 0.05 % CHAPS with APC–streptavidin, 32 nM and 120 nM biotinylated, short-chain (diC6) phosphoinositides and 35 nM Lance chelate-labeled GST-PH domain. Alternatively the TR-FRET sensor complex contains 21 nM Lance chelate-labeled anti-GST antibody and unlabeled PH domain in a final volume of 50 µl. All assays contain CHAPS at low concentration to prevent loss of lipids by adsorption onto plastic surfaces.

For all assays plates are read with the following settings: excitation 360–35 nm filter, emission 665 nm filter, dichroic filter 505 nm, PMT 1,000 V set to digital sensitivity 2,100 flashes per well, 10-ms interval between flashes, read 50 ms after flash, and integration 1,000 ms.

The TR-FRET assays are conducted in 50 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 5 mM MgCl2, 5 mM DTT, and 0.05 % CHAPS (assay buffer) using a two-component reaction. The first component is a twofold-concentrated sensor complex consisting of 32 nM APC–streptavidin, 120 nM biotinylated diC6PtdIns(3,4,5)P3, and 35 nM Lance chelate-labeled GST–PH domain. Alternatively the sensor complex contains 21 nM Lance chelate-labeled anti-GST antibody and unlabeled PH domain. The first component also contains 100 ng recombinant PI3-kinase γ in a final volume of 25 µl assay buffer. The second component contains the diC8PtdIns(4,5)P2 at two times the required final concentrations and ATP at 100 µM again in 25 µl assay buffer. The assays are started by mixing the two components in a 96-well plate and reading at the required time intervals to obtain rates of reaction.

Evaluation

A novel approach to quantitation of phosphoinositides in cell extracts and in vitro enzyme-catalyzed reactions is based on the use of suitably tagged and/or labeled PH domains as probes (Gray et al. 2003). Stable complexes are formed between the biotinylated target lipid and an appropriate PH domain, and phosphoinositides present in samples are detected by their ability to compete for binding to the PH domain. Complexes are detected using AlphaScreen technology or time-resolved FRET. The assay procedure has been validated using recombinant PI3-K γ with diC8PtdIns(4,5)P2 as substrate and general receptor for phosphoinositide-1 (GRP1) PH domain as a PtdIns(3,4,5)P3-specific probe. This PI3K assay is robust and suitable for throughput screening,. The approach is adaptable to lipid phosphatases as demonstrated by assays for PTEN, a phosphoinositide 3-phosphatase, which is measured using the same reagents but with diC8PtdIns(3,4,5)P3 as substrate. PtdIns(3,4,5)P3 present in lipid extracts of growth factor-stimulated Swiss 3T3 and HL60 cells is also detectable at picomole sensitivity. Gray and coworkers (2003) therefore proposed that similar procedures should be capable of measuring any known phosphoinositide present in cell and tissue extracts or produced in PK and PP assays by using one of several well-characterized protein domains with appropriate phosphoinositide-binding specificity.

PtdIns(3,4,5)P3 Measurements

Alternatively to the above method of quantification of phosphoinositides by using phosphoinositide-binding proteins, PtdIns(3,4,5)P3 can be measured directly by prior radiolabeling as described by Blero and coworkers (2005). For this, cells (1.5×106), which have been pretreated with compounds/drug candidates, are cultured in 10 % serum overnight. Cells are washed twice in medium without serum and twice in medium without either phosphate or serum. They are labeled for at least 4 h in medium with [32P]orthophosphate (250 μCi/ml) but without serum. Cells may be stimulated with compounds/drug candidates during the labeling period, in addition or alternatively. The reaction is terminated by 5 ml cold PBS. Cells are lysed in 3.75 ml of 2.4 N HCl. Lipids are extracted in 3 ml methanol and 4.5 ml CHCl3. After TLC and deacylation of the phosphoinositides, separation is performed by HPLC on Whatman SAX columns (Pesesse et al. 2001). Radioactivity is estimated with an online detector (e.g., Raytest, Germany). PtdIns(4,5)P2 is also determined by labeling the cells with [3H]inositol as reported by Serunian and coworkers (1991). The various 3-phosphorylated phosphoinositide standards have to be prepared in insulin-stimulated CHO-IR cells or in platelets as reported (Blero et al. 2001; Giuriato et al. 2003).

Measurement of Recombinant SHIP2 Activity

For the identification and characterization of compounds/drug candidates which directly interfere with the activity of SHIP2 and may represent future antidiabetic drugs, the activity of SHIP2 can be assayed with cells transfected with recombinant SHIP2 by HPLC analysis of their phosphoinositides after radiolabeling in vivo according to the procedure of Wada and coworkers (2001).

In Vivo Generation of 32P-Labeled Phosphoinositides and HPLC Analysis

The same numbers of 3T3-L1 adipocytes transfected with LacZ, wild-type WT-SHIP2, or phosphatase-defective ΔIP-SHIP2 are phosphate-starved overnight in phosphate-free DMEM, followed by serum starvation for 3 h. [32P]orthophosphate (0.1 mCi/ml) is then added, and the cells are cultured for an additional 2 h. Following the labeling period, the cells are incubated without or with compounds/drug candidates for 15 min. The reaction is terminated by washing once with ice-cold PBS, followed by the addition of methanol/1 N HCI (1/1). The labeling of the cells with [32P]orthophosphate is conducted at the same time in all three sets of transfected cells. Phospholipids are then extracted with chloroform. The extracted lipid is deacylated and subjected to amino exchange HPLC using a Partisphere strong anion exchange column as

described previously (Serunian et al. 1991; Funaki et al. 1999). The PI(3,4)P2 and PI(3,4,5)P3 levels in the same sample for each cell line are measured within a single HPLC run. The radioactivity is detected with an online radiochemical detector.

Modification of the Method

Lin and coworkers (2003) reported about the separation of phospholipids, such as phosphoinositides, in microfluidic chip devices and its application to homogeneous high-throughput screening assays for lipid-modifying enzymes. The method takes advantage of the high-separation power of the microchips that separate lipids based on micellar electrokinetic capillary chromatography (MEKC) and the high sensitivity of fluorescence detection. The assay format consists of two steps: an on-plate enzymatic reaction using fluorescently labeled substrates followed by an on-chip MEKC separation of the reaction products from the substrates. The utility of the assay format for screening is demonstrated using phospholipase A2 but may be extended to other phospholipases and lipid phosphatases with the advantages of avoidance of the use of radioactive substrates and tedious separation/washing steps and the detection of both substrate and product simultaneously.

Generic Assay for Protein Kinases (PK) and Phosphatases (PP) Based on Phosphate Release

Purpose and Rationale

PK and PP produce or consume inorganic phosphate (Pi). Detection of an increase or decrease of the Pi concentration in the environment of these enzymes is a common way to monitor the enzymatic activity. Assays capable of measuring a change in the inorganic phosphate concentration serve as uniform readout for the activity of these enzymes (Ellen Chan and Swaminathan 1986; Brune et al. 1994). A number of colorimetric and fluorometric assays have been reported for the determination of inorganic phosphate (Itaya and Ui 1966; Kirkbright et al. 1972; Sebbon and Fynn 1973). Most of these assays are based on complexation reactions with the free phosphate. In addition, some methods for phosphate measurements have been developed based on the enzymatic conversion of phosphate and a substrate to a UV-absorbing product (Webb 1992; Ungerer et al. 1993). The disadvantages of all these methods are that they are slow, lack sensitivity, or are sensitive to interfering compounds (Ellen Chan and Swaminathan 1986; Tashima 1975; Hwang 1976).

To circumvent these problems, Brune and coworkers (1994, 1998) developed a continuous-flow phosphate assay. As an improvement Schenk and coworkers (2003) introduced a probe for the rapid measurement of Pi, in particular to follow its release in real time from enzymes such as phosphatases. The probe consists of a phosphate-binding protein (PBP) labeled with a fluorophore, N-(2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC). Upon binding of Pi under physiological conditions, a ~13-fold increase in fluorescence is observed. The binding of Pi to MDCC-PBP is very fast, has a high affinity, and is very sensitive. These characteristics make the reaction between Pi and MDCC-PBP very suitable for dynamic Pi detection assays and thus for application in a continuous-flow phosphate assay. A further increase in sensitivity of a continuous-flow generic fluorescent assay for PK and PP was achieved by Schenk and coworkers (2003) by coupling to liquid chromatography (LC).

Cellular PTP Assays

The development of cell-based assays for the identification as well as evaluation of the biological activity of PTP inhibitors which positively affect insulin signaling and reduce insulin resistance (e.g., PTP1B, see above) is hampered by a number of issues. The typical functional readout for PTP1B inhibition has been to measure IR

phosphorylation (see section "IR Tyrosine Phosphorylation"). Yet, the increase in IR phosphorylation due to PTP1B inhibition is usually variable, dependent on cell type, and at most two- to three-fold over controls, thus making it unsuitable for screening. Furthermore, because many of the current potent PTP1B inhibitors contain a phosphonate or a phosphate mimetic, they are highly charged, and therefore cell permeability becomes an issue. Without a robust cellular functional readout and questionable cell permeability of PP inhibitors, it becomes difficult to reconcile the reason for the lack of activity of PTP inhibitors in cell-based assays. In order to circumvent some of these issues, yeast and insect cell-based PTP1B assays have been developed.

Yeast-Based Assay

Purpose and Rationale

Yeast has been used as a model system for the screening of various compounds/drug candidates. It provides a cellular environment to prevent PP oxidation, and measuring yeast growth is both an easy and sensitive screening assay (Melese and Hieter 2002). In addition, yeast cell permeability has been shown not to be an issue in concentrating compounds internally, but like mammalian cells, the rate-limiting factor in internalization of compounds has been due to the function of efflux pumps. v-Src, a tyrosine kinase from the Rous sarcoma virus, is lethal when overexpressed in the yeast *Saccharomyces cerevisiae* (Brugge et al. 1987). The lethality of v-Src has been attributed to the hypertyrosine phosphorylation of multiple yeast proteins leading to mitotic dysfunction, but the exact target responsible for the toxicity remains to be identified (Kornbluth et al. 1987; Florio et al. 1994; Trager and Martin 1997). Yeast can be rescued from the v-Src lethality by coexpressing the catalytic domain of PTP1B (Florio et al. 1994). This rescue is presumably due to the activity of PTP1B in dephosphorylating the various yeast proteins phosphorylated by v-Src. Based on these observations Montalibet and Kennedy (2004) developed and optimized a yeast-based assay to screen for PTP1B inhibitors.

Procedure

Yeast with leucine and uracil deficiency markers are transformed with the plasmids coding for v-Src and the appropriate PTP (here PTP1B), and the colonies formed 3 days after are transferred to leucine and uracil dropout media containing raffinose as the sole carbon source and are grown overnight. Serial dilutions of the inhibitors are arrayed in 96-well plates containing leucine and uracil dropout media with 4 % galactose. The plates are inoculated with the overnight yeast cultures at a final concentration of 10 [9] per ml in the absence or presence of increasing concentrations of compounds/drug candidates (Hammonds et al. 1998). The wells are overlaid with mineral oil, incubated at 30 °C, and read periodically at 600 nm for 4 days. The growth curves generated are used to obtain EC50-values for the putative PTP1B inhibitors.

Evaluation

Based on the previous observation that coexpression of PTP1B can rescue yeast from v-Src lethality, Montalibet and Kennedy (2004) optimized the expression levels of PTP1B and v-Src such that inhibition of PTP1B activity results in growth interference. PTP1B catalytic activity was shown to be absolutely required for yeast growth since the catalytically inactive PTP1B mutants C215S and D181A failed to overcome the lethality of v-Src. A certain stoichiometry between PTP1B and v-Src expression is required to obtain a robust growth phenotype and at the same time to ensure sensitivity toward inhibitors. Screening can be carried out in 96-well plates and growth of the liquid culture measured by absorbance at 600 nm. The PTP inhibitor, vanadate, specifically inhibited PTP1B-dependent growth in a concentration-dependent manner with an EC50 of 0.92 mM. This simple yeast growth interference assay may be useful for

throughput screening for inhibitors of PTP1B, in particular, and PTP, in general.

Insect Cell-Based Assay

Purpose and Rationale

Cromlish and coworkers (1999) described an intact cell-based assay for the discovery of cell-permeable, selective PTP inhibitors utilizing the baculovirus expression system, where a PTP of interest is expressed in Sf9 insect cells and a direct readout of the PP activity within these cells is obtained from the amount of hydrolysis of the cell-permeable substrate, para-nitrophenylpyrophosphate (pNPP).

Procedure

Baculoviruses recombinant for the cDNA coding for the desired PTP (e.g., PTP1B) is prepared using the Bac-to-Bac Baculovirus Expression System and recovered from the supernatant medium from transfected Sf9 insect cells after three rounds of amplification to up to a total viral stock volume of 500 ml. Sf9 cells are infected with the recombinant or control baculovirus (Cromlish and Kennedy 1996), collected 29 hpi by centrifugation (48 × g, 5 min), washed once in assay buffer (Hanks' solution buffered with 15 mM HEPES/KOH, pH 7.4), and recentrifuged (21 × g, 10 min). The cells are resuspended gently in assay buffer and examined using a hemocytometer and microscope for cell density as well as trypan blue exclusion. Assays are performed using a pipetting robot, programmed to mix gently after each addition in two hundred microliter Hanks' solution containing the cells. Following a 15-min incubation with compounds/drug candidates at 37 °C, the cells are challenged with 1–50 mM of tissue culture-grade pNPP for 15 min. The cells are centrifuged (410 × g, 3 min, 4 °C). One hundred microliter samples of the supernatants are transferred to fresh clear polystyrene 96-well plates, and the amount of hydrolysis of pNPP is determined spectrophotometrically at OD of 405 nm. The pNPP hydrolysis window obtained between PTP-expressing cells and control-infected cells represents the amount of phosphatase activity due to the PTP of interest.

Evaluation

Inhibitions are calculated by comparing pNPP hydrolysis of the inhibitor-treated PTP-expressing cells with that of DMSO-treated PTP-expressing cells. For the PTP Sf9 cell-based assay to be useful in screening for PTP inhibitors, it is necessary to harvest the cells at a time point when sufficient amounts of the protein of interest are produced, yet the cells are healthy and viable prior to the deleterious effects associated with the later stages of the viral infection.

Bacterial Cell-Based Assay

Purpose and Rationale

Senn and Wolosiuk (2005) developed a high-throughput screening assay for the detection of phosphatase activity in bacterial colonies overexpressing mammalian phosphatases. Unlike the insect cell-based method, this procedure is compatible with the use of physiological substrates but nevertheless is versatile, since it is based on the detection of the common product of all phosphatase reactions, Pi. In this method, substrates diffuse from a filter paper across a nitrocellulose membrane to bacterial colonies located on the opposite face, and then the reaction products flow back to the paper. Finally, a colorimetric reagent discloses the presence of orthophosphate in the filter paper.

Procedure

Escherichia coli cells are transformed with vectors coding for the corresponding PP under a strong inducible promoter and after appropriate dilution grown directly over a nitrocellulose

membrane that lay on Luria-Bertani (LB) agar supplemented with 100 μg/ml ampicillin. When the diameter of colonies is 1 mm (~24 h), the expression of the PP is induced by spreading 0.2 ml of inducer (e.g., 40 mM IPTG, 10 % agarose) onto the agar surface. After 3 h, the nitrocellulose membrane (pore size 0.2 pm; Bio-Rad, USA) is withdrawn from the Petri dish and placed with bacteria facing up for 10 min on a Whatman filter paper soaked in water. After this washing step has been repeated twice, the nitrocellulose membrane is similarly placed for 5–10 min on another Whatman filter paper previously soaked in the corresponding solution for catalysis. The catalytic solution employed to detect the PP activity is 50 mM Tris/HCl (pH 7.8), 10 mM MgCl2, and 0.1–1 mM phosphopeptide designed according to the consensus sequence for the relevant PP. The nitrocellulose membrane is withdrawn and can be stored for additional PP assays. The filter paper is first dipped gently for 2 s into the reagent (1.2 N sulfuric acid, 0.5 % ammonium molybdate, 2 % ascorbic acid) of Chen and coworkers (1956) for the assay of Pi and subsequently placed onto an absorbent paper to remove the excess of liquid until blue spots appeared.

Evaluation

The bacterial cell-based procedure is sensitive enough to detect endogenous phosphate activity of bacteria under some experimental conditions. Although this feature appears to be useful for searching bacterial phosphatases, it can be a drawback when studying cloned mammalian PP. In those cases, *E. coli* strains with diminished phosphatase activity at the pH of interest should be used. In addition, plasmids of a high number of copies could be employed to reduce the time of catalysis and the background signal generated by chromosomal phosphatases. In most cases, the differentiation between the cloned PP activity and background phosphatase activity should be feasible.

Many currently used high-throughput procedures for the detection of compounds modulating PP activity are based on non-physiological substrates holding a colored or fluorescent moiety that facilitates the analysis of the reaction (Wahler and Reymond 2001) but generally impairs the intrinsic activity (Goddard and Reymond 2004). The bacterial cell-based assay for phosphatase activity offers the advantages of the use of specific protein or peptide substrates and the possibility of testing different experimental conditions using the same bacterial lawn. This feature may be helpful for the evaluation of the selectivity of PP.

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Expression, Phosphorylation, Activity, and Interaction of Insulin Signaling Components

Purpose and Rationale

Signal transduction from the cell surface to the nucleus was found to occur predominantly by serine/threonine- and tyrosine-specific PK participating in cascades involving phosphorylation of multiple substrates. The role of protein phosphorylation in signal transduction is well characterized in the case of the insulin signaling cascade in insulin-responsive target cells, such as liver, muscle, and adipose cells (for reviews see Chen 2006; Myers et al. 1994; White 1998, 2003).

In the past decade, it has been increasingly recognized that insulin resistance is associated with chronic, low-grade systemic inflammation.

These cellular inflammatory responses are thought to be mediated by serine/threonine phosphorylation by PK, such as IKKfi (Yuan et al. 2001; Gao et al. 2002), JNK1 (Hirosumi et al. 2002), PKCθ (Yu et al. 2002), or direct interaction with inhibitory proteins, such as SOCS (Mooney et al. 2001; Steppan et al. 2005; Ueki et al. 2004), of key components of insulin signaling, such as the IRS proteins, which thereby undergo inactivation or downregulation. The molecular mechanism of insulin-like compounds/drug candidates can be studied in appropriate assay systems monitoring the phosphorylation state or protein–protein interaction of the individual insulin signaling components.

Preparation of Cytosolic Extracts

Procedure

Cultured Adipocytes

Cultured mouse or human adipocytes are incubated with insulin/compounds/drugs in 12- or 24-well plates for 10–20 min, then washed twice with 50 mM HEPES/KOH (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM EDTA, 10 mM glycerol-3-phosphate, 10 mM Na4P2O7, 2 mM Na3VO4, and 100 mM NaF. Cells are solubilized in the above buffer containing 1 % (by vol.) Triton X-100, 0.1 % sodium deoxycholate, 10 % glycerol, and protease inhibitors (20 µg/ml leupeptin, 10 µg/ml pepstatin A, 50 µg/ml aprotinin, 10 µM E-64, 0.5 mM PMSF = lysis buffer) by scraping with a Teflon policeman (adherent cells). Total lysates are centrifuged (25,000 × g, 20 min, 18 °C). The infranatant is aspirated taking care to avoid contamination by the upper fat layer and recentrifuged to obtain the defatted cell lysate (cytosolic extract).

Primary Rat Adipocytes

Adipocytes (1–3 × 106 cells/ml) are incubated with insulin/compounds/drug candidates and then separated from the medium by flotation and lysed in 1 ml of chilled buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM Na3 VO4, 50 mM Na4P2O7, 10 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 20 µg/ml leupeptin, 10 µg/ml pepstatin, and 25 µg/ml aprotinin by ten strokes in a loose-fitting Teflon-in-glass homogenizer at 15 °C. The fat-free homogenate, prepared by centrifugation (500 × g, 3 min, 15 °C), is supplemented with Triton X-100 (1 % final conc.) and sodium deoxycholate (0.5 % final conc.), incubated for 30 min at 4 °C, and finally centrifuged (13,000 × g, 10 min).

Liver

Portions of frozen liver (0.2–1 g wet weight), which have been perfused as described (see K.5.2.1), are homogenized in a buffer containing 50 mM HEPES/KOH (pH 7.4), 140 mM NaCl, 250 mM sucrose, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA, 2.5 mM Na3VO4, 10 mM glycerol-3-phosphate, 20 mM NaPPi, 20 mM NaF, 1 mM phenylphosphate, 5 µM okadaic acid (sodium salt), 1 % Nonidet P-40, 10 % glycerol, and protease inhibitors (10 µg/ml leupeptin, 5 µg/ml pepstatin A, 75 µg/ml aprotinin, 100 µM benzamidine, 2 µg/ml antipain, 10 µg/ml soybean trypsin inhibitor, 5 µM microcystin, 5 µM E-64, 0.2 mM PMSF) using an Ultraturrax T25 basic (three 10-s cycles at 2,000 rpm on ice) and then a tight-fitting Teflon-in-glass homogenizer (five strokes at 500 rpm on ice). The total homogenate is centrifuged (48,000 × g, 30 min, 4 °C). The supernatant is carefully removed to avoid contamination with the upper fat layer and recentrifuged. The fat-free supernatant obtained is stored in liquid N2 and used as cytosolic extract (3–5 mg protein per ml).

Diaphragm

After incubation with insulin/compounds/drug candidates, the hemidiaphragms (80–100 mg wet weight) are rapidly liberated from the rib cage, rinsed once with homogenization buffer (25 mM Hepes/KOH, pH 7.4, 140 mM NaCl, 10 % glycerol, 1 mM EDTA, 1 mM sodium vanadate, 50 mM sodium pyrophosphate, 100 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 5 µg/ml antipain, 25 µg/ml aprotinin), frozen in liquid N2,

and then homogenized in 2 ml of ice-cold homogenization buffer in a porcelain mortar on ice. After centrifugation (1,500 × g, 10 min, 4 °C), the fat-free supernatant is supplemented with Triton X-100 (0.5 % final conc.), incubated (30 min, 4 °C), and centrifuged (18,000 × g, 20 min, 4 °C) to yield the cytosolic extract.

Skeletal Muscle

Rats at week 10 of age and housed in a temperature-controlled room (22 °C) with a 12:12-h lightdark cycle with free access to chow and water are deeply anesthetized with an i.p. injection of pentobarbital sodium (50 mg/kg). Intact epitrochlearis muscles (type II muscle consisting primarily of both type IIa and IIb fibers) and strips of soleus muscles (consisting primarily of type I fibers) are prepared (~30 mg). Soleus strips and epitrochlearis muscles are incubated for 60 min at 37 °C in 3 ml of oxygenated (95 % O2/5 % CO2) KHB containing 14 mM NaHCO3 and then for 10 min with KHB supplemented with 8 mM glucose, 32 mM mannitol, and 0.1 % BSA in the presence of insulin/compounds/drugs. Thereafter, the muscles are rinsed for 10 min at 37 °C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1 % BSA, then trimmed of fat and connective tissue, quickly frozen between aluminum blocks cooled with liquid N2, and weighed. Frozen muscles are homogenized in 8 vol. of ice-cold lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM glycerolphosphate, 10 mM NaF, 2 mM Na3VO4, 2 mM EDTA, 1 % TX-100, 10 % glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, 2 mM PMSF). Lysates are incubated on ice for 20 min and then centrifuged (13,000 × g, 20 min, 4 °C) to yield the cytosolic extract.

Immunoprecipitation

Up to 1-ml portions of cytosol (equal amounts of protein) are precleared (30-min incubation at 4 °C, 2-min centrifugation at 10,000 × g) with protein A-/G-Sepharose (50 mg, Pharmacia) and then supplemented with typically 2–10 μg/ml appropriate antibodies: anti-IRS-1 (rabbit, polyclonal, protein A-purified, raised against a peptide corresponding to amino acids 1,220–1,235 of rat IRS-1, Upstate Biotechnology), anti-IRS-2 (raised against peptide mixture corresponding to amino acids 618–747 and 976–1,094 of mouse IRS-2), anti-IRβ (raised against a peptide corresponding to the 100 carboxy-terminal amino acids of human IR, Upstate Biotechnology), and anti-GSK-3β (mouse monoclonal, protein G purified, raised against a peptide corresponding to amino acids 203–219 of Drosophila GSK-3β, Upstate Biotechnology, preadsorbed on protein A-Sepharose). After incubation (4–20 h, 4 °C, end-over-end rotation) and centrifugation (10,000 × g, 2 min, 4 °C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 20 mM glycerol-3-phosphate, 10 mM NaPi, 2.5 mM Na3VO4) containing 1 % Nonidet P-40 (omitted for sequential immunoprecipitation), then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.1 % NP-40 and once with 1 ml of immunoprecipitation buffer containing 150 mM NaCl and twice with immunoprecipitation buffer lacking detergent and salt. The washed immune complexes are finally suspended in 50 μl of Laemmli buffer (2 % SDS, 5 % 2-mercaptoethanol), heated (95 °C, 2 min), and centrifuged. The supernatant samples are analyzed by SDS-PAGE using four to twelve percent gradient Bis-Tris precast gels, pH 6.4, morpholinoethanesulfonic acid (MES)/SDS running buffer under reducing conditions (e.g. Novex). For sequential immunoprecipitation, the supernatant samples (50 μl) are supplemented with 1 ml of immunoprecipitation buffer containing 1 % NP-40 and 1–10 μl of the relevant antiserum. After incubation (12 h, 4 °C), 50 μl of protein A-Sepharose (100 mg/ml in immunoprecipitation buffer) is added and the incubation continued (4 h, end-over-end rotation). The immune complexes are collected, washed, and processed for SDS-PAGE.

Immunoblotting

Immunoblotting is performed as described previously by Towbin et al. (1979), Frick and coworkers (1998), and Müller and coworkers (2000) with minor modifications. Briefly, after SDS-PAGE and electrophoretic transfer of the proteins (2 h, 400 mA in 20 % methanol, 192 mM glycine, 25 mM Tris, 0.005 % SDS) to PVDF membranes (Immobilon, Millipore, Germany), the blocked membrane (1 h in blotting buffer containing 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween 20, 0.1 % Brij, 0.01 % NP-40 and supplemented with either 1 % ovalbumin or 1 % BSA or 5 % nonfat dried milk according to manufacturer's instructions and the unspecific background signal obtained) is incubated (2 h, 25 °C) with the appropriate antibodies (often 1:200–1:5,000). The membranes are washed (four times with Tris-buffered saline (TBS) containing 1 % (by vol.) Nonidet-P40 and 0.5 % Tween 20, twice with TBS containing 0.5 % Tween 20, three times with TBS containing 0.05 % Tween 20, twice with TBS). After incubation (1 h, 25 °C) of the membranes with either [125I]protein A (5 μCi/ml, Amersham Pharmacia) or secondary goat anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase (HRP, Santa Cruz Biotechnology) in the same blocking medium, the membranes are washed five times and then subjected in case of the radioactive method to autoradiography (Kodak X-Omat AR) or phosphorimaging (Molecular Dynamics, Storm 860) or in case of the nonradioactive method to an enhanced chemiluminescence (ECL, Amersham Pharmacia or Renaissance Chemiluminescence Detection System, NEN/DuPont) detection system.

Evaluation

The band intensities on the autoradiographs are quantified on a scanning densitometer. The ECL blots are visualized by a LumiImager (Roche) and quantified using ImageQuant software (Roche). The recovery in the amounts of immunoprecipitated protein is corrected (data on fold or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting. Each experiment should be performed with samples from at least four different cell incubations or tissue preparations with antibody incubations/blots performed in triplicate, each.

Immune Complex Kinase Assay

Purpose and Rationale

In case of high expression and/or high specific activity, the relevant PK can be assayed directly using total cytosolic extracts from cells/tissues which have been incubated with compounds/drug candidates, provided the assay used displays sufficient specificity (e.g., due to specific substrate peptide). However, in most cases the relevant kinase has to be enriched by specific immunoprecipitation (see section "Immunoprecipitation") using antibodies which do not interfere with the subsequent measurement of the PK activity within the immune complex. This immune complex kinase assay also eliminates the need for specific peptide or protein substrates not accepted by PK which may be contained in the total cytosolic extracts used and lead to unspecific activity measurements.

The immune complexes prepared as described (see ▶ Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms) are washed in kinase buffer (50 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 5 mM MnCl2, 1 mM MgCl2, 0.5 mM DTT, 20 mM NaF, 1 mM Na3VO4) and then suspended in 25 μl of kinase buffer. The PK reactions are started by addition of ATP (unlabeled or 32P- or 33P-labeled, final conc. Five micrometer, 0.2 mCi/ml to up to 100 μM, 0.5 mCi/ml) and incubated (often 3–15 min, 22 °C) in the absence (autophosphorylation) or presence of recombinant substrate protein (often 0.1–1 μg) or peptide (5–25 μg) in a final volume of 50 μl. Autophosphorylation reactions are terminated by addition of 50 μl of ice-cold twofold stop buffer (100 mM HEPES/KOH, pH 7.4, 300 mM NaCl, 200 mM ATP, 0.1 % Triton X-100) and washing

of the beads (10,000 × g, 2 min, 4 °C) twice with 1 ml of stop buffer and once with 1 ml of stop buffer lacking detergent and salt prior to addition of 20 µl of Laemmli sample buffer and boiling (95 °C, 5 min). Substrate phosphorylation reactions are terminated by addition of 20 µl of fourfold concentrated Laemmli sample buffer and boiling. The phosphoproteins contained in the supernatant of a spin (10,000 × g, 5 min) are separated on SDS-PAGE (10 % Bis-Tris resolving gel, morpholinopropanesulfonic acid/SDS running buffer) and analyzed by phosphorimaging directly (use of [32/33P]ATP) or after immunoblotting with anti-phosphotyrosine or anti-pan-phosphoserine/threonine antibodies (use of unlabeled ATP). Under these conditions the PK reactions are linear with time for the assay period. Protein concentration is determined by the BCA protein assay with crystalline BSA as standard. Phosphorimaging is performed with a phosphorimager Storm 860 and quantitatively evaluated using ImageQuant software (Molecular Dynamics).

Evaluation

Differences in recovery in the amounts of immunoprecipitated protein during a specific experiment are corrected in each case (data on fold or % stimulation) for the amount of protein actually applied onto the gel by homologous immunoblotting. The data should be confirmed by running independent experiments with different batches of cytosolic fractions prepared from independent cell/tissue incubations, each, with two to five parallel independent immunoprecipitation/kinase assay analyses.

Phosphatidylinositol-3′-Kinase (PI3-K)

Portions of the anti-IRS-1/IRS-2 immune complexes (see section "Immunecomplex Kinase Assay") are assayed for PI3-K activity by incubating in 100 µl of 20 mM Tris/HCl (pH 7.0), 50 µM [γ-32P]ATP (5 µCi), 10 mM MgCl2, 2 mM MnCl2, 100 mM NaCl, 2 mM EDTA, 0.5 µM wortmannin (for control incubations, only) containing 10 µg of phosphatidylinositol and 1 µg of phosphatidylserine (Avanti Polar Lipids) for 15 min at 22 °C. After addition of 20 µl of 8 M HCl and 160 µl of a 1/1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates (Silica Gel 60, Merck, Darmstadt) coated with 1 % oxalate and developed in chloroform/methanol/water/ammonia (60/47/11.3/3.2, by vol.). Radiolabeled phosphatidylinositol-3-phosphate (PI3-P, average Rf value of 0.41 under these conditions) is visualized by autoradiography and quantitated by phosphorimage analysis. For calculation of wortmannin-sensitive PI3-K, all values are corrected for PI3-P radiolabeled in the presence of wortmannin.

Phosphoinositide-Dependent Kinase-1/2 (PDK-1/2)

Akt-1/2 represents the major substrate of PDK-1/2 within the insulin signaling cascade (see "▶ Assays for Insulin and Insulin-Like Regulation of Energy Metabolism"). Its phosphorylation can therefore be used as measure for specific increases in PDK-1/2 activity in response to insulin/compounds/drug candidates. For this, cytosolic extracts are prepared (see section "Preparation of Cytosolic Extracts") from cells/tissues which have been incubated with insulin/compounds/drug candidates. After centrifugation (150,000 × g, 60 min, 4 °C), 1-ml portions of the supernatant are immunoprecipitated with 10 µg anti-Akt-1/2 antibodies coupled to protein G-Sepharose beads. The immunoprecipitates are washed and finally suspended in 50 µl of Laemmli sample buffer (2 % SDS), heated (95 °C, 2 min), and centrifuged (12,000 × g, 2 min). After dilution with 1 ml of immunoprecipitation buffer containing 10 µg/ml aprotinin, 20 µg/ml pepstatin, 10 µg/ml leupeptin, 1 mM PMSF, and subsequent centrifugation again (12,000 × g, 2 min), the supernatant is supplemented with 20 µg of a kit containing four different monoclonal anti-phosphoserine antibodies (Biomol, Hamburg, Germany; clones 1C8, 4A3, 4A9, 4H4) and the monoclonal anti-phosphothreonine antibodies

(clone 1E11). After incubation (4 h, 4 °C), the antibody complexes are precipitates during a 2-h incubation with 50 µl of 50 mg protein A-Sepharose per ml of 50 mM HEPES/KOH (pH 7.4) and 0.1 % Triton X-100. The collected immunoprecipitates (12,000 × g, 2 min) are washed three times with 1 ml each of 50 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 100 mM NaF, 0.2 mM Na3VO4, 0.1 % SDS, and 1 % Triton X-100 and finally twice with buffer lacking Triton X-100. Phosphoproteins are eluted from the washed precipitates by incubation with 500 µl of 50 mM HEPES/KOH (pH 7.4) containing 50 mM p-nitrophenylphosphate for 30 min at 4 °C. The supernatants obtained by centrifugation are precipitated with TCA. The acetone-washed pellets are suspended in 50 µl of Laemmli sample buffer and analyzed by SDS-PAGE. Phosphorylated Akt-1/2 is detected by immunoblotting with anti-Akt-1/2 antibodies as described above. In case of high expression/phosphorylation of Akt, immunoblotting with the anti-AktpS473 (PDK-1) or pT307 (PDK-2) antibody (1:400) may be sufficient for the direct detection of Akt phosphorylation and consequently for evaluation of PDK-1/2 activity.

Protein Kinase B (Akt-1/2)

Cytosolic fractions are prepared from cells/tissues which have been incubated with insulin/compounds/drug candidates. Nine hundred microliters of cytosolic extract is incubated with 100 µl of anti-Akt-1/2 antibody (raised in rabbits by immunization with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence, 10 µg pre-coupled to 10 mg of protein A-Sepharose in 100 µl of lysis buffer for 2 h at 4 °C). The immunoprecipitates are collected by centrifugation (12,000 × g, 2 min, 4 °C), washed twice with 1 ml each of Akt assay buffer (20 mM Mops, pH 7.0, 1 mM EDTA, 1 mM EGTA, 10 mM Na4P2O7, 50 mM NaF, 1 mM Na3 VO4, 1 µM microcystin, 1 µM okadaic acid, 10 µg/ml pepstatin, 25 µg/ml leupeptin, 1 µg/ml antipain, 10 µg/ml aprotinin, 0.1 mM PMSF, 0.01 % Brij35, 5 % glycerol) containing 1 % Nonidet P-40, 0.5 M NaCl, and three times with 1 ml each of assay buffer lacking salt and detergent. The immunoprecipitates are finally resuspended in 45 µl of PKB assay buffer containing 10 mM MgCl2, 1 mM DTT, 2.5 µM PKA inhibitor peptide (IP20), and either the synthetic substrate peptide, Crosstide (100 µM final conc.), which is based on the sequence surrounding the serine phosphorylation site of GSK-3 (Ser21 of GSK-3α and Ser9 of GSK-3β) or alternatively histone 2B (150 µg/ml). The assay is initiated by the addition of 5 µl of [γ-32P]ATP (50 µM, 4 µCi) and terminated after incubation for 15 min at 30 °C by placing the test tubes on ice. For determination of Crosstide phosphorylation, the 10-µl portions of the samples are adsorbed on p81 phosphocellulose paper and extensively washed as described for the MAPK assay (see section "Mitogen-Activated Protein Kinase (MAPK)"). The radioactivity associated with the paper is counted by liquid scintillation counting. For determination of histone 2B phosphorylation, the samples are separated on a 12 % SDS-PAGE. The autoradiogram is quantitated by densitometry. Exposure times are chosen which guarantee that the intensity of the bands is linearly related to the quantity of protein contained in the bands.

Glycogen Synthase-3β (GSK-3β)

GSK-3β activity is determined using immune complexes from cytosolic extracts of treated cells/tissues (see sections "Preparation of Cytosolic Extracts" and "Immunoprecipitation") with phospho-glycogen synthase peptide 2 (P-GS 2) as a substrate (Eldar-Finkelman et al. 1996; Wang et al. 1994). The GSK-3β immune complexes (with antibodies raised in sheep by immunization with a synthetic peptide corresponding to amino acids 471–483 of rat GSK-3β and coupled to BSA) are precipitated with 2 mg of anti-sheep IgG coupled to agarose in 50 µl of lysis buffer (under rotation for 2 h at 4 °C). The beads are collected by centrifugation (12,000 × g, 2 min), washed once with 1 ml of 100 mM Tris/HCl (pH 7.4) containing 0.2 % Nonidet P-40, 0.5 M NaCl, and 0.5 M LiCl, once with 10 mM Tris/

HCl, and twice with GSK-3 assay buffer (20 mM HEPES/KOH, pH 7.4, 10 mM MgCl2, 1 mM DTT, 10 mM NaF, 10 mM Na4P2O7) and then suspended in 20 µl of assay buffer containing 0.4 mg/ml BSA, 30 µM [γ-32P]ATP (3,000 Ci/mmol, 1 mCi/ml), and 20 µM P-GS2. After incubation (15 min, 30 °C), the reactions are terminated by addition of 20 µl of 20 % TCA and centrifugation (10,000 × g, 5 min). Then, 15-µl portions of the supernatant are spotted on 1 × 1-cm pieces of Whatman P81 phosphocellulose paper. Twenty seconds later, the filters are washed five times with 0.75 % phosphoric acid (for at least 5 min each time) and once with acetone. The dried filters are counted for radioactivity in the presence of 5 ml of scintillation fluid (e.g., ACS, Amersham Pharmacia). 32Pi incorporation into the negative control peptide (glycogen synthase peptide 2 [Ala21]) is subtracted from values obtained using P-GS 2. No activity is typically measured with immunoprecipitates using nonimmune IgG. In some experiments recombinant PP2A inhibitor (PP2AI, 1 µg, Calbiochem) is used as substrate instead of P-GS1. In this case the PK reaction (conditions as above) is terminated by rapid centrifugation (12,000 × g, 1 min, 4 °C) and separation of the supernatant from the immune complex pellet. The supernatant of a precipitation (10 % TCA for 1 h on ice, 15,000 × g, 15 min, 4 °C) containing the phosphorylated PP2AI is suspended in 50 µl of twofold Laemmli sample buffer, heated (95 °C, 5 min), and centrifuged (12,000 × g, 2 min). The supernatants are run on SDS-PAGE, and phosphorylated PP2AI and GSK-3 are visualized by autoradiography and quantitated by phosphorimaging.

MAPK/ERK Kinase (MEK)

MEK activity is determined using immune complexes from cytosolic extracts of treated cells/tissues (see sections "Preparation of Cytosolic Extracts" and "Immunoprecipitation") with recombinant MAPK/ERK2 (Upstate Biotechnology) as substrate. The assay mix contains 4 mM MOPS buffer (pH 7.5), 5 mM glycerol-3-phosphate, 1.25 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM DTT, 10 mM MgCl2, 0.15 mM ATP, and 0.04 µCi/pl [γ-32P]ATP. The concentration of MAPK/ERK2 (0.5 ng/pl) is chosen to approximate the concentration of MAPK/ERK2 that would be assayed in rat adipocyte cell lysates.

Mitogen-Activated Protein Kinase (MAPK)

MAPK activity is determined using immune complexes from cytosolic extracts of cells/tissues treated with compounds/drug candidates (see sections "Preparation of Cytosolic Extracts" and "Immunoprecipitation") with myelin basic protein (MBP) as substrate. Adipocytes incubated with insulin/compounds/drugs are washed with KRP-HEPES by flotation and then lysed in 1 ml of 50 mM HEPES/KOH (pH 7.2), 100 mM NaCl, 2 mM EDTA, 1 % Nonidet P-40, 0.5 mM Na3VO4, 40 mM p-nitrophenylphosphate, 10 mM glycerol-3-phosphate, 10 mM NaF, 0.2 mM PMSF, 25 µg/ml leupeptin, and 25 µg/ml aprotinin by incubation for 30 min on ice and vigorous vortexing three times for 10 s at 10-min intervals. Lysates are cleared by centrifugation (12,000 × g, 10 min, 4 °C). The infranatant below the fat cake is removed using a syringe, recentrifuged, and used for measurement of MAPK by addition of 250-µl portions to 250 µl of protein A-Sepharose (50 mg/ml of 50 mM HEPES/KOH, pH 7.2,100 mM NaCl), which had been pre-coupled with 10 µl of rabbit anti-p42MAPK antiserum (raised against rat p42MAPK, Upstate Biotechnology) and 5 µl of rabbit anti-p44MAPK antiserum (raised against the carboxy-terminal 14 amino acids of rat p44MAPK) and incubation for 2 h at 4 °C. The beads are collected by centrifugation (12,000 × g, 2 min, 4 °C), washed three times with 1 ml each of lysis buffer and three times with 1 ml each of 10 mM HEPES/KOH (pH 7.4) and 10 mM MgCl2, and then resuspended in 45 µl of kinase buffer (50 mM glycerol-3-phosphate, pH 7.3, 5 mM Na4P2O7, 10 mM NaF, 0.5 mM EDTA, 15 mM MgCl2, 2 mM DTT, 4.4 mM PK inhibitor peptide) containing 0.5 mg/ml MBP. The kinase reaction is initiated by the addition of 5 µl of

0.5 mM [γ-32P]ATP (700 mCi/mmol). Following a 10-min incubation at 30 °C, reactions are terminated by spotting 10 μl of the reaction mixture onto p81 phosphocellulose papers (Whatman), which are immediately immersed in 0.85 % orthophosphoric acid under stirring and washed once in 95 % ethanol for 5 min. Papers are dried and 32P is quantitated by liquid scintillation counting.

Phosphoproteomics

General Considerations

The above methods for the detection of the phosphorylation state of insulin signaling components encompassing the IR and its downstream elements enable their individual analysis, only. Taking into account the expenditure in time and costs, their simultaneous analysis in a phosphoproteomic approach may be of great advantage. Innovations in the area of 2-D gel electrophoresis, protein analysis, and computer databases are moving proteomics from futuristic possibilities into common laboratory procedures. The main aim of classic proteome studies is the separation of complex protein mixtures in order to visualize the relative levels of as many proteins as possible. Improvements of analytical techniques for protein identification, such as peptide mass fingerprinting, have enabled analysis of changes in proteomes on a large scale. However, obtaining information on co- and posttranslational modifications within the analyzed proteome remains a major challenge. A wide variety of posttranslational modifications, such as phosphorylation, glycosylation, acylation, methylation, and acetylation, are known to play key roles in regulating the function, localization, binding specificity, and stability of target proteins. To analyze this additional layer of protein diversity and to reveal its complexity, the traditional protein-by-protein approach clearly will not suffice to meet the huge magnitude of variation created by posttranslational modifications. Therefore, development of proteome-based technology is required to analyze posttranslational modifications of proteins, in general and phosphorylation, in particular.

Purpose and Rationale

Since protein phosphorylation is an essential posttranslational process in a variety of cellular processes, it is not surprising that perturbations in the equilibrium of PK and PP activities are fundamentally involved in metabolic diseases, such as type II diabetes and obesity. These pathogenetic changes in protein phosphorylation are preferably analyzed in parallel ("phosphoproteomics") rather than sequential fashion (e.g., immunoblotting, immunoprecipitation).

Procedure

Various techniques can be used to detect phosphorylated proteins. In many approaches detection of phosphorylated proteins separated on 2-D gels is activated by incubating cells with [32P] or [33P] orthophosphate. However, when radiolabeling is used to detect phosphoproteins, it has to be considered that constitutively phosphorylated proteins with slow phosphate turnover rates, which hence only incorporate small amounts of radioactive phosphate, may be poorly detected. Furthermore, care is necessary when comparing ratios of phosphorylated threonine, serine, and tyrosine residues. Similarly, different metabolic phosphorylation/dephosphorylation rates cause unequal incorporation of [32P] into different types of amino acids. The use of poly- and monoclonal antibodies directed against phosphoamino acids represents an alternative procedure to detect phosphorylated proteins blotted onto a membrane. This method is very sensitive since antibodies can detect as little as a few fmol of epitope. Among other strategies, antibodies recognizing phosphotyrosine (pY), phosphothreonine (pT), and phosphoserine (pS) have been generated by cross-linking of a phosphoamino acid-containing hapten to keyhole limpet hemocyanin or BSA. Anti-pT, pY, and pS antibodies are commercially available, and particularly pY antibodies are now widely used. It was found that different anti-pY antibodies

bind to essentially the same proteins in immunoblotting analysis, independent of the exact nature of the immunogen against which the antibodies were raised. Little to no cross-reactivity to non-phosphorylated tyrosine, pT, pS, AMP, or ATP has been observed. The sensitivity of the anti-pY antibody is very high, since tyrosine phosphorylation in normal and unstimulated cells and tissues is 100- to 1,000-fold less abundant than pS, accounting for only 0.02 % of phosphoamino acids. However, phosphorylation-specific antibodies may not detect certain phosphorylated proteins due to steric hindrance of the recognition site, which is especially true for anti-pT and pS antibodies. In addition, lack of antigenicity conferred by pT and pS (in contrast to pY) has precluded rigorous and unquestionable detection of threonine and serine phosphorylation in proteins. More often pT and pS can be identified specially in the context of a larger epitope, only, thus precluding whole proteome analysis. However, many groups of substrates have overlapping epitopes, for example, those with a proline in the +1 position of a phosphorylated Ser or Thr (the archetypal MAPK or CIK sites) for which monoclonal antibodies have recently been introduced. Once more common phosphorylation motifs have been determined, more antibodies will become available, thus facilitating phosphorylation analysis of the whole proteome (= phosphoproteomics; for a review see Kaufmann et al. 2001).

Extensive reviews cover the most critical tasks of lysis of mammalian cells, sequential extraction (Molloy et al. 1999), pre-fractionation (Cordwell et al. 2000), sample preparation (Herbert 1999; O'Farrell 1975), sample solubilization for isoelectric focusing (Rabilloud et al. 1997) and two-dimensional gel electrophoresis (Görg et al. 2000), and finally detection of phosphoproteins by immunoblotting (see sections "Detection by Immunoblotting" and "Immunoblotting") and to total proteins by chemical staining (Dunn 1999; Birkelund et al. 1997).

Evaluation

Detection of phosphorylated proteins within complex protein mixtures separated by 2-D gel electrophoresis followed by immunoblotting represents a relatively simple and sensitive method. Antibodies directed against pT, pS, and pY have been successfully used in proteome studies to identify and characterize phosphoproteins. A strategy is recommended that allows high-throughput identification of phosphorylated proteins. The protein-by-protein approach for elucidating complex biological regulatory processes mediated by protein phosphorylation can now be replaced by analysis of the phospho-proteome under varying conditions.

However, the described methodology has its limits. Especially specific anti-pS and anti-pT antibodies are not able to recognize all proteins harboring phosphates on these residues. Therefore, immunoblotting analysis should be combined with radiolabeling techniques to obtain highly accurate and complete pictures of the phospho-proteome. A major challenge in creating phospho-proteome maps remains the low abundance of many phosphorylated key regulatory proteins, such as signal transduction molecules. Improvement of the sensitivity of protein identification techniques, such as peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, will be the key to overcome this obstacle.

Multiplex Bead Immunoassay

Purpose and Rationale

The introduction of methodologies that allow the simultaneous measurement of interrelated markers, e.g., phosphoproteins within a common signal transduction pathway, has revolutionized the drug discovery and evaluation process. Monitoring multiple proteins in a single sample and experiment saves time, labor costs, and sample volume. The multiplex bead immunoassay developed by BioSource Inc. (CA, USA) in conjunction with the Luminex xMAP™ detection system and fluorescently encoded microspheres enables the simultaneous measurement of up to 100 different biological markers depending on the

availability of appropriate (phosphosite-specific) antibodies. Each microsphere is labeled with a distinguishable fluorophore that allows it to be assigned or gated to a particular region by the scanner. Antibodies specific for the (phospho-) protein of interest are covalently linked to beads of a unique fluorescent region. The combination of different beads allows the user to simultaneously measure various protein markers of interest. The reagent and antibody bead kits offered by BioSource are intended for the quantitative determination of the (phospho-)protein in total cellular lysates and tissue homogenates.

The multiplex bead immunoassay is a solid phase sandwich immunoassay, which is designed to be analyzed with a Luminex 100™ instrument. The spectral properties of 100 distinct bead regions can be monitored with this instrument, a capability that affords this assay system the potential for measuring up to 100 different marker (phospho-)proteins in a single sample. Beads of defined spectral properties conjugated to marker-specific capture antibodies and samples (including standards of known marker concentration, blank controls, etc.) are pipetted into wells of a filter bottom microplate and incubated for 2 h. During this first incubation, the marker (phospho-) proteins bind to the capture antibodies on the beads. After washing the beads, marker (phosphosite)-specific detector antibodies are added and incubated with the beads for 1 h. During this second incubation, the marker-specific detector antibodies recognize their (phosphorylated) epitopes and bind to the appropriate immobilized marker (phospho-)proteins. After removal of excess detector antibodies, an anti-rabbit R-phycoerythrin (RPE) conjugated secondary antibody is added for 30 min. During this final incubation, the anti-rabbit RPE binds to the detector antibodies associated with the immune complexes on the beads, forming four-membered solid phase sandwiches. After washing to remove unbound anti-rabbit RPE, the beads are analyzed with the Luminex 100™ instrument. By monitoring the spectral properties of the beads and the amount of associated RPE fluorescence, the concentration of one or more analytes can be determined.

The multiple bead immunoassay has been initially introduced for the determination of the concentration of a panel of cytokines circulating in human serum (e.g., of patients suffering from primary Sjogren's syndrome or bacterial infection; Szodoray 2004; Pickering 2004), but can meanwhile be applied successfully for the analysis of phosphorylation state of a number (currently seven) of key components of the insulin signal transduction cascade undergoing either tyrosine phosphorylation (IR, IGF-1 receptor, IRS-1) or serine/threonine phosphorylation (GSK-3, Akt, p70S6 kinase, p42/44 MAPK). Using this method the quantitative evaluation of changes in their phosphorylation state in response to treatment of insulin target cells requires one single-well reaction, only, rather than seven separate immunoprecipitation reactions followed by seven immunoblotting and fluorescent/luminescent imaging procedures. Thus, this method will significantly facilitate the throughput characterization of compounds/drug candidates with insulin-like and/or insulin-sensitizing activity and concomitantly increase its accuracy and resolution.

Protein Interaction Analysis

Purpose and Rationale

In addition to protein phosphorylation, protein–protein interaction represents the major molecular mechanism for insulin signal transduction in insulin target cells (see "▶ Assays for Insulin and Insulin-Like Regulation of Energy Metabolism" and section "Expression, Phosphorylation, Activity and Interaction of Insulin Signaling Components"), as exemplified by binding to and activation by IRS of PI3-K. Protein–protein interaction can be measured by (i) coimmunoprecipitation of one partner and subsequent immunoblotting of the coimmunoprecipitate for presence of the binding partner, (ii) pulldown of one partner via its tag (e.g., GST, His) and subsequent immunoblotting of the recovered material for presence of the (tagged) binding partner, and (iii) the optical phenomenon called surface plasmon resonance (SPR). More

than one decade after its introduction (Jönsson et al. 1991), the latter method (iii) combined with a miniaturized flow system and a sophisticated surface design has gained a lot of attractiveness since it enables many biomolecular interactions to be investigated conveniently in real time and does depend neither on the availability of a pair of compatible antibodies as is the case for coimmunoprecipitation (i) nor on the generation of recombinant tagged or labeled binding partners as is required for pulldown (ii) and furthermore allows kinetic and real time rather than sole steady-state analysis of the interaction.

SPR defines the characteristics of proteins in terms of their specificity of interaction with other molecules, the rates at which they interact (association and dissociation) and their affinity, which can be determined from the level of binding at equilibrium (seen as a constant signal) as a function of sample concentration as well as from kinetic measurements. Even the formation of multimolecular complexes can be monitored.

The natural phenomenon of SPR is utilized by the Biacore protein interaction analysis system (Biacore AB, Sweden) to deliver high-quality data in real time, without the use of labels. The SPR phenomenon occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index, the glass of a sensor surface (high refractive index) and a buffer (low refractive index). In Biacore systems, a sensor chip and microfluidics system form flow cells through which samples flow over the sensor surface. As molecules from the injected sample bind to the immobilized molecules, an alteration in refractive index occurs that is proportional to the change in mass concentration at the surface. Using the SPR phenomenon, these changes are detected in real time, and data is presented as a sensorgram (SPR response plotted against real time).

Procedure

In a typical experiment one of the components (binding partner, ligand) in a biospecific pair is immobilized on the sensor chip surface (Löfas et al. 1995), and the counterpart (the analyte) which interacts with the lig- and is in solution and passed over the gold film in a continuous flow. In the flow system (Sjölander and Urbaniczky 1991), samples and reagents can be introduced over the sensor surface. Automation of flow properties and sample injection gives the experiment control over temperature, flow, and concentration properties as well as permitting reaction conditions on the surface to be changed easily.

The surface properties of the sensor chips are central to Biacore technology. The surface of the gold film that is needed for SPR detection is modified to avoid protein adsorption to the detector surface itself. This is achieved by a self-assembled monolayer of alkanethiols to which dextran is coupled by epoxy chemistry (Johnsson et al. 1991; Löfas 1995). The surface properties are of great importance for the evaluation of results from real-time biomolecular interaction analysis. Unwanted interactions can be identified and to some extent compensated for by proper control surfaces in multidetection approaches (Karlsson and Stählberg 1995). Nordin and coworkers (2005) analyzed small-molecule interactions with PK using SPR biosensors.

Evaluation

A range of methods based on Biacore technology have been developed for different purposes over the past years which have been reviewed by Malmqvist and Karlsson (1997). Biacore's system facilitates the elucidation of disease mechanisms and the discovery of drug candidates by characterizing native and recombinant protein interactions. The system enables the definition of potential drug targets and the development of assays to characterize the interaction of target proteins with low-molecular-weight compounds. Other applications may include the selection of protein therapeutic candidates (e.g., recombinant insulin analogs) according to their on/off rates of binding to the target (e.g., IR) as well as the detection and characterization of immune responses during preclinical and clinical development.

Regarding application of Biacore technology to PK inhibitor interaction, Nordlin and coworkers (2005) found the immobilization conditions to be critical, and they developed several strategies for preserving the binding capacity for inhibitors and ATP. The composition of the assay buffer is developed for optimized kinase assay performance. The assays include kinetic characterization of inhibitor binding to PK and analysis of binding characteristics in the presence of ATP for identification of ATP-competitive binders. The effect of PK phosphorylation on the binding of ATP and inhibitors can also be investigated, as is the PK isotype specificity. Furthermore, a complementary activity assay revealed the correlation between binding to the PK and inhibition of the PK. Thus, the biosensor technology offers a useful tool in PK-targeted compound identification and drug evaluation.

O-Linked Glycosylation (O-GlcNAc) of Insulin Signaling Components

General Considerations

Hyperglycemia is a consequence of type II diabetes and constitutes a self-perpetuating condition that contributes to poor metabolic control by impairing both insulin action and insulin secretion (Kruszynska and Olefsky 1996; Karam 1996; Yki-Järvinen 1992). The deleterious effects of hyperglycemia have been collectively referred to as "glucose toxicity" (Rossetti et al. 1990, 1995; Rossetti 1996), the most prominent one of which is insulin resistance. Studies of humans and animals with spontaneous or experimentally induced insulin resistance have revealed much about the multiple pathways that can lead to insulin resistance. However, the primary events that trigger insulin resistance remain incompletely understood. Experimental induction of insulin resistance in isolated cells or tissues can be a useful approach for the identification of early events because it provides considerable experimental control.

Marshall and coworkers provided ample evidence that excessive flux of glucose through the hexosamine biosynthesis pathway (HBP) triggers various adaptive responses that restore intracellular glucose homeostasis. One such response is reduction of glucose uptake through desensitization of the insulin-responsive glucose transport system (GTS). Since the glucose-sensing/signal transductional capability of the HBP constitutes only one function of this ubiquitous pathway, this regulatory arm of the HBP has been named the hexosamine signaling pathway (HSP) (Marshall and Rumberger 2000; Marshall 2002).

In exploring the functional role of the HSP and the underlying mechanisms, glucosamine (GlcN) is often used as a pharmacological tool since it can readily undergo internalization through the GLUT1/4 and directly enter the HBP at the level of GlcN-6-P (Marshall et al. 1991a, 2004). Thus, GlcN was found to mimic glucose-induced desensitization in adipocytes and was shown to be >40 times more potent than glucose. Although it was originally thought of that glucose and GlcN were causing desensitization through a common mechanism, this premise was later questioned when actinomycin D was found to completely block glucose-induced desensitization but not GlcN-induced desensitization (Marshall et al. 1991b; Han et al. 2003). Thus, glucose-induced desensitization is mediated through a mechanism involving changes in gene expression, whereas GlcN-induced desensitization occurs through an alternate and unidentified mechanism. In conclusion, Marshall and colleagues hypothesized that flux through the HBP serves as a glucose sensor in adipocytes, and potentially other insulin target cells, and by some unknown negative feedback loop regulates glucose transport provoking glucose-induced insulin resistance.

Purpose and Rationale

The studies of Hebert and coworkers (1996), Cooksey and McClain (2002), and Gazdag and coworkers (2000) demonstrated an inverse relationship between cellular UDP-GlcNAc levels and insulin action, but did not reveal a specific

mechanism for the association. UDP-GlcNAc is a substrate for O-GlcNAc transferase (uridine diphospho-N-acetylglucosamine/polypeptide-N-acetylglucosaminyltrans-ferase) that transfers single N-acetylglucosamine moieties to the hydroxyl group of serine and threonine residues on cellular target proteins in a dynamic and reversible process known as O-GlcNAcylation (Wells et al. 2001). The extent of cellular O-GlcNAcylation appears to be regulated, at least in part, by the cellular concentration of the UDP-GlcNAc substrate (Kreppel and Hart 1999). McClain and coworkers (2002) recently found direct evidence that O-GlcNAc transferase plays an important role in insulin action by overexpressing O-GlcNAc transferase in skeletal muscle and fat in transgenic mice and inducing insulin resistance.

O-GlcNAcase (β-N-acetylglucosaminidase) is responsible for specific removal of single O-GlcNAc modifications from cellular proteins. Both O-GlcNAc transferase and O-GlcNAcase exhibit nuclear and cytoplasmic localization and are highly conserved (Dong and Hart 1994). Wells and coworkers (2001) have hypothesized that O-GlcNAcylation of proteins is a type of posttranslational modification that may be analogous to PK and PP regulation of phosphorylation and thus compete for similar or adjacent phosphorylation sites in a reciprocal manner. It seems possible that, in addition to flux through the HBP, regulation of either O-GlcNAc transferase (McClain et al. 2002) or O-GlcNAcase (Vosseller et al. 2001, 2002) may influence insulin action. O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) is a potent and nontoxic inhibitor of O-GlcNAcase in various cell lines (Haltiwanger et al. 1998). Arias and coworkers (2004) found that prolonged incubation in PUGNAc results in increased protein-O-linked glycosylation and insulin resistance in rat skeletal muscle. The following assays represent model systems for the induction and analysis of glucose-induced insulin resistance which is mediated by O-GlcNAcylation and can be modified by compounds/drug candidates.

Induction of O-Glc-NAc Modification in Adipocytes, Myocytes, and Muscles

Primary Adipocytes

Isolated adipocytes are obtained from the epididymal fat pads of male Sprague–Dawley rats (180–225 g) by collagenase digestion as described above with modifications introduced by Marshall and coworkers (2005a). After digestion, cells are washed three times in HEPES-buffered balanced saline solution (HBSS) consisting of 25 mM HEPES (pH 7.6), 120 mM NaCl, 0.8 mM MgSO4, 2 mM CaCl2, 5.4 mM KCl, 1 mM NaH2PO4, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 % BSA. Cells are then diluted to a final concentration of 5 × 105 cells/ml (12 ml HBSS per 1 g initial fat weight). From a common pool of cells, 200-μl portions of the adipocyte suspension in 12 × 75-mm sterile polystyrene tubes are incubated at 37 °C in the absence or presence of insulin (10 nM final conc.) to stimulate the glucose transport system and to facilitate the subsequent uptake of GlcN. After 20–30 min, GlcN is added, and adipocytes are further incubated at 37 °C.

3T3-L1 Adipocytes

3T3-L1 adipocytes are cultured for 16 h in DMEM containing 4 mM glucose in the absence of serum and containing PUGNAc (100 μM). After 16 h, cells are refed with identical media for 3 h. For GlcN and/or chronic insulin/glucose treatment of cells, 3T3-L1 adipocytes are cultured for 16 h in DMEM containing 4 mM glucose in the absence of serum with 0.2 % BSA and containing 5 mM GlcN/5 mM HEPES (pH 7.6) and/or insulin (1 nM). After 16 h, cells are washed twice and refed with identical media used during 16-h treatment, but lacking insulin. Cells are then washed twice with Krebs–Ringer's phosphate buffer (KRP) and stimulated in KRP with various concentrations of insulin for the indicated time at 37 °C.

Myocytes

C2C12 cells are cultured to confluence in growing conditions in DMEM containing 25 mM glucose and 10 % fetal bovine serum (FBS) in collagen type I-coated culture plate according to the procedure described by Fujita and coworkers (2005). When cell confluence is attained, cells are cultured for 4 days with DMEM containing 25 mM glucose, 2 % horse serum, and with or without 10 mM GlcN. After 4 days, the change in cell morphology induced by GlcN is observed by an optical microscope.

Muscles

Epitrochlearis muscles are quickly excised from rats (140–160 g) anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) upon loss of pedal reflexes and then transferred into the tissue incubation medium. Muscles are incubated (18 h) in flasks containing 1.5 ml of low-glucose (5.5 mM) DMEM supplemented with CaCl2 (final conc. 2.5 mM), NaHCO3 (final conc. 25 mM), insulin (final conc. 0.6 nM), and BSA (0.1 %). Insulin is included for the initial 18 h of incubation because this prevents the increase in basal glucose transport observed with prolonged incubation of isolated skeletal muscle. Flasks are placed in a shaking water bath at 35 °C and continuously gassed with 95 % O2/5 % CO2. The media are replaced with fresh DMEM every 6 h during the initial 18-h incubation. To minimize bacterial contamination during prolonged incubation, 100 μU/ml penicillin and 100 μg/ml streptomycin are added to the DMEM, and Teflon air filters are used to minimize aerosol contaminants.

Following the 18-h incubation in supplemented DMEM, muscles are rinsed in KHB supplemented with 0.1 % BSA, 100 pU/ml penicillin, 100 μg/ml streptomycin, 2 mM sodium pyruvate, and 5 mM mannitol for two 30-min periods at 30 °C for removal of glucose and DMEM. During the rinse steps, the KHB buffer is supplemented with 0, 0.6, or 12 nM insulin. Muscles treated with inhibitors of GFAT in the initial incubation steps continued to be exposed to up to 100 μM inhibitor during the subsequent rinse steps. Muscles are then rapidly blotted, trimmed, clamp frozen with aluminum tongs cooled to the temperature of liquid N2, and stored at −80 °C until further processing. Total frozen muscles are weighed, transferred to pre-chilled glass tissue grinding tubes and homogenized in ice-cold lysis buffer (1 ml/muscle) containing 20 mM Tris/HCl (pH7.4), 150 mM NaCl, 1 % NP-40, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2.5 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na3VO4, 20 mM glycerol-3-phosphate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 mM PMSF in the absence or presence of up to 100 μM of PUGNAc. Homogenates are transferred to microfuge tubes, rotated for 1–2 h at 4 °C, and then centrifuged (12,000 × g, 15 min, room temperature) to remove insoluble material. Following homogenization, processed muscle samples are used for determination of the levels of GlcNAc-6-phosphate and UDP-GlcNAc as well as the amount of O-GlcNAc-modified proteins.

Assay for Glutamine/Fructose-6-Phosphate Amidotransferase (GFAT)

Fluorometric Method

GFA activity is assayed according to previously described methods (Yki-Järvinen et al. 1997) with modifications. Cells corresponding to 5–15 mg protein are homogenized for 10 s on ice and sonicated for 10 bursts in a Branson 250 sonifier in 10 ml of homogenization buffer (50 mM sodium phosphate, pH 7.5, 100 mM KCl, 1 mM EDTA). The sample is then centrifuged (60,000 × g, 15 min, 4 °C), and the supernatant is used for assay of GFA activity. All samples are assayed immediately after homogenization. Fifty microliters of homogenate is incubated (45 min, 37 °C) in a reaction mix (final volume 100 μl) containing 12 mM fructose-6-phosphate, 12 mM glutamine, 40 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 1 mM DTT in the absence or presence of 0.5 mM of UDP-GlcNAc. The reaction is

terminated with 50 µl of 1 M perchloric acid, vortexed, and centrifuged (15,000 × g, 15 min, 4 °C). The deproteinized supernatant (145 µl) is then treated with 258 µl of a 1/4 mixture of tri-N-octylamine/1,1,2-trifluoroethane, vortexed, and centrifuged (15,000 × g, 1 min). The aqueous phase is then filtered through a 0.2-pm filter, and 50 µl is derivatized with an equal volume of O-phtaldialdehyde (OPA) solution (4 mg OPA dissolved in 50 µl of ethanol and added to 5 ml of 0.1 M sodium borate and 10 µl mercaptoethanol). Immediately thereafter samples are separated over a reverse phase C18 column (25 cm × 4.6 mm) equilibrated with 15 mM sodium phosphate (pH 7.2), 5 % acetonitrile, and 5 % isopropanol. Absorbance of the sample eluent is analyzed using a fluorescent detector, and the peak area is integrated. OPA-derivatized GlcN6P standards are run separately to determine the retention time and to generate a standard curve for correlation of area to activity. The correlation coefficient between the concentration of GlcN6P standards and the area under the GlcN6Ppeak is typically 0.999 or higher. The recovery of samples spiked with GlcN6P prior to derivatization is usually more than 95 %.

Radiometric Method

A highly sensitive and rapid method for the separation of radiolabeled fructose-6-phosphate from glucosamine-6-phosphate has been developed by Broschat and coworkers (2002), which can be used in a disposable column or a 96-well formate and is >10-fold more sensitive than the HPLC method. The column assay has a broad range of linearity with low variation between samples. When performed in the 96-well formate, the assay is linear with time and enzyme concentration and greatly improves the rapidity and accuracy with which GFAT activity can be measured.

Research Formate

Cells are homogenized as described for the fluorometric assay using the cytosolic fraction obtained by 100,000 × g spin for 1 h. The final assay conditions contain 20 µM fructose-6-phosphate (300,000 cpm), 400 µM glutamine, 20 mM imidazole buffer (pH 6.8), 1 mM EDTA, 10 mM KCl, 1 mg/ml BSA, and 1 mM DTT in a total volume of 50 µl. All assays are conducted at room temperature. Just prior to the assay, the samples prepared in stabilizing buffer containing glucose-6-phosphate (Zhou et al. 1995) are buffer-exchanged on PD-10 columns equilibrated in the GFAT assay buffer. The reaction is stopped by dilution of the enzyme reaction with 1 ml of 10 mM NaHCO3 (pH 3.0). Separation of GlcN-6-phosphate from substrate is performed immediately at room temperature. Unbound [14C]GlcN-6-phosphate is collected in scintillation vials and quantified by scintillation counting.

Screening Formate

Strong anion exchange resin (AG1X8, 400 mesh, formate form) is prepared prior to the assay and stored for 2 weeks at room temperature or at 4 °C for longer periods. The resin is suspended in batch in a 5-volume excess of 10 mM NaHCO3 (pH 3.0) and allowed to settle for 20 min. The fines and excess buffer are decanted, and the procedure is repeated until no fine material remained, usually three to four times. The pH of the formate buffer above the resin should be unchanged by the resin after the equilibration is complete. GFAT activity is measured in a total volume of 50 µl in U-bottomed deep-well 96-well plates. The substrates, 20 µM [14C]fructose-6-phosphate (75,000 cpm per well) and 400 µM glutamine, are mixed, and the reaction is terminated by the addition of a 150-µl suspension of Dowex AG1X8 (400 mesh) in 10 mM NaHCO3, containing 50 µl resin and 100 µl buffer by volume. Suspension of the resin is maintained using a stir plate prior to transfer. The resin is allowed to settle for 5 min, after which a 50-µl aliquot of the supernatant is removed using a programmable liquid transfer device and transferred to a 96-well topcount plate. After addition of 150 µl of scintillation cocktail, the plates are sealed,

shaken for 5 min, and counted for radioactivity in a topcount reader.

Assay for O-GlcNAc Transferase

Cells corresponding to 2–5 mg protein (bicinchoninic acid method using BSA as standard) are homogenized in 6 × volume (µl/mg) of homogenizing buffer containing 20 mM HEPES/KOH (pH 7.0), 10 mM MgCl2, 0.4 mM Na3VO4, 1 mM EDTA, 4 µM benzamidine, 0.5 mM PMSF, 1 µg/ml pepstatin, 1 ng/ml leupeptin, 0.5 µg/ml antipain, and 250 mM sucrose (HBS) in a glass homogenizer for 30 s on ice. The homogenate is centrifuged (100,000 × g, 25 min, 4 °C). The supernatant (cytosol) is mixed with an equal volume of 30 % PEG8000 and incubated on ice for 30 min to precipitate proteins. The precipitated material is pelleted by brief centrifugation (10,000 × g, 2 min). The supernatant is discarded, and the pellet is washed once with HBS and resuspended in half the original volume of HBS. O-GlcNAc transferase is measured as described by Haltiwanger and coworkers (1992) with slight modifications. Both methods derive their specificity by utilizing peptide substrates that are modeled after O-glucosaminylation motifs of known O-GlcNAcylated proteins. The method recommended utilizes an Arg-tagged peptide which binds firmly to phosphocellulose paper. Reactions are conducted in 45-µl mixtures containing 1–5 µg of cytosolic protein in 20 mM HEPES/KOH (pH 8.0), 250 mM sucrose, 0.5 mM EDTA, 5 mM peptide substrate (TITSETPSSTTTQITKR), and 0.2 µCi UDP-[6-3H]-N-acetylglucosamine(25 Ci/mmol). The reactions are incubated (60 min, 25 °C). At the end of the incubations, 40-µl portions are spotted onto 1-cm2 Whatman P81 ion exchange papers. The paper squares are immediately immersed in 50 mM formic acid, washed extensively with 4 × 4 ml of 50 mM formic acid per square, and then counted for radioactivity in 5 ml scintillation cocktail per square. All assays should be conducted in the linear range for time, peptide concentration, and homogenate protein.

Measurement of GlcN-6-P Levels

For determination of the GlcN-6-P levels according to the procedure of Marshall et al. (2005b), adipocytes or myocytes after treatment with compounds/drug candidates are transferred to 1.5-ml microfuge tubes and washed three times with ice-cold, BSA-free HBSS. During the final wash, the cell volume is reduced to 150 µl, and 0.4 ml of cold perchloric acid (0.3 M) is added to the cells for 10 min. The mixture is then centrifuged (20,800 × g, 10 min, 4 °C), and the deproteinated metabolite extract (420 µl) is transferred to a new microfuge tube. After the extract is neutralized by adding a small amount of K2CO3 (5 M), extracts are frozen and stored at −20 °C. The concentration GlcN-6-P is measured in the extracts by HPLC as previously described (Marshall et al. 2004, 2005a) by a method that entailed derivatization of GlcN-6-P with O-pthaldialdehyde (OPA) so that it can be detected fluorometrically (following separation by reverse phase HPLC).

Measurement of UDP-GlcNAc Levels

This assay is performed essentially as described previously by Robinson and coworkers (1995). To avoid degradation of UDP-GlcNAc, isolated tissue samples are frozen immediately in liquid nitrogen, and then the procedure for the extraction of UDP-GlcNAc is carried out as quickly as possible. Frozen muscle or liver tissue (~150 mg) is homogenized at 4 °C in five volumes of PBS. A small aliquot of tissue homogenate is used for protein assay. One hundred percent (w/v) TCA is added to tissue homogenate (10 % final conc.). Samples are mixed well, and the precipitates are pelleted by centrifugation (10,000 × g, 15 min, 4 °C). To eliminate TCA from the supernatant, two volumes of diethylether are added and mixed well. After centrifugation (1,000 × g, 3 min, room temperature), the diethylether phase is discarded. This TCA elimination step is repeated ten times. The aqueous samples are dried up and dissolved in 30 µl distilled water. The extracts are

centrifuged (20,000 × g, 10 min, room temperature), and high-pressure liquid chromatography (HPLC) is performed on a SAX anion exchange column (4.6 × 250 mm, e.g., Whatman Partisil) eluted with 40 mM ammonium phosphate at a flow rate of 1 ml/min. The amount of UDP-GlcNAc in the samples is quantified by ultraviolet absorption (A254) and calculated from the height of peak against standard series of UDP-GlcNAc. The retention time of the peak of UDP-GlcNAc is 12.0 min.

Detection of O-GlcNAc-Modified Proteins

Immunological Detection

For immunological detection of O-GlcNAc-modified proteins according to the procedure introduced by Vosseller and coworkers (2002), whole-cell lysates for immunoblotting are prepared by scraping 3T3-L1 cells from 6-cm culture dishes in 400 µl of 1 % SDS, transferring to Eppendorf tubes, and boiling for 10 min. Laemmli sample buffer is added directly to lysates for SDS-PAGE. Lysates for immunoprecipitations are obtained by scraping cells in 1 % Nonidet P-40 buffer containing 15 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, protease inhibitors, and 1 µM PUGNAc to inhibit deglycosylation during lysis. Protein concentrations in lysates are determined by using the Bio-Rad protein reagent. The anti-O-GlcNAc antibody used is a mouse monoclonal IgM from ascites designated 110.6 (Comer et al. 2001). For these immunoprecipitations, antibody is bound and covalently coupled by using dimethyl pimelimidate (Pierce) to agarose-conjugated anti-IgM. For immunoblotting, secondary antibody is horseradish peroxidase-conjugated goat anti-mouse IgM (µ chain-specific). In all cases, immunoblots are developed with ECL reagent and imaged on hyperfilm (Amersham Pharmacia). Immunoprecipitations with indicated antibodies are performed overnight at 4 °C and captured with protein A/G-Sepharose (Santa Cruz Biotechnology) unless otherwise indicated, washed in RIPA buffer (1 % Nonidet P-40 buffer described above, but including 0.1 % SDS, 0.25 % sodium deoxycholate, and not containing protease inhibitors or PUGNAc), and followed by washing in TBS. Proteins are boiled off Sepharose beads in Laemmli sample buffer for SDS-PAGE with precast minigels (Bio-Rad). The separated proteins are transferred to polyvinylidene difluoride (Immobilon-P, Millipore), blocked at least 1 h in TBS containing 4 % BSA and 0.1 % Tween 20, and probed with indicated antibodies overnight at 4 °C. After binding of appropriate horseradish peroxidase-coupled secondary antibodies, ECL detection is used. In cases where densitometry is used to quantitate Western blot signals, the Flourchem imaging system and software (Alpha Innotech, CA) are used.

Enzymatic Detection

Terminally O-GlcNAc-modified membrane proteins are identified by the galactosyltransferase probe method using purified UDP-Gal-GlcNAc-Galβ-(1–4) galactosyltransferase (Gal-transferase) and UDP-[3H]galactose as described by Roquemore and coworkers (1994) with modifications introduced by Buse and coworkers (2002). Briefly, 0.5 or 1 mg of total crude membrane proteins in 50 mM HEPES (pH 7.4), 150 mM NaCl, and 0.5 % CHAPS is incubated for 1 h at 37 °C with the addition of 10 mM HEPES (pH 7.3), 10 mM galactose, 5 mM MnCl2, 1 mM DTT, 25 mM 5′-AMP, 20 µCi/ml UDP-[6-3H]galactose (40 Ci/mmol), and 1 U/ml galactosyltransferase (previously autogalactosylated for 30 min at 37 °C in 50 mM Tris/HCl, pH 7.3, 5 mM MgCl2, 1 mM mercaptoethanol, 0.4 mM UDP-galactose, and 0.1 mg/ml aprotinin). Enzyme reactions are terminated by the addition of 10 mM EDTA. Immunoprecipitation of galactosylated membranes is described below.

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Insulin-Like Signal Transduction via Plasma Membrane Microdomains (Caveolae and Lipid Rafts)

General Considerations

Caveolae

Caveolae were discovered as non-coated small flask-shaped invaginations (25–100 nm) of the plasma membrane by electron microscopy of endothelial cells (Fan et al. 1983; for a review see 1993a, 1993b, 1998; Anderson and Jacobs 2002; Shaul and Anderson 1998). It is possible that caveolae are formed from non-invaginated lipid rafts in the presence of the principal structural caveolae protein, caveolin (Kurzchalia et al. 1994; Lisanti et al. 1994; Parton 1996; Schlegel et al. 1998; Müller and Frick 1999, 2000; Müller and Welte 2002). Caveolae and the so-called lipid rafts are believed to be enriched in the bulk membrane lipids, such as phosphatidylinositol, cholesterol, sphingomyelin, and glycolipids, such as glycosyl-phosphatidylinositol and the ganglioside GM1 (for reviews see Rietveld and Simons 1998; Bickel 2002). Due to the high concentration of cholesterol and sphingomyelin, caveolae and lipid rafts are much more resistant to solubilization by detergents than are other parts of the plasma membrane and intracellular membranes (Brown and Rose 1992; Brown and Brown and London 1997, 1998; Harder et al. 1998; Müller 2002; Edidin 2003). The concentrations of various lipids in caveolae and rafts suggest great variations between different cell types, but also reflect the different experimental approaches used. Moreover, caveolae constitute between cell types a very variable, and usually unknown, fraction of the plasma membrane. Figures for enrichment of various components in caveolae compared to the total plasma membrane therefore reflect, to a large extent, this property of the particular cell type under investigation. In addition to the high content of cholesterol and sphingolipids, caveolae are stabilized by one or more caveolin isoforms. The caveolins are a family of 21- to 25-kDa integral membrane proteins that from the coat structure of caveolae (Glenney 1992; Okamoto et al. 1998; Smart et al. 1999). An unusual hairpin membrane domain results in both the amino and carboxy termini facing the cytoplasm. Caveolins avidly bind cholesterol, which may be the basis for their association with the liquid-ordered phase of the plasma membrane. In adipocytes caveolin-1 is the most prominent isoform (Rothberg et al. 1992), and its ablation leads to a total loss of caveolae.

Caveolae are believed to be involved in a number of cellular processes, such as receptor-mediated uptake and organization of transmembrane signaling (Shaul and Anderson 1998). Caveolae and lipid rafts harbor a number of components of various intracellular signal transduction pathways including G-protein-coupled

receptors, heterotrimeric G proteins, RTK (Src family tyrosine kinases), components of the Ras-mitogen-activated protein kinase (MAPK) pathway, protein kinase C isoforms, and endothelial nitric oxide synthase (eNOS) (Schlegel et al. 1998; Shaul and Anderson 1998; Smart et al. 1999). As a consequence, they may function as locations for the direct physical interaction of signaling elements where the cross talk between the corresponding signaling pathways takes place (Okamoto et al. 1998). Lisanti and coworkers demonstrated direct binding of a common domain within caveolin, the caveolin-scaffolding domain (CSD) to the corresponding caveolin-binding domain (CBD) of a variety of signaling proteins which are thereby kept in a basal inactive state, however competent for future activation (Couet et al. 1997a; Ju et al. 1997; Mineo et al. 1998; Müller 2001b; Moffett et al. 2000). The inhibitory interaction between caveolin and signaling molecules should be accessible for modulation in response to extracellular/intracellular signals. Activation of signaling pathways engaging CBD-harboring signaling molecules requires their relief from binding to/inhibition by caveolin both in short and long term (Couet et al. 1997a, 1997b; Kobzik et al. 1996).

Lipid Rafts

Discovery of the intracellular molecules and pathways that mediate insulin action has preoccupied many investigators for many years, but controversy and confusion remain. An emerging concept to explain such signaling specificity in the face of molecular promiscuity is that specialized scaffold, anchor, and adapter proteins segregate signaling molecules into specific cellular compartments via protein–protein interactions (Mastick et al. 1998). One level of spatial organization proposed for the plasma membrane is that of the liquid-ordered phase. According to a model proposed by Simons and Ikonen (1997) and Simons and Toomre (2000), lipids in the liquid-ordered phase pack together to form dynamic "rafts" in the plasma membrane, and these lipid rafts either recruit or exclude specific molecules, including signaling proteins. The biophysical and biochemical microenvironment of the rafts (e.g., membrane fluidity) may influence the function of the raft proteins. Also, the physical segregation of proteins into such "microdomains" may regulate the accessibility of those proteins to regulatory or effector molecules. The structural bases suggested for protein association with rafts include glycophosphatidylinositol (GPI) anchors (e.g., folate receptor), dual fatty acylation by myristylation and palmitoylation (e.g., Src family tyrosine kinases), transmembrane domain structure (e.g., influenza hemagglutinin polypeptide), cholesterol binding (e.g., caveolins), and protein–protein interactions (e.g., caveolin-interacting proteins). On the basis of their ability to sort proteins, rafts have been implicated in a number of cell functions, including signal transduction.

Signaling Function of Lipid Rafts and Caveolae

A pathway for insulin activation of glucose transport has been identified in insulin-sensitive cells which involves rafts/caveolae and the in- and out-movement of signaling proteins (Baumann et al. 2000; Ribon et al. 1998). Thus, rafts/caveolae seem to harbor signaling components for insulin-stimulated glucose transport and regulate their activity by interaction with caveolin and additional caveolar structural proteins that may serve as scaffolding proteins for concentrating and activating/inhibiting specific insulin signaling components. In addition, Baumann (2001) and coworkers presented a large body of evidence that a signal generated at the lipid rafts of adipocytes in response to insulin is required for insulin-stimulated glucose transport, in addition to that elicited by the PI3-K pathway (see section "Lipid Raft- and Caveolae-Based Assays in Insulin and Insulin-Like Signal Transduction").

Interestingly, compounds which manage to modulate the interaction of caveolin/caveolae with CBD-containing signaling proteins and, in consequence, the activation state of the corresponding downstream signaling cascades have been described. A study by Müller and coworkers (2001a, b) demonstrated that in isolated rat adipocytes, the non-RTK, pp59Lyn, and pp125Fak are downregulated by localization in

caveolae/binding to caveolin and upregulated by release from caveolae/dissociation from caveolin in response to phosphoinositolglycans (PIG), degradation products of GPI membrane protein anchors (Jones and Varela-Nieto 1999; Müller and Frick 1999), and the sulfonylurea drug, glimepiride (Langtry and Balfour 1998). The short-term redistribution of these kinases from caveolae/rafts induces insulin-independent activation of the metabolic insulin signaling cascade. These findings suggest that the concentration of signaling proteins at caveolae/caveolin may be regulated by diverse physiological and pharmacological stimuli thereby integrating cross talk to various signal transduction pathways.

Purpose and Rationale

In rat adipocytes and in 3T3-L1 adipocytes, the IR is localized to caveolae (Müller et al. 2002c; Gustavsson et al. 1999). The immediate downstream signaling molecule, IRS-1, on the other hand, has been reported not to be associated with the plasma membrane in rat adipocytes. In rat adipocytes the disruption of caveolar integrity by cholesterol depletion using β-cyclodextrin reversibly makes the cells insulin resistant by inhibiting the IR from phosphorylating IRS-1. On the other hand, insulin signaling for mitogenic control via MAPK, ERK1, and ERK2 is not impaired in the rat cells (Gustavsson et al. 1996). The importance of caveolae for insulin action is reinforced by the finding that, in response to insulin stimulation, the insulin-regulated glucose transporter, GLUT4, is translocated to caveolae for glucose uptake (Gustavsson et al. 1996; Kandror et al. 1995) and that some of the downstream signaling for enhanced glucose uptake may take place in caveolae.

In addition, destruction of caveolae causes a reduced uptake of long-chain fatty acids in 3T3-L1 cells and HepG2 cells (Pohl et al. 2002; Meshulam et al. 2006), and the major structural protein of caveolae, caveolin, and the caveolae-localized protein, CD36, both bind fatty acids. Caveolin has been implicated in TAG metabolism through its association with lipid bodies and LD in 3T3-L1 adipocytes and other cell lines (Fujimoto et al. 2001; Ostermeyer et al. 2001; Pol et al. 2001) and its mediation of the interaction between perilipin and the catalytic subunit of PKA which is required for β-adrenergic-dependent phosphorylation of perilipin and activation of lipolysis in adipocytes (Cohen et al. 2004). Caveolin-1–/– mice that are deficient in caveolin and caveolae have, moreover, been found to have deranged lipid metabolism and atrophied adipose tissue (Drab et al. 2001; Razani et al. 2001, 2002a, for a review see Razani et al. 2002b). Furthermore, caveolin-1–/– mice show a 90 % decrease in IR expression selectively in adipose tissue, greatly reduced insulin-induced phosphorylation of PKB and insulin resistance (Capozza et al. 2005; Oshikawa et al. 2004; for a review see Ishikawa et al. 2005). These findings indicate that intact caveolae are important for normal insulin signaling and (insulin) regulation of metabolism of carbohydrates and lipids in adipocytes. The following methods enable the analysis of the role of rafts/caveolae in insulin and insulin-like signal transduction in adipocytes as well as of their participation in the insulin-like activity of compounds/drug candidates.

Preparation of Plasma Membranes

Lipid rafts are routinely prepared from the plasma membranes of differentiated mammalian cells, preferably primary and cultured adipocytes or lung endothelial cells, due to their relative abundance.

Sucrose Gradient-Based Method

Basal and insulin/compound-stimulated adipocytes are homogenized in 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, 25 mM NaF, and 1 mM pyrophosphate with protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 4 mM iodoacetate, and 50 mM PMSF) using a motor-driven Teflon-in-glass homogenizer at room temperature. Subsequent procedures are carried out at 4 °C.

Cell debris and nuclei are removed by centrifugation at (1,000 × g, 10 min). A plasma membrane-containing pellet, obtained by centrifugation (16,000 × g, 20 min), is resuspended in 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, and protease inhibitors. The plasma membranes are purified by sucrose density gradient centrifugation (Avruch and Wallach 1971).

OptiPrep-Step Gradient-Based Method

Purification of plasma membranes from total membranes is performed by fractionation using the OptiPrep gradient (AXIS-Shield) according to the procedure of Pohl and coworkers (2005). Monolayers of confluent 3T3-L1 adipocytes are washed once in PBS to remove the culture medium and then once in the homogenization buffer (10 mM Tris/HCl, pH 7.4, 25 mM sucrose, 0.5 mM EDTA). Thereafter, cells are scraped in 3 ml of homogenization buffer and centrifuged (1,000 × g, 5 min). The pellet is resuspended in 2 ml of homogenization buffer. Cells are lysed by ten passages through a fine syringe needle followed by treatment with a tight-fitting Dounce homogenizator. The homogenate is centrifuged (1,000 × g, 10 min). The resulting postnuclear supernatant is centrifuged (100,000 × g, 40 min). The resulting total membrane pellet is resuspended in 1 ml of homogenization buffer containing 25 % (w/v) iodixnol and loaded onto a nine-step OptiPrep gradient, which consisted of 25, 22, 19, 16, 13, 10,7,4, and 1 % (w/v) iodixnol. After centrifugation (200,000 × g, 3 h, 4 °C, Beckman SW41Ti), 18 fractions are collected from the bottom of each centrifuge tube. A portion of each fraction can be analyzed by SDS-PAGE and immunoblotting for markers of the plasma membranes. Fractions 5, 6, and 7 are used as purified plasma membranes according to their enrichment of Na+/K+-ATPase, CD36, and caveolin-1.

Affinity Partitioning-Based Method

All work including the two-phase systems is done at 4 °C in a well-tempered cold room as two-phase partitioning is critically dependent on temperature. Male Sprague–Dawley rats, weighing 200–300 g, are starved overnight and killed by decapitation. Lungs are excised and thoroughly perfused with ice-cold homogenization medium (0.25 M sucrose, 5 mM Tris/HCl, pH 8.0). They are cut into pieces and squashed in a garlic press. Four grams of lungs are transferred to a Potter–Elvehjem homogenizer containing a 30-g two-phase system including the lung tissue. The two-phase system consisted of 5.7 % (w/w) dextran T500 (bottom phase) and 5.7 % (w/w) PEG 3350 (top phase) in 15 mM Tris/H2SO4 (pH 7.8) (final concentrations in the system). Homogenization is done by five up and down strokes by a motor-driven homogenizer with a Teflon pestle at 1,000 rpm. The homogenate is transferred to a Dounce homogenizer and further homogenized by ten strokes with a loose-fitting pestle followed by ten strokes with a tight-fitting one. After homogenization, the phases are separated by gentle centrifugation (270 × g, 5 min, Sorvall SS-34 rotor). The PEG-rich top phase is collected, and the dextran-rich bottom phase re-extracted with fresh top phase by mixing and phase separation as above. The two top phases collected are combined and further subjected to affinity partitioning. The fresh top phase for re-extraction is obtained from a similar 30-g two-phase system as above, replacing the lung tissue with 4 g of 15 mM Tris/H2SO4 (pH 7.8). After weighing in the components, the system is thoroughly mixed and allowed to settle overnight before collecting the top phase to be used for re-extraction. The phases for affinity partitioning are obtained as follows: a 20-g system containing 6.0 % (w/w) PEG 3350 and 6.0 % (w/w) dextran T500, including 1.3 mg WGA coupled to dextran T500, in 15 mM borate/Tris (pH 7.8) (final concentrations), is weighed in. After thoroughly mixing the system and letting settle overnight, the phases are collected individually, the WGA–dextran-containing bottom phase to be used for the initial affinity partitioning step and the PEG-enriched top phase for re-extraction (Persson et al. 1991). The combined top phases from the partitioning step above are mixed thoroughly with the WGA-containing bottom phase by 20 inversions, vortexing, and another

20 inversions. After phase separation through gentle centrifugation as above, the top phase is removed and the bottom phase re-extracted in the same manner with the fresh top phase obtained from the pre-equilibrated 20-g system. The final bottom phase is collected, diluted ten times with 0.1 M N-acetylglucosamine in 5 mM Tris/HCl (pH 8.0) to dissociate membranes from WGA–dextran, and centrifuged (100,000 × g, 90 min). The plasma membrane pellet is resuspended in homogenization medium.

Preparation of Lipid Rafts and Caveolae

Purpose and Rationale

Typical preparations of lipid rafts use 1 % Triton X-100 to extract whole cells, and the low-density, detergent-resistant material is separated from other solubilized membrane fractions by centrifugation on a 5–30 % sucrose gradient (Brown and Rose 1992). Subsequently, lipid rafts have been prepared using a variety of other detergents, including Lubrol WX, Lubrol PX, Brij58, Brij96, Brji98, Nonidet P40, CHAPS, and octylglucoside (for a review see Macdonald and Pike 2005). Although preparations of detergent-resistant membranes are readily isolated, several observations have raised concerns that extraction of cells with detergent may be generating clusters of raft lipids and proteins that did not exist in the intact cell. For example, examination of cells grown on coverslips and then extracted with Triton X-100 reveals a continuous membrane sheet pock-marked by large holes (Mayor and Maxfield 1995). Because rafts are thought to be $<$50 nm in diameter (Anderson and Jacobson 2002; Edidin 2003), the residual "detergent-resistant" membranes probably form from individual rafts that coalesced as a result of the detergent treatment.

To avoid the complications associated with preparing rafts using detergent extraction procedures, several methods have been established for isolating rafts from cells fractionated in the absence of detergent. Song and coworkers (1996) sonicated cells in a pH 11 sodium carbonate buffer and isolated caveolae by centrifugation of the lysate in a discontinuous 5 %/35 %/45 % sucrose gradient. Although this procedure is relatively easy, the spin time of 16–20 h is long, and the resulting raft fraction is significantly contaminated with other membranes. A more careful fractionation of lipid rafts (Smart et al. 1995), which is based on lysis of the cells in an isotonic buffer containing EDTA and purified plasma membranes on a Percoll gradient. The plasma membranes are then sonicated and the lipid rafts isolated by flotation through a 10–20 % gradient OptiPrep. This method results in the production of a relatively clean raft preparation. However, it is time-consuming and yields are poor. Furthermore, there is significant variability from preparation to preparation and from cell type to cell type. Because of the drawbacks associated with those procedures for isolating detergent-free lipid rafts, Macdonald and Pike (2005) developed an improved method that allows the rapid isolation of purified rafts in good yield. It is based on lysis of cells by shearing in an isotonic buffer containing calcium and magnesium and apparently allows isolation of a highly purified raft fraction in a single step by flotation in a 0–20 % OptiPrep gradient.

Studies on caveolae structure and function would be facilitated by a convenient high-yield isolation procedure. Most isolation protocols developed involve the detachment of caveolae from crude membranes either by Triton X-100 treatment (Sargiacomo et al. 1993; Chang et al. 1994) or by sonication (Smart et al. 1995) followed by density gradient centrifugation in either sucrose (Sargiacomo et al. 1993; Chang et al. 1994) or OptiPrep gradients (Smart et al. 1995). To obtain highly purified caveolae, however, extensive purification of a suitable plasma membrane fraction is required prior to detachment and centrifugation as was demonstrated using lung endothelial cells (Schnitzer et al. 1995a, b). Alternatively, immunoisolation protocols may be used (Oh and Schnitzer 1999; Abedinpour and Jergil 2003), although these also require prepurification steps.

Procedure

Conventional plasma membrane purification protocols based on centrifugation techniques usually result in low yields. In an alternative approach rat liver plasma membranes are purified by affinity partitioning in a PEG–dextran aqueous two-phase system (see above). Partitioning conditions are chosen to direct the bulk of unfractionated membranes to the PEG-rich top phase and then selectively to attract plasma membranes to the dextran-rich bottom phase by including WGA conjugated to dextran as an affinity ligand. This lectin specifically binds to N-acetylglucosamine and sialic acid residues on the plasma membrane surface. The affinity purification method is rapid and resulted in plasma membranes of high purity and yield (Persson et al. 1991; Persson and Jergil 1992). The possibility of combining two-phase affinity purification of plasma membranes with density gradient centrifugation to isolate caveolae has been explored (Abedinpour and Jergil 2003). Such a method should be generally applicable for the preparation of caveolae from various tissues and cells. The isolation of a vesicular fraction from rat lung highly purified in caveolin, a caveolar marker protein, by this approach is reported.

Carbonate-Based Method

Purified plasma membranes are pelleted and resuspended in 0.5 M Na2CO3 (pH 11) and sonicated with a probe-type sonifier 3 × 20 s. The homogenate is then adjusted to 45 % (w/v) sucrose in 12 mM MES (pH 6.5), 75 mM NaCl, 0.25 M Na2CO3, with protease inhibitors, and loaded under a 5–35 % (w/v) discontinuous sucrose gradient in the same solution and centrifuged (39,000 rpm, 16–20 h, 4 °C, Beckman SW41 rotor). The light-scattering band at the 5–35 % sucrose interface is collected and referred to as standard caveolae fraction. The recovery of caveolin in the caveolae fraction is typically 25 ± 5 % as determined by immunoblotting. Alternatively, for preparation from intact adipocytes, washed cells (0.5 × 107 cells) are suspended in 2 ml of sodium carbonate buffer (0.5 M Na2CO3, pH 11) containing protease inhibitors and homogenized sequentially using a loosely fitting Dounce homogenizer (ten strokes) and a sonicator (3 × 20 s bursts). The homogenate (2 ml) is then adjusted to 45 % sucrose by addition of 2 ml of 90 % sucrose, 50 mM MES/KOH (pH 6.5), and 150 mM NaCl (final pH of the mixture 10.2). A discontinuous sucrose gradient is formed by overlaying this solution with 4 ml of 35 % sucrose and 4 ml of 5 % sucrose, both in the same buffer containing 0.25 M Na2CO3. After centrifugation (see above), 0.85-ml gradient fractions are collected to yield a total of 14 fractions. The individual gradient fractions are pooled into rafts (Fr. 4–7) and non-raft areas (Fr. 10–14). The membranes from each pooled gradient fraction obtained by either method are diluted two- to threefold with 25 mM MES (pH 6.5), 150 mM NaCl, and 1 % TX-100, collected by centrifugation (50,000 × g, 30 min, 4 °C) and resuspended in non-dissociating buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % Nonidet P-40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, and protease inhibitors) or dissociating buffer (= non-dissociating buffer supplemented with 60 mM octylthioglucoside, 0.3 % deoxycholate) as indicated, incubated (1 h, on ice), and used for (co) immunoprecipitation, immunoblotting, or photoaffinity labeling.

Detergent-Based Method

For preparation from intact adipocytes, washed adipocytes (3.5 × 107) are suspended in 1.5 ml of lysis buffer (25 mM MES, pH 6.0, 150 mM NaCl, 5 mM EDTA, 1 % TX-100, 0.2 mM sodium orthovanadate, and protease inhibitors) and incubated for 20 min on ice. The cells are lysed with ten strokes in a manual Teflon-in-glass homogenizer over the course of 1 h at 4 °C. The lysate is centrifuged (1,300 × g, 5 min) to pellet unbroken cells, cellular debris, nuclei, and large unsoluble material. One ml of the postnuclear supernatant is subjected to sucrose gradient centrifugation by

mixing with an equal volume of 85 % sucrose, 25 mM MES (pH 6.0), 150 mM NaCl, and 5 mM EDTA at the bottom of a 12-ml centrifuge tube which is overlayed with 5.5 ml of 35 % sucrose and then 3.5 ml of 5 % sucrose in the same medium. After centrifugation (230,000 × g, 18 h, 4 °C, Beckman SW41 rotor), 0.9-ml fractions are collected from top to bottom, and termed fraction Fr. 1, 2, 3, etc. The bottom fraction is Fr. 12. Fr. 5 appears as a white, light-scattering band under illumination located at 5–7 % sucrose at the 35 % sucrose interface. The rafts contained in Fr. 5 are pelleted by dilution of the sucrose with five volumes of 50 mM HEPES/KOH containing protease inhibitors and centrifugation (200,000 × g, 2 h).

Alternatively, for preparation from plasma membrane fractions, Triton X-100 is added to the purified plasma membrane fraction to a final concentration of 1 % (w/v) (approximately a 100-fold excess of detergent to membrane protein). The sample is incubated for 30 min at 4 °C and centrifuged (100,000 × g, 90 min). The pellet is resuspended in homogenization medium by ten strokes in a tight-fitting Dounce homogenizer. The resulting sample is transferred to a Beckman SW40 ultracentrifuge tube. The sample is adjusted to 40 % (w/v) sucrose by addition of 80 % (w/v) sucrose, before layering 5.5 ml each of 24 and 5 % (w/v) sucrose on top of the sample. All sucrose solutions are made in 5 mM Tris/HCl (pH 8.0). After centrifugation (100,000 × g, 21 h, 4 °C, Beckman SW41 rotor), 12 1-ml fractions are collected by upward displacement. The light-scattering band at the 5–35 % (w/v) sucrose interface is collected as lipid rafts. The recovery of caveolin is typically 15 % as determined by immunoblotting.

Immunopurification

Lipid rafts prepared from rat adipocytes (originating from 500 µl of packed cell volume) are resuspended in 0.5 ml of homogenization buffer (buffer H). One percent Triton X-100, 2 % CHAPS, and 60 mM D-octyl glycoside (final concentrations) are added to 50 µl of resuspended rafts to a final volume of 100 µl. After 1 h on ice, the fractions are centrifuged (104,000 × g, 1 h). The pellets containing detergent-resistant rafts are resuspended in 100 µl buffer H and analyzed. For immunoprecipitations the rafts (corresponding to 750 µl of packed cell volume) and resistant to 1 % Triton X-100 treatment are suspended in 300 µl buffer H and incubated for 4 h with 6 µl caveolin-1 antibody supplemented with 10 µl of packed Protein A-Sepharose during the last hour. Non-bound material is removed, and the Sepharose gel is washed five times in 600 µl buffer H. The bound proteins are eluted by boiling for 15 min in buffer H supplemented with 0.6 % C13E12, 48 mM NaF, 48 mM NaCl, 5 % SDS, 2 % β-mercaptoethanol, and 5 % glycerol. For GLUT-4 analysis, rafts (corresponding to 500 µl packed cell volume) are treated with 1 % C13E12 and centrifuged (7,000 × g, 10 min). The supernatant is incubated for 4 h with 4 µl caveolin-1 antibody supplemented with 7 µl packed protein A-Sepharose during the last hour. The gel is washed and eluted as described above. Each step in the immunoprecipitation procedures is performed at 4 °C. The recovery of immunopurified lipid rafts is normalized by homologous immunoblotting with anti-caveolin antibodies of the same blot following stripping of the membrane.

Evaluation

A generally applicable method for the preparation of highly purified caveolae and/or lipid rafts based on a combination of affinity preparation of plasma membranes and density gradient centrifugation has been developed. To test this approach lung tissue has been chosen as endothelial cells lining vascular ducts are rich in caveolae. In earlier studies caveolae were prepared from plasma membranes of these cells isolated by an in situ silica-coating procedure (Schnitzer et al. 1995b). Alternatively, a crude lung homogenate can be used as starting material, preparing a plasma membrane-enriched fraction by affinity partitioning (Abedinpour and Jergil 2003). To improve purity the affinity step is preceded by homogenization of the tissue directly in a conventional aqueous two-phase system followed by phase separation

to remove material disturbing the affinity step. A similar approach was elaborated successfully for the purification of plasma membranes from the liver (Persson and Jergil 1992; Ekblad and Jergil 2001), suggesting that the method might be applicable also to other tissues. Lung membranes have been shown similarly to liver membranes both in the conventional and in the affinity two-phase step. The overall yield of the plasma membrane marker enzyme, 5′-nucleotidase, is somewhat lower from the lung (30–35 %) than from the liver (50–70 %), however. This is primarily due to a low recovery of 5′-nucleotidase in the affinity step rather than an unfavorable distribution of the enzyme in this step or the preceding conventional two-phase step. The enrichment of 5′-nucleotidase (eightfold) compares favorably with the five- to tenfold enrichment of various plasma membrane markers in the earlier isolation procedure involving silica coating of lung endothelial plasma membranes. The silica-coating procedure isolates the luminal part of the plasma membrane (Schnitzer et al. 1995b), while all regions of the plasma membrane are isolated by the affinity partitioning procedure (Persson et al. 1991; Persson and Jergil 1992; Ekblad and Jergil 2001).

The plasma membrane fraction obtained by the affinity procedure is suitable for the isolation of a fraction highly enriched in the caveolar marker, caveolin, after dispersion of the membranes in Triton X-100 and flotation in a sucrose density gradient. The enrichment of caveolin is approximately 20-fold in this density gradient step, slightly higher than the 13-fold enrichment observed when caveolae-rich vesicles are isolated from silica-coated endothelial plasma membranes (Schnitzer et al. 1995b). However, both the affinity partitioning method presented here and the silica-coating procedure result in plasma membrane material suitable for obtaining highly purified caveolae by sucrose gradient centrifugation regardless of the enrichment of caveolin attained.

Caveolae may be isolated also by a detergent-free method, gradient centrifugation by sonication of affinity purified plasma membranes. Although the yield is much lower, this nevertheless shows that the separation also works for the detergent-free preparation of caveolae-enriched material. It may be possible to optimize shearing of the plasma membrane preparation and the gradient separation to increase the yield, particularly since much vesicular material containing caveolin remained in the high-density part of the gradient. The caveolin-enriched fraction isolated after Triton X-100 treatment consists of morphologically similar closed vesicles of the same size (50–100 nm) as that of caveolae previously isolated from lung endothelial cells (Schnitzer et al. 1995a, b; Stan et al. 1997). Notably no vesicles in the size range 200–700 nm are observed. Such vesicles are common in Triton-insoluble membranes prepared from crude lung homogenates, being either non-caveolar membranes or membranes occasionally having attached caveolae (Schnitzer et al. 1995b). They are not present, however, in caveolar preparations obtained by Triton X-100 treatment of endothelial plasma membranes isolated by the silica-coating procedure followed by sucrose gradient centrifugation. The thin-section micrographs indicated that many of the isolated vesicles are not smoothly rounded but had an irregular shape.

Lipid Raft- and Caveolae-Based Assays in Insulin and Insulin-Like Signal Transduction

Purpose and Rationale

For studying signal transduction processes which involve caveolae, these structures are isolated as rafts by biochemical methods (carbonate or detergent extraction) from primary or cultured differentiated cells, such as muscle and fat cells, after incubation with external stimuli. Rafts can be prepared either directly from total cells or total cell lysates or (preferably) isolated (purified) plasma membranes. The use of rafts from total cells or lysates may obscure subtle changes in signaling since the (considerable) fraction of rafts present in membranes of the Golgi apparatus

(where their biogenesis takes place) may respond differently (or not at all) toward external stimuli compared to the plasma membrane rafts. Subsequent analysis for the presence in/cofractionation with caveolae, direct interaction with caveolin, and changes in activity of signaling proteins in response to the external stimuli requires the methods of enrichment/purification of rafts, photoaffinity labeling (of GPI proteins), coimmunoprecipitation with caveolin (of signaling proteins), immunoblotting (of signaling proteins), and immune complex kinase assays (in case of kinase activity of signaling proteins).

Coimmunoprecipitation of Lipid Raft Proteins

Total cell lysates (25–50 μg protein) or rafts in non-dissociating buffer (10–15 μg protein) are precleared (1 h, 4 °C) with protein G/A-Sepharose (50 mg/ml) in a total volume of 100 μl and then supplemented with appropriate antibodies (e.g., pp125Fak, 2 μg/sample; IRS-1, 1:50 dilution; IRS-2, 10 μg/sample; pp59Lyn, 5 μg/sample; caveolin, 0.7 μg/sample; IRβ, 1:175 dilution) preadsorbed on protein G-Sepharose (monoclonal antibodies) or protein A-Sepharose (rabbit antibodies) in a total volume of 100 μl. After incubation (4 h, 4 °C, end-over-end rotation) and centrifugation (3,000 × g, 2 min, 4 °C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate) containing 0.2 % Nonidet P-40 and 0.3 % deoxycholate, then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.2 % Nonidet P-40 and once with 1 ml of immunoprecipitation buffer lacking salt and detergent and finally suspended in 50 μl of Laemmli sample buffer (4 % SDS, 115 mM Tris/HCl, pH 6.8, 1 mM EDTA, 10 % glycerol, 4 mg/ml bromophenol blue) supplemented with 1.2 % β-mercaptoethanol (except for anti-caveolin immunoprecipitates), heated (95 °C, 2 min), and centrifuged. The supernatant samples are analyzed by SDS-PAGE (4–12 % Bis-Tris precast gel, pH 6.4, MES/SDS running buffer under reducing conditions) and subsequent immunoblotting (see sections "Immunoblotting" and "Immunoblotting of Lipid Raft Proteins"). The centrifugation conditions for collection of the protein A/G-Sepharose-bound immune complexes do not lead to sedimentation of nondissociated rafts to any significant degree according to immunoblotting with anti-caveolin antibody. The coimmunoprecipitation of proteins with caveolin from non-dissociated rafts is specific for raft-associated components, such as the GPI protein, Gce1, and the dual-acylated non-RTK, pp59Lyn, since the use of nonimmune IgG instead of anti-caveolin antibody or of dissociated DIGs and anti-caveolin antibody does not result in immunoprecipation of Gce1 and pp59Lyn.

Immunoblotting of Lipid Raft Proteins

Immunoblotting is performed as described (see sections "Detection by Immunoblotting" and "Immunoblotting") with minor modifications. After SDS-PAGE and transfer of the proteins to PVDF membranes (2 h, 400 mA in 20 % methanol, 192 mM glycine, 25 mM Tris, 0.005 % SDS), the blocked membrane (1 h in 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween 20, 0.1 % Brij, 0.01 % NP-40) with 1 % ovalbumin and 1 % BSA (anti-phosphotyrosine, anti-pp59Lyn, anti-IRβ) or with 5 % nonfat dried milk (anti-caveolin, anti-IRS-1/IRS-2) is incubated (2 h, 25 °C) with antibodies against IRS-1 (1:500), IRS-2 (1:750), caveolin (1:2,000), pp59Lyn (1 μg/ml), or IRβ (2 μg/ml) diluted in the same medium and then washed five times with the same medium. After incubation of the membranes (1 h, 25 °C) with horseradish peroxidase-coupled goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody diluted in the appropriate blocking buffer (1:5,000 or 1:2,500) and subsequent washing with detergent-containing (two times) and

detergent-free (two times) buffer (20 mM Tris/HCl, pH 7.6, 150 mM NaCl), the labeled proteins are visualized by the enhanced chemiluminescence method.

Immune Complex Assay for Lipid Raft PK

Immune complex kinase assays are performed as described (see sections ▶ Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms and "Immunecomplex Kinase Assay") with minor modifications. Immune complexes are suspended 30 µl of kinase buffer (50 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1.25 mM MnCl2, 12.5 mM MgCl2, 1.25 mM EGTA, 0.5 mM DTT, 1 mM Na3VO4) containing [γ-32P]ATP (final conc. 40 µM, 0.2 mCi/ml) or 1 mM ATP and incubated (e.g., for pp59Lyn: 15 min, 22 °C) in the presence of 1 µg heat-denatured enolase. Phosphorylation is terminated by addition of 10 µl of fourfold concentrated Laemmli buffer and boiling. The phosphoproteins are separated on SDS-PAGE (10 % Bis-Tris resolving gel, MOPS/SDS running buffer) and analyzed for phosphotyrosine by phosphorimaging ([γ-32P]ATP) or immunoblotting (ATP). Under these conditions the kinase reactions are linear with time for the assay period. IRS-1 immune complexes are assayed for PI3-K by incubating (10 min, 22 °C) in 50 µl of 20 mM Tris/HCl (pH 7.0), 50 µM [γ-33P]ATP (5 µCi), 10 mM MgCl2, 2 mM MnCl2, 100 mM NaCl, 2 mM EDTA, and 0.5 µM wortmannin (for control incubations, only) containing 10 µg of phosphatidylinositol (PI) and 1 µg of phosphatidylserine. After addition of 10 µl of 8 M HCl and 160 µl of a 1/1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates coated with 1 % oxalate and developed in chloroform/methanol/water/ammonia (60/47/11.3/3.2, by vol.). Radiolabeled PI3-P is quantitated by phosphorimage analysis. For calculation of wortmannin-sensitive PI3-K, all values are corrected for PI3-P radiolabeled in the presence of wortmannin. SDS-PAGE and subsequent immunoblotting for caveolin and the PK assumed to localize permanently or transiently at lipid rafts (e.g., protein kinase C) are performed as described (section "Immunoblotting of Lipid Raft Proteins").

Electron Microscopic Analysis of Caveolae

Purpose and Rationale

Several laboratories have localized important elements of the insulin signal transduction machinery to caveolae or rafts, including the IR itself. Baumann and coworkers (2000) and Chiang and coworkers (2001) have reported the discovery of a novel insulin signaling pathway in adipocytes that relies on proper localization of signaling molecules to lipid rafts and that is required for insulin-stimulated glucose uptake. Using a detergent-based method of lipid raft preparation, Mastick and coworkers (1995) found only trace levels of insulin receptor in the rafts of 3T3-L1 adipocytes. Müller and coworkers (2001a, b) characterized the composition of rafts from isolated rat adipocytes. The rafts and non-rafts were prepared by both detergent and detergent-free methods from total cell lysates and from plasma membranes, respectively. Representative fractions were analyzed by immunoblotting for known raft and non-raft proteins. Despite the methodological differences, there was good agreement between the proteins enriched in rafts and non-rafts by the two procedures. By both methods, the β-subunit of the IR was relatively depleted from the rats and relatively enriched in the non-rafts. However, Gustavsson and coworkers (1999) reached the opposite result by double immunogold transmission EM and detergent-free isolation of rafts. In these experiments, caveolin and both subunits of the IR were found to colocalize in ~50-nm-round structures that corresponded to individual caveolae and in clusters of these structures. This colocalization was confirmed by immunofluorescence microscopy in paraformaldehyde-fixed 3T3-L1 adipocyte plasma membrane sheets. The immunofluorescence labeling pattern appeared as doughnutlike spots of 0.3–0.5 µm diameter, a size that was noted to be similar to that of the caveolae clusters

visualized by EM. Biochemically, the IR is cofractionated with caveolin in detergent-free isolation of rafts from rat adipocyte plasma membranes. The addition of insulin was not associated with a significant change in localization of the IR, as assessed biochemically or by immunofluorescence. Even low concentrations of Triton X-100 (0.1 %) completely solubilized the β-subunit of the IR from the caveolin-enriched fractions. This observation may explain why Mastick and coworkers (1998) found only trace amounts of the IR in rafts prepared on the basis of detergent insolubility. The molecular basis for the (partial or transient) localization of IR at lipid rafts may rely on the interaction of IR with caveolin-1, which is assumed to differentially modulate insulin signaling, as has been demonstrated by Nystrom and coworkers (1999).

Procedure

Samples of appropriate fractions from the sucrose gradient are mixed with ten volumes of 3 % paraformaldehyde and 1.5 % glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (final conc.). The sample is incubated (40 min, 4 °C) followed by centrifugation (100,000 × g, 90 min). The pellet is washed twice with 0.1 M sodium cacodylate (pH 7.4), treated with 1 % OsO4 for 1 h, and washed twice with 0.1 M sodium cacodylate. Samples were stepwise dehydrated in ethanol, treated with 1 % uranyl acetate in ethanol, and embedded in Epon. Thin sections (50 nm) were stained with uranyl acetate and led by standard procedures and examined and micrographed in an electron microscope.

Samples of the caveolae-enriched fractions are also examined following negative staining after fixation with 2 % paraformaldehyde. The material is attached to carbon and pioloform-coated nickel grids for 20 min. The grids are washed three times for 5 min with phosphate-buffered saline before staining with 1 % uranyl acetate for 2 min and micrographing in an electron microscope.

Evaluation

An insulin-independent pathway has been described that mimics the metabolic actions of insulin in adipocytes (Müller et al. 2001a, b). This pathway can be stimulated in adipocytes by exogenous phosphoinositolglycans (PIG), cleavage products of the glycosyl-phophatidylinositol (GPI) anchor of GPI-linked proteins, which have initially been proposed to act as soluble mediators of insulin action, but do not share structural and functional similarity with the typical second messengers regulating glycogen metabolism in response to adrenergic hormones (Wasner et al. 2003). PIG treatment of isolated rat adipocytes leads to concentration-dependent increases in tyrosine phosphorylation of IRS-1, IRS-1-associated PI3-K activity, and glucose uptake in an insulin-independent fashion. A critical mechanism in this pathway may involve the dissociation of the non-RTK, Lyn, and Fak, from caveolin-1 (Müller et al. 2000), their activation by tyrosine phosphorylation, and their redistribution from rafts to non-raft membranes (Müller et al. 2001b, 2005). Incubation of adipocytes with PIG, as with insulin, is followed by tyrosine phosphorylation of caveolin-1. The same research group succeeded in the characterization and identification of a specific binding protein for synthetic PIG in the lipid rafts of the adipocyte plasma membrane (Müller et al. 2002a, b). The involvement of lipid rafts in insulin-mimetic signaling by PIG was demonstrated by complete abrogation of redistribution of signaling proteins and IRS-1 tyrosine phosphorylation in response to PIG challenge of rat adipocytes, which have been depleted of lipid rafts by prior cholesterol removal from the plasma membrane (Müller et al. 2002c). The physiological role of this pathway and its potential cross talk with the insulin signaling pathway in caveolae remain to be determined (Müller 2002). These questions will be facilitated by molecular cloning and inactivation or overexpression of the gene coding for this PIG receptor in 3T3-L1 adipocytes.

Several laboratories have investigated whether there is overlap between membrane compartments that contain caveolin and GLUT4. Using biochemical isolation techniques, Scherer and coworkers (1994) detected a transient increase in GLUT4 immunoreactivity in rafts after insulin stimulation of 3T3-L1 adipocytes. Also, insulin stimulation led to an increase in plasma

membrane caveolin and a corresponding decrease in caveolin detected in low-density microsomes. These investigators detected caveolin in GLUT4 vesicles immunoisolated from rat adipocytes. Kandror and coworkers (1995) conducted similar studies but arrived at a different conclusion. They found that intracellular caveolin and GLUT4 were detectable on vesicles of similar size and buoyant density and that these vesicles translocated to the plasma membrane in response to insulin. However, in contrast to the findings of Scherer and coworkers (1994), only limited amounts of caveolin protein were present in immunoabsorbed GLUT4 vesicles, which suggested that the intracellular pools of these proteins, although biophysically similar, were distinct. Gustavsson and coworkers (1996) isolated subcellular membrane fractions from rat adipocytes and performed glucose uptake assays on intact adipocytes at various times during an insulin pulse. On basis of the results obtained, the authors propose a model such that, upon insulin stimulation, GLUT4 moves first to the PM and then to detergent-resistant plasma membrane microdomains (caveolae?), where GLUT4-mediated glucose transport occurs. Taken together, at this point the weight of the published data supports localization of a significant subset of plasma membrane IR and GLUT4 to caveolae in adipocytes, but the issue is not settled (for a review see Bickel 2002).

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Glycosyl-Phosphatidylinositol-Specific Phospholipase (GPI-PL) and Insulin-Like Signaling

General Considerations

Insulin-responsive mammalian cells and tissues express a number of GPI proteins, the majority of them with their GPI anchor embedded in the outer leaflet of the plasma membranes as well as some lipases, the (G)PI-PLC/D, which may control downregulation of GPI protein cell surface expression and simultaneously upregulation of the soluble protein moiety released into circulation (Romero et al. 1988; Low 1989, 1990; Thomas et al. 1990; Romero and Larner 1993; Satiel 1990; Saltiel et al. 1986; Müller and Bandlow 1991; Varela-Nieto et al. 1996; Nosjean et al. 1997; Jones and Varela-Nieto 1999; Müller et al. 2005). In fact, soluble forms of GPI proteins have been detected in human plasma, such as 5′-nucleotidase, Thy-1, alkaline phosphatase, and CD16 receptors, and GPI-PLC have been found in human neutrophils, bovine brain, rat intestine, and a human carcinoma cell line. The GPI anchor consisting of three mannose residues and a non-acetylated glucosamine, one end of which amide-linked to the protein moiety via a phosphoethanolamine bridge and the other end glycosidically linked to the 6-hydroxyl group of PI, is cleaved by (G)PI-PLC/D releasing diacylglycerol or phosphatidic acid, respectively, and leaving a terminal inositolglycan structure at the protein moiety harboring (PIG) or lacking a (cyclic) phosphate residue (for reviews see Chan et al. 1988; Cross 1990; Ferguson 1991; Müller 2000).

In eukaryotes, some GPI-PLC/D are upregulated by glucose as well as certain hormones, growth factors, and drugs (e.g., insulin, glimepiride) in rodent adipocytes, myocytes, and human endothelial cells (Müller et al. 1993, 1994a, c; Movahedi and Hooper 1997). In particular, the antidiabetic drug, glimepiride, which lowers blood glucose predominantly by stimulation of insulin release from pancreatic β-cells and, in addition, to a minor degree by mimicking metabolic insulin action in peripheral tissues, such as activation of glucose transport in muscle cells and inhibition of lipolysis in adipocytes (Müller et al. 1994d; Bähr et al. 1995), has been demonstrated to activate a GPI-PLC. Upon treatment of primary or cultured rodent adipocytes with pharmacological concentrations of this sulfonylurea, a potent amphiphilic to hydrophilic conversion of a subset of GPI proteins by activation of a GPI-PLC has been observed (Müller et al. 1993, 1994b). The functional and physiological significance of the insulin- and glimepiride-regulated lipolytic cleavage of GPI proteins in mammalian cells is not well understood at present, albeit initially a role as soluble mediators for metabolic insulin action has been proposed (Farese 1990; Fonteles et al. 1996; Gaulton and Pratt 1994; Larner 1987; Lazar et al. 1994; Lisanti et al. 1989; Mato 1989; Low and Saltiel 1988; Lawrence et al. 1986; Müller 2002a; Romero et al. 1990; Shashkin et al. 1997; Saltiel et al. 1988).

Purpose and Rationale

Recently, a synthetic inositol derivative, GPI-2350, has been described which inhibits the well-characterized bacterial, trypanosomal, and serum (G)PI-PLC/D with high potency and selectivity (Müller et al. 2005). Using GPI-2350, which

almost completely downregulates the insulin- and glimepiride-inducible GPI-PLC in intact rat adipocytes, a major role for this enzyme in metabolic insulin signaling and action in these cells could be excluded. In contrast, activation of the GPI-PLC turned out to be indispensable for the insulin-mimetic effects of glimepiride via the recently described IR-independent cross talk from lipid rafts to the IRS-1 protein (see above). Based on the criteria for lipid rafts, certain GPI-anchored, acylated and transmembrane signaling proteins have been found to be enriched in rafts vs. non-raft areas of the plasma membranes. Furthermore, rafts are not homogenous in composition and structure since subspecies of higher (hcRafts) and lower cholesterol (lcRafts) content can be distinguished from one another on the basis of their lower and higher buoyant density, respectively (Müller 2005; Müller et al. 2005). Strikingly, the stimulus-induced redistribution of certain GPI-anchored as well as acylated signaling proteins from hcRafts to lcRafts (Müller et al. 2001a, b) is blocked by GPI-2350. This finding hints to a role of the mammalian GPI-PLC in the control of the localization and activation of signaling proteins within rafts during signal transduction across the plasma membrane of adipocytes. The following assays enable the analysis of the role of the GPI-PL for insulin-like signaling and metabolic action in insulin target cells as well as of the effects of compounds/drug candidates on the activity of the GPI-PL. The GPI-PL may turn out as a promising target for future antidiabetic drugs (Müller and Frick 1999; Müller 2002b; Müller and Welte 2002).

Activity Assay for GPI-PL

Procedure

Adipocyte GPI-PLC

Adipocyte GPI-PLC (5–25 μg PM or hc/lcRafts) assayed as the conversion of GPI protein, acetylcholinesterase (AchE) from its amphiphilic version (containing the intact GPI anchor) into its hydrophilic version (containing the lipolytically cleaved GPI anchor) is determined according to a protocol adapted from Müller and coworkers (1994b) by incubation with 10 μl of bovine erythrocyte AChE solution (12 U/ml in 10 mM Tris/HCl, pH 7.4, 144 mM NaCl, 0.1 % TX-100) in 100 μl of 20 mM HEPES/KOH (pH 7.8), 144 mM NaCl, 0.1 % TX-100, and 0.2 mM MgCl2 for 1 h at 25 °C and subsequent termination by adding 5 μl of glacial acetic acid and then 0.4 ml of 10 mM Tris/HCl (pH 7.4) and 144 mM NaCl. Each reaction mixture is subjected to TX-114 partitioning (see below). The GPI-PLC activity is calculated as the amphiphilic to hydrophilic conversion of the GPI protein substrates from the ratio of the activities of hydrophilic 5′-Nuc or AChE measured in the TX-114-depleted phase and the total activity measured before partitioning and corrected for the nonenzymatic background in the TX-114-depleted phase (accounting for 10–20 % of the total activity) as revealed by blank incubations lacking (G)PI-PLC.

TX-114 Partitioning

Pelleted hc/lcRafts (10–50 μg protein) or GPI-PL reaction mixtures (0.5 ml) are separated into amphiphilic and hydrophilic proteins using partitioning between TX-114-enriched and depleted phases according to Bordier (1981) and Pryde and Phillips (1986) by suspending in 1 ml or mixing with 0.5 ml, respectively, of ice-cold 25 mM Tris/HCl (pH 7.4) and 144 mM NaCl containing 1 or 2 % TX-114. After incubation for 1 h on ice, the mixture is layered onto a cushion of 0.4 ml of 0.25 M sucrose and 25 mM Tris/HCl (pH 7.4) on ice. Phase separation is induced by warming up to 37 °C and subsequent centrifugation (10,000 × g, 1 min). After re-extraction of the lower TX-114-enriched phase, aliquots of the pooled upper TX-114-depleted phase are measured for AChE activity or precipitated (15 % polyethylene glycol 4000) for SDS-PAGE analysis.

Evaluation

Concentration–response curves are established using various concentrations of standard (human

insulin) and the test compounds, e.g., sulfonylureas, allowing calculation of EC50 values and potency ratios.

GPI-PL-Dependent Translocation of GPI Proteins Within Lipid Rafts

Procedure

Photoaffinity Labeling of the GPI Protein, Gce 1

Solubilized plasma membranes and rafts (5–10 µg protein) are incubated (30 min, 4 °C) with 50 µCi 8-N3-[32P]cAMP (0.5 nmol) in 50 µl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM MgCl2, 2 mM MnCl2, 1 mM isobutyl-methylxanthine, 1 mM DTT, 1 mM AMP, and protease inhibitors in the wells of microtiter plates (96-formate) and then irradiated with UV light (254 nm, 8,000 µW/cm2) at a distance of 0.5 cm for 1 min (Müller et al. 1993, 1994a, b). Subsequently, the photoaffinity labeling reaction is quenched by addition of 100 µl of the same buffer containing 10 mM cAMP. Gce1 is precipitated (5 % trichloroacetic acid, 1 h on ice, 10,000 × g, 15 min) and solubilized in sample buffer for SDS-PAGE. Protein concentration is determined using the BCA protein determination kit from Pierce. Autoradiographs and direct photoimages are processed and quantified by computer-assisted video densitometry (e.g., Storm 860 PhosphorImager system, Molecular Dynamics). The recovery in the amounts of immunoprecipitated protein has to be corrected (data on fold or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting.

Preparation of lc/hcRafts

Pretreated and subsequently washed adipocytes are homogenized in lysis buffer (25 mM MES, pH 6.0, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM glycerol-3-phosphate, 1 mM sodium orthovanadate, and protease inhibitors) using a motor-driven Teflon-in-glass homogenizer (ten strokes). Plasma membranes are obtained by differential centrifugation of the defatted postnuclear infranatant (= cytosolic fraction) and then purified by two sequential centrifugation steps through sucrose and Percoll cushions as described above and finally suspended in 25 mM Tris/HCl (pH 7.4), 0.25 M sucrose, and 1 mM EDTA at 2 mg protein/ml. hc/lcRafts are obtained by the detergent method and discontinuous sucrose gradient centrifugation as reported above. The light-scattering opalescent bands at the 0.5–0.65-M (fractions 3 + 4) and 0.8–0.9-M (fractions 5 + 6) sucrose interfaces are collected as hcRafts and lcRafts, respectively, with density measurement using the refractive index. hc/lcRafts are collected by centrifugation (50,000 × g, 30 min, 4 °C) after threefold dilution of the pooled gradient fractions with 25 mM MES (pH 6.5), 1 % TX-100, and 150 mM NaCl and then characterized (immunoblotting, enzyme assays) by enrichment/deprivation of relevant marker proteins as described above or subjected to TX-114 partitioning (see section "Activity Assay for GPI-PL"). For immunoprecipitation of caveolin, rafts are solubilized (1 h, 4 °C) in 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % TX-100, 60 mM β-octylglucoside, 0.3 % deoxycholate, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 1 µM microcystin, and protease inhibitors and centrifuged (50,000 × g, 30 min). For direct immunoblotting, rafts are solubilized in twofold Laemmli sample buffer and centrifuged (10,000 × g, 5 min). The supernatants are used.

Evaluation

The following working hypothesis for signal generation in and transduction across the PM of rat adipocytes on the basis of the redistribution of lipid-modified signaling proteins, such as GPI proteins and acylated tyrosine kinases, within rafts has been deduced from the present experimental findings (Müller 2005). (1) Amphiphilic GPI proteins with intact GPI anchor as well as hydrophilic GPI proteins with lipolytically cleaved GPI anchor preferentially accumulate at hcRafts vs. lcRafts. (2) Only uncleaved GPI

proteins redistribute from hcRafts to lcRafts in response to glimepiride and insulin. Apparently, the GPI anchor functions as a signal for the recruitment to rafts vs. non-raft areas, but does not determine the targeting to lcRafts or hcRafts. The current working model implicates that the PIG core structure of the GPI anchor functions as the only hcRafts-targeting signal (via recognition through the receptor protein, p115) for a subset of GPI proteins which are amenable to stimulus-induced redistribution to lcRafts. The present findings strongly suggest the expression of three distinct subspecies of rafts in the plasma membranes of rat adipocytes: (i) TX-100- and Lubrol-WX-insoluble hcRafts, (ii) TX-100-soluble and Lubrol-WX-insoluble lcRafts in the basal state, and (iii) TX-100- and Lubrol-WX-insoluble lcRafts upon glimepiride challenge. This is more compatible with the model of heterogenous rafts rather than that of layered rafts or homogenous rafts with selective lipid extraction as has been recently proposed for their heterogenous structure as well as their biogenesis and maintenance (Macdonald and Pike 2005). Accordingly, layered rafts are constituted of rings ranging from a liquid-ordered cholesterol- and glycosphingolipid-enriched core through less ordered regions into the liquid-disordered non-raft areas of the residual PM, the core of which is left as hcRafts after TX-100 solubilization, whereas core and immediately surrounding areas are left unsolubilized by Lubrol-WX. Homogenous rafts are liquid-ordered domains of a uniform mixture of the protein, and lipid constituents are surrounded by the liquid-disordered phase of the plasma membranes which are selectively solubilized by TX-100 and Lubrol-WX (Macdonald and Pike 2005). Both models can hardly explain the existence of TX-100-insoluble lcrafts or their generation from hcrafts in response to glimepiride. In contrast, heterogenous rafts of different lipid and protein composition could coexist as separate structural entities in the membrane and be differentially solubilized by TX-100 and Lubrol-WX. It is conceivable that the glimepiride-induced translocation of certain GPI-anchored and acylated signaling proteins from distinct hcRafts accompanied by the limited loss of cholesterol results in the de novo generation of lcRafts of increased buoyant density retaining TX-100 insolubility to a partial degree. Alternatively, the translocated raft protein components may be recruited directly from discrete hcRafts into preexisting Lubrol-WX-insoluble (TX-100-soluble) lcRafts thereby rendering them partially insoluble in TX-100.

In conclusion, the adipocyte GPI-PL has now been recognized as a critical component specifically operating in the insulin-like signaling cascade triggered by glimepiride rather than in insulin signaling. Furthermore, insulin-like signaling by glimepiride is not significantly impaired in the insulin-resistant state as reported previously for rat adipocytes in vitro (Müller and Wied 1993). Therefore, the adipocyte GPI-PLC might represent a useful target for the therapy of type II diabetes mellitus.

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Assays for the Expression and Release of Insulin and Glucose-Regulating Peptide Hormones from Pancreatic β-Cell

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Insulin Release from the Isolated Perfused Rat Pancreas

Purpose and Rationale

The in vitro perfusion of the isolated rat pancreas as described by Anderson and Long (1947), Ross (1972), Grodsky and coworkers (1983, 1984), and Muñoz and coworkers (1995) offers the advantage to study the influence of carbohydrates, hormones, and drugs such as sulfonylureas not only on insulin but also on glucagon and somatostatin secretion without interference of secondary effects resulting from changes in hepatic, pituitary, or adrenal functions.

Procedure

The insulin concentration of the perfusate of the rat pancreas prepared as described (see ▶ Insulin Target Tissues and Cells) is determined with the RIA-gnost kit using rat insulin as standard in every second sample. The determination is done immediately after the end of an experiment.

Insulin Release from the Isolated Perifused Rat Pancreatic Islets

Purpose and Rationale

An assay with isolated pancreatic islets to study the dynamic response and transitions between various metabolic states has been recommended by Idahl (1972). A further description and discussion of the method were given by Malaisse-Lagae and Malaisse (1984).

Procedure

The insulin concentration of the perifusate of the rat islets prepared as described (▶ Insulin Target Tissues and Cells) is determined with the RIA-gnost kit using rat insulin as standard in every second sample. The determination is done immediately after the end of an experiment.

Evaluation

The raw data are expressed in μU insulin per islet/min. Mean and standard error of the mean of each time interval are calculated for graphical representation. The values under exposure to drug are compared with the values under perifusion with glucose only and with the effect of elevated glucose.

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Insulin Release from Cultured β-Cells

Purpose and Rationale

Insulin released into the supernatant of monolayers of cultured β-cells grown in 96-well culture dishes as described above is determined radioimmunochemically (see above) using a guinea pig anti-rat insulin antibody, 125I-labeled human insulin as tracer, and rat insulin as standard. Free and bound radioactivity is separated using an anti-IgG (goat anti-guinea pig) antibody. Typically, the sensitivity of this assay is 17 pM, and the coefficient of variation is less than 3 % at both low and high levels.

Procedure

Insulin Secretion from MIN6 Cells

Cultured MIN6 cells (1 × 105) are replated and cultured (see ▶ Insulin Target Tissues and Cells) for 24 h before being used in secretion assays. Insulin secretion is measured during 1-h static incubations in KRBH(118.5 mM NaCl, 2.54 mM $CaCl_2$, 1.19 mM KH_2PO_4, 4.74 mM KCl, 25 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 10 mM HEPES, pH 7.4) containing 0–20 mM glucose (basal or glucose-induced secretion) and/or compounds/drugs. Samples of the supernatant are assayed for insulin. To determine total insulin content, insulin is extracted using 95/5 ethanol/acetic acid. Insulin is measured using a mouse insulin ELISA kit.

Insulin Secretion from RINm5F Cells

One day prior to the experiment, approximately 1 × 106 RINm5F cells, grown as described above, are seeded into 24-well test plates. At the time of the experiment, the culture medium is aspirated, and the cells are washed with 1 ml of a modified KRBH containing 10 mM HEPES/KOH (pH 7.4), 5 mM $NaHCO_3$, 0.5 % BSA, and 0–20 mM glucose. After preincubation for 30 min at 37 °C with the above buffer, compounds/drug candidates dissolved in DMSO at 10 mM are added (final concentration ranges from 10 nM to 100 μM) and incubated for

30 min at 37 °C. The incubation is stopped by aspiration of the buffer which is stored at −80 °C until measurement of insulin by ELISA or RIA. After the aspiration, the cells are washed with PBS and dissolved in 0.5 ml NaOH (0.1 M). After overnight incubation, the resulting solution is collected for assay of cellular protein content.

Insulin Secretion from INS1 Cells

INS1 cells (5 × 104 per well in 12-well plates) are treated with RPMI 1,640 medium containing 3–20 mM glucose and 0.5 mM FFA or a mixture of oleic and palmitic acids in equal ratio, with a total concentration of 0.5 mM. Control wells contained 0.5 % fatty acid-free BSA in medium. After 24 h, the cells are washed in PBS and preincubated for 2 h in KRBH containing 0–20 mM glucose (119 mM NaCl, 4.75 mM KCl, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.18 mM KH_2PO_4, 20 mM HEPES, pH 7.4, 2.54 mM $CaCl_2$). Glucose responsiveness is evaluated by an initial 30-min incubation in KRH containing 3 mM glucose followed by incubation for 30 min in KRH containing 20 mM glucose. Samples are collected for insulin measurement using RIA or ELISA with CV 4.5 % and minimal detection 0.15 μg/l.

Insulin Secretion from Attached INS-1E Cells

The secretory responses to glucose and other secretagogues are tested in INS-1E cells between passages 54–95 according to the procedure of Merglen and coworkers (2004). Before the experiments, cells are maintained for 2 h in glucose-free culture medium. The cells are then washed twice and preincubated for 30 min at 37 °C in glucose-free KRBH (135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4). BSA (0.1 %) is added as an insulin carrier. Next, cells are washed once with glucose-free KRBH and then incubated for 30 min in KRBH and compounds/drug candidates. Incubation is stopped by putting the plates on ice. The supernatants are collected for insulin secretion, and cellular insulin contents are determined from acid-ethanol extracts. Insulin secretion is measured by RIA or ELISA using rat insulin as standard (see K.4.1).

Perifusion of INS-1E Cell Clusters

Spheroid clusters composed of INS-1E cells are preincubated for 2 h in glucose-free culture medium. After low-speed centrifugation (500 rpm), cell clusters are resuspended in glucose-free KRBH and counted, and about 500 spheroids are distributed per chamber in a 250-μl volume thermostated at 37 °C. The flow rate is set at 0.5 ml/min, and fractions are collected every minute after a 20-min washing period at basal 2.5 mM glucose. At the end of the perifusion, cell clusters are collected, and insulin contents are determined from acid-ethanol extracts.

Total Insulin Content

Cells from 4 wells of 24-well culture dishes are scraped into ice-cold PBS containing 5.5 mM glucose and 0.1 % fatty acid-free BSA and immediately frozen in liquid N2. Frozen cells are sonicated in acidic ethanol (0.2 M HCl in 87.5 % ethanol). The samples are then centrifuged. The supernatant is used for measurement of total insulin content. Insulin in the perifusion fractions and cell extracts is determined by RIA or ELISA (see ► Measurement of Insulin and Other Glucose-Regulating Peptide Hormones).

Lipolysis in β-Cell

Purpose and Rationale

Besides the de novo synthesis of LC-CoA starting with the ACC reaction and malonyl-CoA formation, leading to inhibition of the translocation of LC-CoA into mitochondria, lipid-derived signaling molecules involved in insulin secretion may also originate from the breakdown of TAG stores in β-cells. This may occur through activation of HSL, the enzyme that exerts a major control in TAG hydrolysis in adipose tissue (for a review, see Yeaman 2004).

It was first demonstrated in 1999 that HSL is expressed and active in the β-cells of the

pancreatic islet (Mulder et al. 1999). Two isoforms are expressed in the β-cell, the 84-kDa protein seen in adipocytes and other tissues and an 89-kDa protein (Mulder et al. 1999) encoded by exons 1–9 plus exon A, which is spliced to exon 1, introducing an additional 43 amino acids into the polypeptide (Lindvall et al. 2004).

The role of HSL in the β-cell has yet to be fully established. Two studies have independently looked at insulin secretion in HSL knockout mice (Roduit et al. 2001; Fex et al. 2004). Both demonstrate features of insulin resistance in peripheral tissues, but differ in the effect of the knockout on insulin secretion from the pancreas. In one study, the glucose responsiveness of insulin secretion was impaired (Roduit et al. 2001), both in vivo and in pancreatic islets isolated from the mice. The islets from the HSL knockout mice were totally unresponsive to glucose, but showed a normal response to depolarizing concentrations of KCl. This work is consistent with a key role for HSL in coupling of glucose metabolism to increased insulin secretion, perhaps by generating a lipid messenger such as DAG or fatty acyl-CoA from intracellular stores of TAG (Mulder et al. 1999; Roduit et al. 2001; Yaney et al. 2000). Also consistent with this role is the increased expression of HSL in response to high concentrations of glucose, with the elevated glucose ensuring an appropriate supply of the lipid mediators (Sorhede-Winzell et al. 2001). The second study in HSL null mice demonstrated that insulin secretion was essentially unimpaired, with an increase in islet mass being found, presumably as a result of the insulin resistance in peripheral tissues (Fex et al. 2004). Possible reasons for the apparent discrepancies between the two studies may rely on the genetic background of the mice.

However, recent studies on transgenic mice that overexpress HSL specifically in the β-cell have provided evidence for a role for HSL in mediating the lipotoxicity associated with type II diabetes. These mice showed impaired glucose tolerance and had defective GSIS when fed a high-fat diet, accompanied by lower levels of β-cell TAG than found in control mice fed the same diet (Sorhede-Winzell et al. 2001). One proposed mechanism for this is that HSL provides endogenous ligands for lipid-activated transcription factors, including the PPAR-activated receptors. In this regard, it would appear that, in control animals, the accumulation of intracellular TAG is actually a protective mechanism (Listenberger et al. 2003), with the lipotoxicity being manifested when the storage capacity is exceeded or when the lipid stores are mobilized by HSL. Consistent with this is the observation that long-term feeding with a high-fat diet leads to downregulation of pancreatic HSL levels by as much as 75 %, presumably as a protective mechanism against the generation of these lipid metabolites (Sorhede-Winzell et al. 2001).

Procedure

Lipolysis in cultured β-cells is determined as the release of glycerol into the incubation medium. Since FFA and glycerol are the end products of lipolysis and glycerol is not metabolized by the β-cell due to low expression of glycerol kinase, glycerol release actually reflects the degree of lipolysis in cultured β-cells. Cells grown in 24-well culture dishes are incubated in 5001 of RPMI 1640 medium containing 10 % (by vol.) heat-inactivated fetal calf serum, 50 IU/ml penicillin, 0.25 μg/ml amphotericin B, and 50 μg/ml streptomycin in the presence of increasing concentrations of glucose (2.8–16.7 mM) at 37 °C for up to 4 h in an atmosphere of humidified air/CO_2 (19/1). After certain periods of time, 20-μl portions are transferred to a 96-well culture plate and incubated with glycerol kinase (50 U/ml) and luciferin/luciferase reagent. After addition of 10 μl of 1 mM ATP, the luminescence is measured in a luminometer after 4, 8, and 12 min. The glycerol concentration is calculated from the standard curve.

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Measurement of Ca^{2+} Levels

Imaging

For imaging of cultured β-cells according to the protocol of Toye and coworkers (2005) with the modifications adapted from Freeman and coworkers (2006), MIN6 cells are cultured on 35-mm Fluorodishes (World Precision Instruments) and incubated with DMEM plus 3 μM fura-2-AM (Molecular Probes) for 40 min at 37 °C. They are imaged at room temperature (20–24 °C) using an IonOptix fluorescence system with 340-nm and 380-nm dual excitation. The 510-nm emission ratio is collected at 1 Hz. Background subtraction is performed by measuring fluorescence from a cell-free region in the field of view. Cells are perfused continuously with extracellular solution containing 137 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES/NaOH (pH 7.4), plus glucose or compounds/drug candidates.

Fluorometry

Calcium levels are monitored in cells transduced the day before the experiment with the calcium-sensitive photoprotein aequorin. Measurements of mitochondrial and cytosolic calcium are performed using the corresponding adenovirus constructs, AdCA-mtAeq and AdCA-cyAeq, respectively. The 2-h preincubation period also serves to load cells with the aequorin prosthetic group, i.e., 5 μM coelenterazine [2-(p-hydroxybenzyl)-6-(p--hydroxyphenyl)-8-benzylimidazo[1,2-]pyrazin-3-(7H)-one; Calbiochem]. Calibration is calculated based on the total counts obtained at the end of the trace following permeabilization of the cells with 50 μM digitonin and 10 mM $CaCl_2$ exposure.

Measurement of 86Rb+ Efflux

Purpose and Rationale

The binding of a sulfonylurea to its receptor SUR1, a subunit of the ATP-sensitive K+ channel of β-cells, leads to blockade of the channel activity and concomitant decrease in the K+ efflux (Boyd et al. 1991). The resulting depolarization of the β-cell plasma membrane causes opening of the voltage-gated Ca^{2+} channel (Nelson et al. 1987; Niki et al. 1989, Rorsman and Trube 1986; Nichols et al. 1996). The rise in the intracellular [Ca^{2+}] triggers the exocytosis of insulin. 86Rb+ efflux can be used as marker for K+ efflux and analyzed in response to treatment of cultured β-cells with compounds/drug candidates.

Procedure

RINm5F cells are grown and plated at a density of 200,000 cells/well (24-well tissue culture plates). 86Rb+ efflux experiments are performed at 37 °C and overnight equilibration of cells in RPMI 1640 medium supplemented with 10 % FCS, 0.1 μCi/ml 86RbCl, and 0.2 μCi/ml L-[3H]leucine (internal marker of cell recovery). After removing the medium, cells are preincubated in a medium containing 120 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM KCl, and 20 mM HEPES/NaOH (pH 7.5) supplemented with 0.1 μCi 86RbCl, 0.24 mg/ml oligomycin, 1 mM 2-deoxy-D-glucose, and several concentrations of compounds/drug candidates as well as sulfonylureas (as positive controls). 86Rb+ efflux studies are initiated by removing the preincubation medium and incubating the cells with 200 μl of the same medium/well without 86Rb+, oligomycin, and 2-deoxy-D-glucose. Efflux is stopped by removing this medium and washing the cells three times with 1 ml of 0.1 M $MgCl_2$ at 37 °C. Cells are extracted with 2 × 1 ml of 0.1 N NaOH and counted. Total intracellular concentrations of ATP are measured after extracting the cells with 1 % Triton X-100 by using the luciferase-luciferin technique.

Evaluation

Inhibition is measured as percent of maximum 86Rb+ efflux. Half maximum inhibition constants can be calculated.

Modifications of the Method

Daniel and coworkers (1991) recommended a high-throughput 86Rb efflux assay in human medulloblastoma cells TE671 for screening of potassium channel modulators. Hu and coworkers (1995) cloned a voltage-regulated K channel from human hippocampus for transfection into CHO cells. The authors recommended this method as a high-throughput assay to identify isotype-specific K-channel modulators.

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Measurement of Cell Membrane Potential

Purpose and Rationale

ATP-sensitive potassium (KATP) channels couple cell metabolism to electrical activity of the plasma membrane by regulating membrane K+ fluxes (for a review, see Ashcroft 2005). A reduction in metabolism opens KATP channels, producing K + efflux, membrane hyperpolarization, and suppression of electrical activity. Conversely, increased metabolism closes KATP channels. The consequent membrane depolarization stimulates electrical activity and may thereby trigger cellular responses such as the release of hormones and neurotransmitters or muscle contraction.

Studies on isolated cells and tissues, and more recently on genetically modified mice and patients with mutations in KATP channel genes, have demonstrated that KATP channels play a multitude of physiological roles (Seino and Miki 2004). They contribute to glucose homeostasis by regulating insulin secretion from pancreatic β-cells (Ashcroft and Rorsman 1989; Henquin 2000; Gribble and Reimann 2003; Rorsman et al. 1994; Koster et al. 2000; Seghers et al. 2000; Dunne et al. 2004), glucagon secretion from pancreatic α-cells (Gopel et al. 2000a), somatostatin secretion from D cells (Gopel et al. 2000b), and GLP-1 secretion from L cells (Gribble et al. 2003). In ventromedial hypothalamic neurons, they mediate the counter-regulatory response to glucose (Miki et al. 2001), and in arcuate nucleus neurons, they may be involved in appetite regulation. In these glucose-sensing cells, KATP channels respond to fluctuating changes in blood glucose concentration. In many other tissues, however, they are largely closed under resting conditions and open only in response to ischemia, hormones, or neurotransmitters. In cardiac muscle and central neurons, the resulting reduction in electrical activity helps protect against cardiac stress and brain seizures (Zingman et al. 2002; Yamada et al. 2001). KATP channels are involved in ischemic preconditioning in the heart (Gumina et al. 2003) and the regulation of vascular smooth muscle tone (opening of KATP channels leads to relaxation) (Daut et al. 1994; Chutkow et al. 1996). Given their critical role in regulating electrical excitability in many cells, it is perhaps not surprising that disruption of KATP channel function can lead to disease. To date, mutations in KATP channel genes have been shown to cause neonatal diabetes (Gloyn et al. 2004; Magge et al. 2004) and hyperinsulinemia (Dunne et al. 2004; Glaser et al. 2000; Thomas et al. 1996; Huopio et al. 2000). The effect of compounds/drug candidates on the open/closed state of KATP channel and the electrical activity of β-cell plasma membranes can be assayed with the following assays, which may be helpful for the identification of insulin secretagogues and sensitizers for GSIS as potential drug in the antidiabetic therapy.

Procedure

Rat insulinoma cells RINm5F (Gögelein et al. 1998) or cultured pancreatic β-cells from NMRI mice (Rorsman and Trube 1985; Zünkler et al. 1988) or HIT T15 β-cells (Niki et al. 1989) are used. Whole-cell patch-clamp experiments are performed according to Hamill and coworkers (1981), Rajan and coworkers (1993), and Lindau and Neher (1988) at room temperature with cells

bathed in an external solution of 140 mM NaCl, 4 mM KCl, and 10 mM HEPES (pH 7.4). The patch pipettes are mounted on a suction pipette holder. The outlet is connected to silicon rubber tubing through which the suction is applied. The pipette solution contains 135 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES (pH 7.5), and 0.3 mM NaATP. Pipettes are prepared by pulling from borosilicate glass, coated with silicon rubber, and heat polished at the tip. Pipette resistances are ranging between 4 and 7 MΩ. A patch-clamp amplifier (e.g., EPC 7, List Electronic, Darmstadt, FRG) is used which allows capacitance and series resistance compensation. The series resistance after achieving the whole-cell recording configuration is 5–20 MΩ, and 50 % series resistance compensation is used to keep the voltage error during current flow below 2 mV. The cell membrane potential is held at −70 to −80 mV, and hyper- and depolarizing voltage pulses of 10 mV amplitude are applied alternatively every 2 s. Voltage-dependent currents, i.e., the Ca^{2+} inward current and the delayed K+ outward current, are not activated by these low pulse amplitudes, and, therefore, most of the current is flowing through ATP-dependent K+ channels. Inside-out patches are prepared according to Hamill and coworkers (1981) by disrupting the outer vesicle membrane.

Evaluation

The current and voltage signals are stored on magnetic tape or filtered by a four-pole Bessel filter and displayed on a digital oscilloscope. Outward currents flowing from the cell to the bath are indicated by upward deflections and inward currents by downward deflections. Half maximal effective concentrations are calculated as EC50 values.

Modifications of the Method

Henquin and Meissner (1984), Henquin and coworkers (1984, 1985), and Meissner (1990) measured membrane potential of mouse pancreatic β-cells. Hu and coworkers (2000) compared the effects of nateglinide, a non-sulfonylurea hypoglycemic agent, with sulfonylureas on pancreatic β-cell KATP channel activity with the whole-cell configuration of the patch-clamp technique in primary cultures of rat pancreatic β-cells. Wang and Giebisch (1991) reported a dual modulation of the renal ATP-sensitive K+ channel by PKA and PKC in cells of the cortical collecting duct of rabbit kidneys using the cell-attached and inside-out modification. Shieh and coworkers (2000) characterized KATP channel opener-activated currents in pig and guinea pig bladder smooth muscle cells using the whole-cell patch-clamp technique. Shindo and coworkers (2000) examined with the whole-cell configuration of the patch-clamp technique the effects of a vascular relaxing agent on the heterologously expressed pancreatic-type ATP-sensitive K+ channels SUR1/Ki6.2, SUR2A/Ki6.2, and SUR2B/Ki6.2 in human embryonic kidney 293T cells. Using whole-cell and single-channel patch-clamp recording, Gomora and Enyeart (1999) studied pharmacological properties of a cyclic AMP-sensitive potassium channel, IAC, which is distinctive among K+ channels both in its activation by ATP and inhibition by cyclic AMP. Alternatively, (Merglen et al. 2004) monitored the cell membrane potential using 100 nM of the fluorescent probe bis-oxonol [bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; Molecular Probes]. Filters used for excitation and emission had wavelength optima at 544 and 590 nm, respectively.

Measurement of Mitochondrial Membrane Potential

Procedure

Mitochondrial membrane potential (Δψm) is measured using the fluorescent probe, rhodamine 123. Cells cultured in 24-well plates are maintained for 2 h in 2.5 mM glucose medium at 37 °C before loading with 10 μg/ml rhodamine 123 (Molecular Probes) for 20 min at 37 °C in KRBH. The Δψm is monitored with excitation and emission filters set at 485 and 520 nm,

respectively. Glucose (additions on top of the basal concentration of 2.5 mM) and then the protonophore carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) are added to each well.

Measurement of cAMP Production

Approximately 1 × 106 cultured β-cells (grown as described above) in 0.45 ml buffer (113 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 2.5 mM $CaCl_2$, and 1.2 mM $MgSO_4$) containing 1 % human serum albumin are preincubated for 10 min at 37 °C and then incubated for 10 min after the addition of 2 µl of IBMX (50 mM) in order to prevent the breakdown of cAMP. The reaction is then started by the addition of 50 µl of a peptide solution dissolved in the above buffer (final concentration ranges from 10 pM to 1 µM). After incubation for 10 min at 37 °C, the reaction is stopped by the addition of 200 µl of 12 % TCA. The reaction mixture is sonicated for 30 s at 25 W (e.g., Heat Systems, Ultrasonics) and centrifuged (11,500 × g, 2 min). HCl (25 µl, 1 M) is added to 0.5 ml supernatant. TCA dissolved in the supernatant is removed by diethyl ether (3 × 1 ml), and the resulting supernatant is stored at –80 °C until cAMP assays being performed by the use of a RIA kit.

Measurement of Cytosolic ATP Levels

Cytosolic ATP levels are monitored in cells expressing the ATP-sensitive bioluminescent probe luciferase 1 day after transduction with the specific AdRIP-Luc viral construct according to Maechler and coworkers (1998) and Rubi and coworkers (2001). After the preincubation steps described above, the 24-well plates are transferred to the plate reader in the luminometer mode. The luciferase substrate, 100 µM beetle luciferin (Promega), is added to the KRBH. After a 10-min period in basal 2.5 mM glucose, cells are stimulated with the indicated glucose concentrations, and 20 min later, the mitochondrial poison NaN_3 (2 mM) is added (as a control).

Analysis of Lipotoxicity

General Considerations

There is increasing experimental evidence that sustained elevated plasma and intracellular lipid concentrations result in impaired insulin action and secretion in vivo and in vitro. This has implications for the causal relationship between the pathogenesis of obesity and type II diabetes (Carpentier et al. 2001; Dobbins et al. 2002). It is known that increased plasma FFA concentrations reduce insulin-mediated glucose uptake and metabolism in muscle tissue presumably via the glucose-fatty acid (Randle) cycle (Randle et al. 1963), but the exact mechanism of action of FFA on the β-cell to modulate glucose-stimulated insulin secretion (GSIS) (Zhou and Grill 1994; Roche et al. 1998; Unger and Orci 2002) remains to be elucidated. A complex interrelationship between the metabolism of glucose and lipids in β-cells has been described (Prentki and Corkey 1996), and this critically depends on the duration of exposure (acute or chronic) to the lipid metabolites (Zhou and Grill 1994; Roche et al. 1998). In conclusion, it is now generally accepted that in rodent, β-cells and islet lipids and/or lipid precursors/metabolites interfere with GSIS and β-cell functionality (Maedler et al. 2001; El Assaad et al. 2003; Cnop et al. 2001), but their nature (e.g., TAG, FFA, acyl-CoA, DAG) and molecular mode of action still remain a matter of intense research and debate.

However, serious doubts have been raised against the hypothesis of FFA-induced impairment of GSIS and β-cell death as playing a major role in the pathophysiology of human type II diabetes. Although increased plasma FFA and lipid (as lipoprotein TAG) concentrations and alterations in their composition occur in obesity in humans (Wang et al. 2003), this condition is not invariably linked to the onset of type II diabetes. Fewer than 12 % of obese subjects with elevated

lipid profiles suffer from diabetes (Ford et al. 2002). Furthermore, the concept of glucolipotoxicity (Poitout and Robertson 2002) proposes that, in type II diabetes, the elevation of both glucose and FFA could contribute to the decrease in pancreatic islet function as a result of both β-cell apoptotic death and decreased insulin secretion.

Purpose and Rationale

The following assay systems enable the determination of effects of compounds/drug candidates on the fatty acid-induced (and glucose-induced) modulation of GSIS, cell death and TAG concentration, composition, and localization in cultured β-cells. They may be helpful for the identification and characterization of compounds/drugs with the potential of abrogating the lipo(gluco)toxic effects of lipid (metabolites) in β-cells in response to certain (patho)physiological stimuli (e.g., stress, reactive oxygen species; see ► Monitoring of Diabetic Late Complication).

Procedure

Viability Assay

INS1 cells are plated into 8-well Permanox slides (Gibco) and treated for 24 h with 0.1, 0.25, 0.5, and 1 mM of FFA in 3 or 20 mM glucose. Adjustments to the stock solution are made to ensure a constant BSA concentration. Following incubation, cells are rinsed in PBS, incubated for a further 30 min in PBS containing 10 μM calcein-AM (Sigma) at 37 °C, and fixed in 2.5 % paraformaldehyde for 30 min. Slides are rinsed twice more in PBS and the slides mounted in VECTASHIELD containing propidium iodide (Vector Laboratories). Viability is quantified by counting the propidium-iodide-positive nuclei and calcein-AM-positive cells (average cell count = 60 cells per field) and determining the percentage of propidium-iodide-positive nuclei in at least three fields of view per condition.

TAG and Phospholipid Content and Composition

INS1 or COS7 cells are seeded into 6-well plates at 2 × 105 cells per well and treated for 24 h with 0.5 mM specific fatty acids. Total lipids are extracted from cells in chloroform/methanol (2/1) and lipid fractions are separated using solid-phase extraction (Burdge et al. 2000). The TAG and phospholipid fractions are collected and fatty acid methyl esters prepared using methanolic sulfuric acid. The fatty acid methyl esters are separated by gas chromatography (e.g., Agilent 6890 GC equipped with a DB-Wax 30-m capillary column coated with a polyethylene glycol stationary phase, internal diameter 0.25 mm, film thickness 0.25 μm; Agilent Technologies). Fatty acids are separated according to fatty acyl carbon chain length and degree of saturation. Intact TAG analyses are performed by high-temperature gas chromatography (HTGC, e.g., Hewlett Packard 5,890 series II GC with a DB1-HT 15-m fused silica capillary column, internal diameter 0.32 mm, film thickness 0.1 mm). Hydrogen is the carrier gas and a flame ionization detector is employed to monitor column eluent. HTGC analyses separate the TAG according to their carbon number. The absolute concentrations of individual fatty acids and TAG are determined typically by reference to internal standards (heptadecanoic acid [17:0] for NEFA; tripentadecanoin [15:0] for TAG; phosphatidylcholine and diheptadecanoyl [17:0] for phospholipid).

Light Microscopy

INS1 and cells are incubated in 8-well Permanox or glass slides (Gibco) with compounds/drug candidates for 24 h in the absence or presence of 3 or 20 mM glucose and 1 mM FFA. Cells are washed in PBS and fixed in 2.5 % PFA and then stained for lipids. A stock solution of 0.5 % (saturated) oil red O in propan-2-ol is freshly diluted (three-part stock solution in two-part distilled water) and filtered immediately before use. Cells are stained for 30 min at 37 °C, then briefly destained in 60 % isopropanol, and washed in water before mounting in PBS/glycerol. A freshly prepared 1 % Nile blue solution in distilled water is filtered, and an aliquot is further diluted in water to 0.02 %. Fixed

cells are washed twice in PBS. Nile blue solution (1 %) is added to the cells for 10 min at 65 °C or 70 °C, after which the cells are washed in water and destained in 1 % acetic acid (65 °C) for 30 s. Cells are restained for 15 min in 0.02 % Nile blue at 65 °C or 70 °C before being washed in water and mounted in PBS/glycerol.

Electron Microscopy

INS1 cells for morphological analysis are incubated with compounds/drug candidates in 25-cm^2 flasks for 24 h as described above. Cells are removed and gently prepared as a pellet, fixed in 2.5 % glutaraldehyde (minimum 1 h) at room temperature (or for a 10-min period at 70 °C), postfixed in 2 % osmium, and dehydrated and embedded in Spurr resin. Cells for immunoelectron microscopy are fixed in ice-cold 4 % paraformaldehyde (10 min) followed by 8 % paraformaldehyde at room temperature (50 min), washed in PBS, dehydrated in methanol, and embedded in LR Gold resin. Ultrathin sections are cut onto nickel grids and contrast-enhanced with uranyl acetate and lead citrate. Sections are examined in an electron microscope with an accelerating voltage of 80 kV.

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Interaction with β-Cell Plasma Membranes and KATP Channels

It is now established that increasing the closed probability of the β-cell ATP-dependent potassium channel (KATP) is the major mechanism through which sulfonylureas as well as glucose (via transport, phosphorylation, and metabolism for generation of ATP) stimulate insulin release from pancreatic β-cells (for reviews, see Ashcroft and Ashcroft 1992; Ashcroft 2005; Bryan and Aguilar-Bryan 1997; Gribble and Reimann 2003; Henquin 2000; Inagaki et al. 1996). The resulting reduction in potassium ion efflux causes depolarization of the β-cell plasma membrane which in turn leads to opening of voltage-sensitive Ca^{2+} channels of the L-type. The increased influx of Ca^{2+} and thus the elevated cytosolic Ca^{2+} levels trigger the fusion of insulin-containing secretory granules with the plasma membrane, presumably mediated by Ca^{2+}-/calmodulin-dependent protein kinase and additional unknown mechanisms (Philipson 1995; Aguilar-Bryan and Bryan 1999). The β-cell KATP consists of the regulatory sulfonylurea receptor subunits (SURs), such as SUR1, and the physically associated pore-forming potassium ion inwardly rectifying subunit, KIR6.2, in a multimeric assembly with the SUR and KIR6.2 subunits in 1:1 stoichiometry and four identical SUR/KIR6.2 complexes per functional channel unit (size of the channel holocomplex [SUR/KIR6.2]4 about 1,000 kDa; Skeer et al. 1994; Clement et al. 1997; Bryan and Aquilar-Bryan 1999; Shyng and Nichols 1997). SUR1, which is predominantly expressed in neuronal/pancreatic β-cells, is an ATP-binding cassette (ABC) protein or transport ATPase, which closely resembles members of the multidrug resistance-associated protein family with 17 predicted transmembrane domains (TMDs) and two nucleotide-binding folds (NBF), which bind specifically (Mg^{2+})ATP/ADP (Bryan et al. 1995; Inagaki et al. 1995, 1996; Ueda et al. 1999; Gribble et al. 1997). The KIR6.2 subunits have two TMDs which somehow contribute to the K+ conductivity and selectivity (Babenko et al. 1998). The glibenclamide-binding site of SUR1 is proposed to consist of a benzamido (meglitinide)-binding site on TMDs 1–5 and the sulfonylurea (tolbutamide)-binding site on TMDs 12–17 based on photolabeling with [125I] iodoglibenclamide and chimeric receptors (Ashfield et al. 1999; Babenko et al. 1999; Chutkow et al. 1996; Isomoto et al. 1996; Tanabe et al. 1999; Aguilar-Bryan et al. 1995). Interaction with both the benzamido- and sulfonylurea-binding sites could account for the several thousandfold increases in affinity of glibenclamide versus tolbutamide. SUR2A and SUR2B are splice variants of a single SUR2 gene differing in 42 amino acids at the carboxy-terminus, exclusively, with SUR2A expressed mainly in cardiac/skeletal muscle cells (Chutkow et al. 1996; Inagaki et al. 1996) and SUR2B in vascular/nonvascular smooth muscle cells and brain (Isomoto et al. 1996; Aguilar-Bryan et al. 1998). K+ channel openers (KCOs) bind to SURs at the tolbutamide-binding site encompassing those (flanking) TMDs 12–17 which surround the central core region of the tolbutamide-binding site (Uhde et al. 1999). SURs define the sensitivity of the KATP channel holocomplex for its sensitivity toward both sulfonylureas and KCOs with SUR1 mediating high sensitivity for sulfonylureas/low sensitivity toward KCOs and vice versa SUR2A/SUR2B mediating low sensitivity for sulfonylureas/high sensitivity for KCOs (for a review, see Ashcroft 2005).

The following assays enable the investigation of the direct binding of compounds/drug candidates to the KATP channels and their SUR subunits, which may be helpful for the identification of insulin releasers acting like sulfonylureas or glinides, which have been used successfully in the antidiabetic therapy since decades.

Isolation of Membranes

Procedure

For receptor binding studies, membranes are isolated from adenoma tissue suspension, from cerebral cortex of Wistar rats (Kaubisch et al. 1982; Geisen et al. 1985), or from RINm5F cells (Müller et al. 1994a).

Cerebral Cortex Homogenates

The homogenates are prepared from decapitated Wistar rats. The cortices are immersed in ice-cold buffer, pH 7.5, and are homogenized under cooling in an ULTRA-TURRAX tissue homogenizer. The cell membranes are centrifuged (50,000 × g, 1 h, 4 °C), resuspended in fresh phosphate buffer, and recentrifuged at the same speed. The resulting pellet is suspended in phosphate buffer, the final dilution being 1:50, based on wet weight of the cerebral cortex. One-ml aliquot of this cell suspension is used for the incubations.

β-Cell Adenoma Tissue

The tissue is dissected from rats of the strain NEDH and is homogenized in 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 0.5 mM EDTA, and 100 μM PMSF (50 ml/g tissue) under cooling with ice (3 × 5 s) with an ULTRA-TURRAX homogenizer. After centrifugation (3,000 × g, 5 min), the supernatant is transferred into new tubes and centrifuged (200,000 × g, 1 h, 4 °C). The pellet is washed with 2 × 5 ml of the same buffer, resuspended in 2 vol. of the same buffer, and centrifuged again (75,000 × g, 30 min). The pellet is resuspended again in the membrane buffer.

Membranes from Cultured β-Cells

Insulin-producing cells from RINm5F cell culture (one culture flask) are washed twice with 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, and 0.5 mM EDTA, scraped with 20 ml of the same buffer, and homogenized with ten strokes of a tight fitting Potter-Elvehjem homogenizer followed by sonication (bath sonicator, 4 °C, 10 s, maximal power). After centrifugation (200,000 × g, 45 min, 4 °C), the pellet is suspended in 10 ml of 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 100 mM NaCl, 0.5 mM EDTA, and 200 μM PMSF and recentrifuged (1,000 × g, 10 min). The supernatant is transferred to a new tube and centrifuged (50,000 × g, 30 min, 4 °C). The pellet is washed once with 25 mM HEPES/KOH (pH 7.4) and finally suspended in membrane buffer (25 mM HEPES/KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 μM PMSF, 10 μg/ml soybean/trypsin inhibitor, 10 μM leupeptin, 1 mM iodoacetamide at 5 mg protein/ml). Aliquots are stored at –80 °C.

Binding to Membranes

Purpose and Rationale

Sulfonylureas block KATP channel in the β-cell plasma membrane (Schmid-Antomarchi et al. 1987a, b). Binding to the receptor and depolarization of the membrane initiate a chain of events leading to the release of insulin (Boyd 1992). The high-affinity sulfonylurea receptor is considered to be an integral part of the KATP channel (Aguilar-Bryan et al. 1992). Binding studies of sulfonylureas and other drugs can be performed with isolated pancreatic islets, isolated insulinoma cells, isolated intact membranes, or solubilized membranes from primary or cultured cells or intact cells (Geisen et al. 1985; Gaines et al. 1988; Panten 1989; Panten et al. 1992; Schwanstecher and Panten 1993; Müller et al. 1994a).

Procedure

Filter-binding assays are performed in a total volume of 1 ml containing 5 mg membrane protein, 25 mM MOPS/KOH (pH 7.4), 0.1 mM $CaCl_2$, and labeled sulfonylurea, e.g., [3H]glibenclamide or [3H]glimepiride at concentrations between 0.1 and 20 nM. After incubation for 45 min at 25 °C, the binding reaction is terminated by rapid filtration through Whatman GF/F filters soaked with the same buffer. The filters are washed three times with 5 ml of ice-cold 25 mM HEPES/KOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 200 μM PMSF, 0.5 μg/ml leupeptin, and 0.75 μg/ml pepstatin and two times with 5 ml of ice-cold

HEPES/KOH (pH 7.4), placed in 10 ml of ACSII scintillation cocktail, and after incubation overnight counted for radioactivity in a liquid scintillation counter. Nonspecific binding is determined in parallel samples in the presence of 1 μM unlabeled ligand.

Binding to Cells

Culture

Cultured cells producing the β-cell sulfonylurea receptor, SUR1, include the hamster insulin-secreting tumor cell line, HIT-T15 (passage 65–75; CRL1777 ATCC). Cells are maintained in T-175 culture flasks as monolayers in DMEM containing high glucose supplemented with 10 % fetal bovine serum, 100 U, 7 ml penicillin, and 0.1 mg/ml streptomycin. Cells are grown in 5 % CO_2 at 37 °C, maintained in subconfluent cultures, fed three times a week, and subcultured as needed. To subculture, confluent cells are detached with 0.05 % trypsin/EDTA, resuspended in supplemented DMEM/high glucose, and replated at 1/10 of the original density.

Steady-State Analysis

After growth to 70 % confluency, cells are treated with trypsin (0.02 % trypsin in 0.9 % NaCl/0.2 mM EDTA), washed with 3 × 10 ml KRBH containing 20 mM HEPES/KOH (pH 7.4), 125 mM NaCl, 5 mM KCl, 7.5 mM $NaHClO_3$, 2 mM $CaCl_2$, and 0.8 mM $MgSO_4$, suspended at a density of 2 × 107 cells/ml in the same buffer, and then incubated for 45 min at 4 °C at a density of 4 × 106 cells per 0.5-ml assay volume with labeled sulfonylurea, e.g., [3H]glibenclamide or [3H]glimepiride at concentrations between 0.1 and 20 nM. For determination of nonspecific binding, 1 μM unlabeled ligand is included. The incubation mixture is rapidly filtered on Whatman GF/C filters soaked with ice-cold buffer under reduced pressure. Filtration and washing have to take less than 30 s. The filters are washed three times with 6 ml ice-cold buffer containing 100 μM PMSF, 0.5 μg/ml leupeptin, 0.75 mg/ml pepstatin, 1 μg/ml aprotinin, and 50 mg/ml antipapain dihydrochloride, placed in 10 ml of ACSII scintillation cocktail, and after incubation overnight counted for radioactivity in a liquid scintillation counter.

Kinetic Analysis

For studying the association kinetics, the binding reaction is started by addition of radiolabeled ligand and terminated after predetermined periods of time by rapid filtration. For studying the dissociation kinetics, displacement of radiolabeled ligand at equilibrium (60-min incubation) is initiated by addition of unlabeled drug (final concentration 1 μM) and terminated after given periods of time. For termination, the incubation mixtures are rapidly chilled to 2–4 °C by placing the assay tube in a dry ice/methanol bath for 1 s immediately prior to filtration in a filtration apparatus located in a cool bench. Further processing is performed as described above.

Evaluation

Kd and Bmax values are calculated from Scatchard plot analyses and kon and koff values from kinetic studies. Curvilinear Scatchard plots allow the assumption of more than one binding site. IC50 values for half maximal inhibition of [3H]sulfonylurea binding can be calculated from competition-inhibition plots.

Modifications of the Method

Masuda and coworkers (1995) tested the effect of troglitazone on sulfonylurea receptor binding in rat pancreatic islet cells and in HIT cells. They concluded that troglitazone has a noncompetitive binding site at, or in the vicinity of, the sulfonylurea receptor.

Photoaffinity Labeling of Membranes

Purpose and Rationale

Binding sites of sulfonylureas have been identified in pancreatic β-cell membranes and in other tissues such as brain. The technique of photoaffinity labeling allows the identification and purification of membrane receptors (Bernardi et al. 1988; Yip 1984; Kramer et al. 1994; Aguilar-Bryan et al. 1990; Boyd et al. 1991;

Müller et al. 1994a; Geisen et al. 1985; Gaines et al. 1988; Kaubisch et al. 1982; Rajan et al. 1993; Sakura et al. 1995; Tanabe et al. 1999).

Procedure

Photoaffinity Labeling with Glibenclamide

Samples of membrane suspension are prepared as described above. 600 µg of β-cell membranes, suspended in 100 mM sodium phosphate buffer (pH 7.4), is incubated in a total volume of 200 µl with 40–60 nM (0.3–0.4 µCi) [3H] glibenclamide at 20 °C in the dark for 60 min. Irradiation is performed in a Rayonet RPR 100 photochemical reactor (Southern Ultraviolet Co., CT), equipped with RPR 2,530 Å lamps at a distance of 10 cm from the lamps. After irradiation at 254 nm for 2 min, the membranes are diluted with 1 ml of 10 mM Tris/HEPES buffer (pH 7.4) containing 4 mM EDTA, 4 mM iodoacetamide, and 4 mM PMSF and centrifuged (48,000 × g, 30 min). The resulting pellet is resuspended in 200 µl water and protein precipitated according to Wessel and Flügge (1984).

Differential Photoaffinity Labeling

600 µg of β-cell membranes is incubated with various concentrations (10–9 to 10–4 M) of glibenclamide or the test compounds. After incubation with 60 nM (0.37 µCi) [3H]glibenclamide in the dark, the membranes are photolabeled at 254 nm for 2 min. After washing of the membranes, the proteins are separated by SDS gel electrophoresis.

SDS-PAGE Analysis

The protein precipitates are dissolved in 70 µl of 62.5 mM Tris/HCl buffer (pH 6.8) containing 2 % SDS, 5 % mercaptoethanol, and 0.005 % bromophenol blue by shaking on a mixer for 60 min. After centrifugation (15,000 × g, 10 min), the clear supernatants are subjected to SDS gel electrophoresis on 150 × 180 × 1.5 mm slab gels (Kramer et al. 1988). After fixing and staining, the gels are scanned with a densitometer (e.g., CD50, Desaga, Heidelberg). The radioactivity is determined by liquid scintillation counting after slicing the gels into 2-mm pieces and after digestion of proteins with Biolute.

Evaluation

The radioactive peak of [3H]glibenclamide bound to the receptor protein is decreased depending on the concentration of unlabeled glibenclamide or test compounds. IC50 values can be calculated.

Binding to Recombinant SUR1

Procedure

Heterologous Expression of SUR1

Chinese hamster ovary and COS (1, m6, 7) cell lines do not produce SUR1 and are used for heterologous SUR1 expression. For transient transfection, COS cells are plated at 50–60 % confluence. Cells are transfected using either a DEAE-dextran or a Lipofectamine protocol. Three-day-old cultures of COS cells are trypsinized and replated at a density of 3.5 × 105 cells per 35-mm well (six-well dish) and allowed to attach overnight. Typically, 5 µg of a SUR1 plasmid is mixed with 5 µg of a KIR6.2 plasmid and brought up to 7.5-µl final volume in TBS (8 g/l NaCl, 0.4 g/l KCl, 0.2 g/l Na_2HPO_4, 3 g/l Tris base, 0.2 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, pH 7.5) before addition of DEAE-dextran (30 µl of a 5 mg/ml solution in TBS). The samples are vortexed, collected by spinning in a microfuge, and then incubated (15 min, 22 °C) prior to addition of 0.5 ml of 10 % FBS in TBS. Cells are washed twice with Hanks' balanced salt solution (HBSS), the DNA mix is added, and the cells are maintained in a CO2 incubator at 37 °C. After 4-h incubation, the DNA mix is decanted and the cells shocked for 5 min in 1 ml HBS and 10 % dimethyl sulfoxide, then placed in 2 ml of DMEM/high glucose containing 2 % FBS and 10 µM chloroquine, and kept in the incubator for 4 h. Thereafter, the cells are washed three times with HBSS and incubated in normal growth media until assayed.

Alternatively, Lipofectamine is used for transfection instead of DEAE-dextran. COS cells are plated in six-well dishes and are used at 70–80 %

confluency. Typically 1 μg of a SUR1 plasmid and 1 μg of a KIR6.2 plasmid are mixed with 375 μl of Opti-MEM reduced serum medium (Life Technologies, Inc.) and then added to 375 μl of Opti-MEM containing 9 μl of Lipofectamine. After 1-h incubation, the mixture is supplemented with Opti-MEM to 1-ml final volume and added to the cells, which had been washed twice with 3 ml of Opti-MEM each. After 5-h incubation, the Opti-MEM is replaced with DMEM/high glucose containing 10 % FBS. Transfections are scaled up based on the area of the plates used. For instance, 150-mm plates used for membrane isolations are transfected with 100 μg of each plasmid using the DEAE-dextran protocol. Transfected cells are used for determination of sulfonylurea binding and photolabeling or 86Rb + efflux 36–72 h post-transfection.

Membrane Preparation

Membranes are prepared from 15–25- to 150-mm dishes. Cells are washed three times in PBS (pH 7.4), then scraped in PBS, and collected in 10-ml plastic tubes. The cells are pelleted, resuspended in 10 ml of hypotonic buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA), and allowed to swell for 1 h on ice. Cells are then homogenized, transferred to a 15-ml glass tube, and spun (2,000 × g, 20 min, 4 °C) to remove nuclei and unbroken cells. The supernatant is then transferred to a polycarbonate centrifuge tube, and membranes are collected by centrifugation (50,000 rpm, 90 min, Ti80 fixed angle rotor). The pelleted membranes are resuspended in 5–200 μl of membrane buffer (25 mM Tris/HCl, pH 7.4,2 mM EDTA, 250 mM sucrose, 0.2 mM PMSF, 10 μg/ml leupeptin) and stored frozen in aliquots in liquid N2.

Membrane Solubilization

Membranes are rapidly thawed, resuspended using a Teflon-in-glass homogenizer, and mixed with ice-cold digitonin (25 % w/v, in deionized water, prepared daily) to a final protein concentration of 3 mg/ml and 1 % digitonin. Subsequent steps are performed at 25 °C in the presence of 0.2 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml pepstatin, and 0.2 mM phenanthroline. After 30 min of incubation, solubilized membrane proteins are separated from insoluble material by centrifugation (100,000 × g, 1 h, 4 °C). Alternatively, the thawed and re-homogenized membranes are mixed with the same volume (resulting in 3 mg/ml protein) of ice-cold 0.2 % (w/v) phosphatidylcholine, 20 % (w/v) glycerol, 280 mM KCl, 4 mM EDTA, 0.4 mM PMSF, 20 μg/ml leupeptin, 50 mM HEPES/KOH (pH 7.4), and 2.5 % (w/v) Triton X-100 or CHAPS, incubated on ice for 1 h (under gentle stirring), and then centrifuged (150,000 × g, 1 h, 4 °C). The supernatants are collected and stored frozen in liquid N2.

Partial Purification of SUR1

For purification of the 140-kDa core glycosylated species of SUR1, 4-ml aliquots of digitonin-solubilized membranes are cycled four times over a 1-ml concanavalin A-Sepharose column equilibrated with 40 mM Tris/HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, and 1 % digitonin. The column is washed with 8 ml of the equilibrating buffer and eluted with 4 ml of the equilibrating buffer containing 0.5 M methyl α-methylmannopyranoside. The eluted protein is stored in liquid nitrogen. For purification of the 150-kDa complex glycosylated SUR1, wheat germ agglutinin-Sepharose is used instead of concanavalin A-Sepharose. The procedure is the same as described with the exception of elution of the receptor using 0.3 M N-acetylglucosamine.

The eluate for the lectin columns is cycled twice over a 1-ml column of Reactive Green 19-agarose equilibrated with 40 mM HEPES/KOH (pH 8.5), 1 mM EDTA, and 0.5 % (w/v) digitonin. After washing with 10 ml of equilibrating buffer, and 10 ml of the same buffer supplemented with 0.5 M NaCl, the protein is eluted with 4 ml of equilibrating buffer containing 1.5 M NaCl. The eluate from the Reactive Green-19 purification step is diluted 1:2 with 40 mM HEPES/KOH (pH 8.5), 1 mM EDTA, and 0.2 % digitonin and then cycled twice over a 1-ml phenylboronate-10 Sepharose column. The phenylboronate column is washed with 10 ml of the HEPES buffer, followed by 2 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, and 0.2 % digitonin.

The protein is eluted with 4 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, and 0.15 % (w/v) sodium dodecyl sulfate. Prior to binding measurements, pooled samples from the various column steps are concentrated by centrifugation (3,000 × g, 30 min, 4 °C) through 100,000 molecular weight cutoff filters (Amicon), which have been pretreated with 5 % (v/v) Tween 20 for 14 h at 4 °C.

Interaction with Extrapancreatic Tissues

In addition to β-cells and hypothalamic neurons (Angel and Bidet 1991), SUR1 is also expressed in human adipocytes (Shi et al. 1999). Closure of the KATP by sulfonylurea binding to the adipocyte SUR1 leads to depolarization of the plasma membrane, opening of voltage-dependent Ca^{2+} channels, and concomitant increase of the cytosolic Ca^{2+} concentration. Thus, adipocytes and determination of intracellular Ca^{2+} may be used for assaying the potency of sulfonylureas with regard to binding to SUR1.

Prior to fluorometric Ca^{2+} measurement, the adipocytes are washed three times (by flotation) with HBSS (140 mM NaCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 4 mM $NaHCO_3$, 5 mM glucose, 2 mM sodium pyruvate, 2 mM glutamine, 20 mM HEPES, 1 % BSA). The cells are then loaded with fura-2-acetoxymethyl ester (10 μM) in the same buffer for 45 min at 37 °C in the dark with continuous shaking. For removal of extracellular dye, the cells are washed three times with HBSS and resuspended in HBSS at 3.5 × 105 cells/ml. Cytosolic Ca^{2+} is determined using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. After the establishment of a stable baseline in the absence or presence of the KATP channel openers, diazoxide and nitrendipine, the response to sulfonylureas is determined. Digitonin (25 μM) and Tris/EGTA (100 mM) are used to measure maximal and minimal fluorescence to calibrate the signals. Cytosolic Ca^{2+} is calculated by the equation of Grynkiewicz and coworkers (1985).

Direct effects of sulfonylurea agents on glucose transport (Cooper et al. 1990; Rogers et al. 1987; Müller 2000), insulin-induced decrease in 5′-nucleotidase activity in skeletal muscle membranes (Klip et al. 1987), and diacylglycerol-like activation of protein kinase C (Cooper et al. 1990) were studied in BC3H1 myocytes, rat cardiomyocytes (Bähr et al. 1995), and rat diaphragms (Müller et al. 1994b). Augmentation of the effects of insulin and insulin-like growth factors I and II on glucose uptake by sulfonylureas (Wang et al. 1987), coordinate regulation of glucose transporter function, and gene expression by insulin and sulfonylureas (Wang et al. 1989), glyburide-stimulated glucose transport via PKC-mediated pathway (Davidson et al. 1991), were studied in the same cell line. For glimepiride, a sulfonylurea of the third generation, but not for sulfonylureas of the second or first generation (glibenclamide or tolbutamide, respectively), its direct interaction with lipid rafts at the plasma membranes of rat adipocytes, presumably via spontaneous intercalation between raft glyco (sphingo)lipids, has been reported (Müller and Geisen 1996; Müller and Welte 2002). This is associated with activation of the GPI-PL (see ▶ Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes) and the translocation of certain GPI proteins and non-RTK from high cholesterol-containing rafts to low cholesterol-containing rafts accompanied by their activation. This redistribution of signaling components which ultimately leads to the IR-independent tyrosine phosphorylation of IRS-1 and downstream signaling to the glucose transport system in rat adipocytes (Müller et al. 2001; for reviews, see Müller 2002, 2005) may represent the molecular basis for the so-called extrapancreatic activity of certain sulfonylurea drugs, i.e., the blood glucose lowering in the absence of additional insulin release. The effects of compounds/drug candidates on the redistribution and activation of lipid raft proteins in rat adipocytes can be assayed as described (see ▶ Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes).

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Measurement of Glucose Absorption

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Inhibition of Polysaccharide-Degrading Enzymes

General Considerations

Starch as the predominant ingredient of human food is rapidly degraded in the gastrointestinal tract by salivary and pancreatic α-amylase to maltose which is further hydrolyzed by maltase localized in the brush border of the small intestine to glucose. Glucose is immediately absorbed leading to hyperglycemia and consequently to hyperinsulinemia. Both phenomena are undesirable in diabetics and in obese patients. The inhibition of the digestion of starch leads to a decrease and a retardation of glucose absorption. In nature, α-amylase inhibitors are found in wheat and other grains (Shainkin and Birk 1970). Several inhibitors of amylase and α-glucosidase have been developed (Bischoff 1991). Animal experiments with high doses of absorbable α-glucosidase inhibitors indicate that lysosomal storage of glycogen may occur (Lembcke et al. 1991).

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Assay for α-Amylase

Purpose and Rationale

α-Amylase activity can be measured by determination of the reducing groups arising from hydrolysis of soluble starch by isolated pancreatic α-amylase according to the protocol of Rick and Stegbauer (1970). The reduction of 3,5-dinitrosalicylic acid to nitroaminosalicylic acid produces a color shift which is followed photometrically by changes in the absorbance at 546 nm. Inhibition of starch hydrolysis by an α-amylase inhibitor results in a diminished absorbance at 546 nm in comparison with the controls.

Procedure

Commercially available pancreatic α-amylase (Merck, Germany) is used. Various concentrations of the α-amylase inhibitor are dissolved in 1 ml 20 mM Sorensen buffer, pH 6.9, and 10 mM NaCl. 0.1 ml pancreatic α-amylase solution in 0.4 % BSA is added. After prior incubation at 25 °C, the enzymatic reaction is started by addition of 1.0 ml soluble starch solution. The reaction is stopped after 10 min with 1.0 ml of dinitrosalicylic acid reagent. The mixture is heated in a boiling water bath for 10 min and, after cooling, measured at 546 nm against the reagent blank.

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Assay for α-Glucosidase

Purpose and Rationale

The inhibition of glucosidase can be measured in vitro using glucosidase from porcine small intestinal mucosa.

Procedure

Glucosidase is prepared from rat or porcine small intestinal mucosa or porcine pancreas. The enzyme activity is assayed according to Dahlqvist (1964). The inhibitory activity is determined by incubating a solution (20 μl) of an enzyme preparation with 80 mM sodium phosphate buffer, pH 7.0 (500 μl) containing 37 mM sucrose or maltose or 3.7 mM isomaltose, and a solution (20 ml) containing various concentrations of the inhibitor at 37 °C for 20 min. The reaction mixture is heated for 2 min in a boiling water bath to stop the reaction. The amount of liberated glucose is measured by the glucose oxidase method. Matsuo and coworkers (1992), Ikeda and coworkers (1991), and Glick and Bray (1983) studied the effect of intestinal disaccharidase inhibitors on obesity and diabetes.

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Everted Sac Technique for Assaying α-Glucosidase

Purpose and Rationale

The everted sac technique allows to study the effects of intestinal enzymes on substrates in an incubation vial.

Procedure

Male rats weighing 120–140 g are sacrificed and the small intestines are removed by cutting across the upper end of the duodenal junction. The intestine is stripped of the mesentery and the entire intestinal content is rinsed with cold saline solution. The intestine is divided into 7–8 cm segments and turned inside out using a Pasteur pipette. The everted intestine is ligated at one end with a cotton thread and a second ligature is placed loosely around the opposite end ready for tying. A 1 ml syringe with Krebs-Henseleit buffer is introduced into the lumen sac. The end of the sac is ligated and placed in a 25 ml Erlenmeyer flask containing 6 ml of 1 % starch, dissolved in Krebs-Henseleit buffer, with or without various concentrations of the α-amylase inhibitor. Pork α-amylase (4,000 U/g starch) is also included in the 6 ml starch solution. Following gassing with 95 % O2/5 % CO2, the flask is tightly capped and incubated in a shaking bath at 37 °C for 120 min. The reaction is terminated by the addition of 10 μl 1 N HCl. At the end of the incubation period, the sac is removed from the flask and the inner fluid is collected by cutting one end of the sac. The final volumes of the solute in the serosal and the mucosal side and the level of glucose liberation are measured.

Evaluation

Glucose liberated in the presence of various concentrations of the α-amylase inhibitor is expressed as a percentage of glucose found without the inhibitor. Dose-response curves can be drawn plotting percent inhibition versus concentration of the inhibitor.

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Dietary glucose crosses the apical membrane of the enterocyte by SGLT1 and exits across the basolateral membrane through the facilitative transporter GLUT2 (Thorens et al. 1990). Plasma glucose is maintained at ~5 mM. When the glucose concentration in the lumen is lower than that in the plasma, SGLT1 transports glucose uphill against its concentration gradient. This transcellular pathway is powered by a downhill gradient of Na+ across the apical membrane, maintained by the basolateral Na+/K±-ATPase. The cloning of SGLT1 by Wright's laboratory (Hediger et al. 1987) and GLUT2 by Thorens' laboratory (Thorens et al. 1990) began the molecular biological era of intestinal nutrient transport.

Assays for GLUT2 Transport Activity

General Considerations

Diabetes management is directed toward the control of postprandial fluctuations or excursions in blood glucose, which reflect the relative rates of delivery from the intestine and disposal to the tissues. While much is known about the impact of disposal mechanisms on blood glucose, rather less is known about delivery mechanisms, including gastric emptying, membrane hydrolysis, and sugar absorption itself.

Intestinal sugar absorption is increased in experimental diabetes (Csaky and Fischer 1981; Debnam et al. 1990; Thomson 1981). Knowledge of sugar absorption has been based primarily on the mechanism of Na+/glucose cotransport by Na+/glucose cotransporter (SGLT1) and its long-term regulation by diet (Ferraris 2001). Recently, however, we have discovered a new pathway of sugar absorption, the apical GLUT2 pathway, which operates within minutes when high concentrations of primary digestion products, namely, sugars, disaccharides, and α-dextrins, are presented to or generated at the apical membrane of the small intestine. This process is reversed when these sugars are absent from the lumen in vivo. The first indication of the existence of this mechanism was obtained in a study of the increase in fructose absorption in experimental diabetes (Corpe et al. 1996).

Purpose and Rationale

Recent experimental evidence hints to a refined model for intestinal glucose absorption (for a review, see Kellett and Brot-Laroche 2005; Kellett and Helliwell 2000) whereby a long-term diet containing high-glycemic-index sugars is priming the rapid induction of the translocation to and insertion into the apical plasma membrane of GLUT2 by simple dietary sugars. Most importantly, the apical GLUT2 component of absorption seems to be several times greater than the active component at high glucose concentrations. According to this hypothesis, apical GLUT2 and SGLT1 act in tandem to cover the entire concentration range with SGLT1 playing an important regulatory role in the control of apical GLUT2. Apical GLUT2 is not only tightly regulated by long- and short-term supply of dietary sugars but also by local and endocrine hormones, cellular energy status, stress, and diabetes; regulation occurs through a network of intracellular signaling pathways. Accordingly, apical GLUT2 can provide a major route of sugar absorption by which absorptive capacity is rapidly and precisely upregulated to match the dietary intake of sugars during assimilation of a meal (for a review, see Kellett and Brot-Laroche 2005). Apical GLUT2 provides a safety factor by preventing high sugar loads from reaching the colon (Cheeseman 2002). Moreover, the consequent rapid delivery of sugar

into the blood might increase the postprandial excursions, especially when glycemic control is poor. Apical GLUT2 is therefore a potential therapeutic target for novel dietary or pharmacological approaches to control intestinal sugar delivery and thereby improve glycemic control. The following assays enable the analysis of effects of compounds/drug candidates on the regulation (via insertion into the apical membrane) and transport activity of GLUT2 and may be helpful for the identification and characterization of a new class of glucose absorption inhibitors as putative future antidiabetic drugs.

Perfusion of Jejunal Loops

Procedure

Helliwell and coworkers (2000) described the perfusion of jejunal loops. Male Wistar rats (240–260 g) fed ad libitum on standard diet with free access to water are anesthetized with an intraperitoneal injection of a mixture of 1.0 ml of Hypnorm (Janssen, UK) and 0.4 ml of Hypnovel (Roche Diagnostics) per kg of body weight. The intestine is exposed by a laparotomy. A 30 cm segment just below the ligament of Treitz is cannulated, flushed free of nutrients, and perfused with a single-pass system at a flow rate of 0.5 ml/min with a modified Krebs bicarbonate saline (118 mM NaCl, 4.74 mM KCl, 1.2 mM KH2PO4, 1.25 mM CaCl2, and 24.8 mM NaHCO3) segmented with 95 % O2/5 % CO2. The effluent is collected every 5 min using a fraction collector. The jugular vein is cannulated with a Silastic tube and infused with saline at a flow rate of 3 ml/h. Radiolabeled substrate is added to the perfusate, and radiolabeled PEG4000 is included as a nonabsorbable marker to allow for the correction of fluid movements. At the end of the perfusion period, the intestine is removed, weighed wet, and then dried overnight before reweighing. For the addition of phloretin, the luminal solution required the use of modified Krebs bicarbonate saline (see above) to keep the inhibitor in solution. Phloretin is initially made up as a stock solution in DMSO and 200 µl of stock and then added to 150 ml of perfusate to give a final concentration of 1 mM. Final DMSO concentration is therefore 0.13 % (v/v), which has been demonstrated previously to have no effect on tissue permeability. Phloretin can be used in solution for the length of the experiment.

Compound Application

Because compounds take some time to become effective in whole tissue and because there is a limit to the time of viability for isolated loops in vitro, the following procedure for the treatment of jejunum with drugs has been adopted. The jejunum is first perfused luminally in vivo with 5 mM D-fructose (plus 1 mM β-hydroxybutyrate as an energy source) in the presence or absence of compound. The perfusion system is a gas-segmented, single-pass system with perfusate and gas flow rates of 0.75 and 0.38 ml/min, respectively. The jejunum is perfused with compound for 30 min. At the end of the in vivo treatment period, the cannulated loop is then excised and perfused in vitro using the gas-segmented, recirculated flow system: 5 mM D-glucose or fructose and the compound at the end of the in vivo treatment period are perfused luminally through the jejunum for 60 min; the flow rates of perfusate and gas were 7.0 and 3.4 ml/min, respectively. Control perfusions are performed in which no compounds are present. These show that there is no falling off in the glucose/fructose uptake rate after the initial approach to the steady state, confirming that this approach to compound treatment and perfusion ensured that the jejunum is viable for the whole experimental period from the start of the in vivo to the finish of the in vitro perfusion. Samples (0.05 ml) are taken from the perfusate at 5 min intervals throughout the in vitro perfusion. After measurement of glucose/fructose concentration with a COBAS automated analyzer (Roche Diagnostics) using a test kit from the same supplier, the amount of glucose/fructose remaining in the perfusate is calculated, with correction for losses in perfusate volume caused by water transport. Because the perfusate is recirculated, the concentration of fructose decreases with time; it is therefore necessary to limit the period over which the rate of transport is

measured to one in which the concentration decreased by no more than 20 %. Over such periods, plots of luminal perfusate content versus time are effectively linear. The rate of glucose/fructose transport, expressed as μmol/min• (g of dry weight)–1, is therefore determined by linear regression analysis as the average rate of disappearance over this period from the luminal perfusate in vitro.

Dually Perfused In Situ Jejunum

Dual perfusion of the jejunum is typically performed as described by Hirsh and Cheeseman (1998). Male Sprague-Dawley rats (200–350 g) are fed a standard chow diet and water ad libitum. Before the start of the experiment, food is withdrawn for approximately 24 h to minimize intestinal luminal contents during surgery. All rats are anesthetized prior to surgery using sodium pentobarbital given by intraperitoneal injection (60 mg/kg body weight) and placed on a heated (37 °C) surgical table. After performing a laparotomy, the blood supply to the spleen, rectum, colon, cecum, stomach, and ileum is tied off and the tissues are removed. The vasculature to the pancreas and duodenum is also ligated. A 35 cm segment of the jejunum, starting 5 cm distal from the ligament of Treitz, is isolated and the luminal contents are removed by gently flushing with 20 ml of warm saline (0.9 %), and the jejunum is cannulated at both ends. The lumen is perfused with a Krebs bicarbonate saline solution (120 mM NaCl, 4 mM KCl, 2.5 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 1 mM CaCl2) using a pump. The solution containing 5 mM 3-O-MG or 5 mM 3-O-MG and 5 mM D-fructose is maintained at 37 °C and gassed with 95 % O2, 5 % CO2. Isotopically labeled hexoses, 10 μCi of either 3-O-methyl-D-[1-3H] glucose or both 3-O-methyl-D-[1-3H]glucose and D-[U-14C]fructose, are added to the luminal circuit immediately after portal vein cannulation. The single-pass luminal circuit is perfused at a flow rate of 1.6 ml/min, and the solution is segmented by 95 % O2/5 % CO2 gas bubbles. The gas bubbles are introduced into the luminal perfusate through a Y piece at a flow rate which ensured that bubbles occupied the diameter of the perfusion tube. This not only exposes the tissue to a saturating gas partial pressure but also helps to mix the solution in the tissue lumen. After a single-pass through the segment of jejunum, the luminal perfusate is discarded. The aorta, proximal to the superior mesenteric artery, is ligated just prior to insertion of a cannula into the superior mesenteric artery. The single-pass vascular circuit is perfused at a rate of 1.6 ml/min with fresh Krebs bicarbonate saline solution, containing 5 mM D-glucose, 0.034 mM streptomycin sulfate, 5 mM L-glutamine, 1,120 USP heparin units, and 10 % w/v Ficoll 70 as a plasma expander, which is maintained at 37 °C and gassed with 95 % O2/5 % CO2 maintaining the pH at 7.4. Once the vascular circuit is established, the rat is euthanized and the vascular perfusate is collected via cannula placed in the hepatic portal vein. The effluent is collected continuously for up to 80 min using a fraction collector.

Washout Studies in the Dually Perfused Jejunum

This procedure is similar to the one used by Boyd and Parsons (1979). This washout model is useful for indirectly indicating the locus of compound/drug action. When the labeled 3-O-MG is washed out in the presence of an equimolar concentration of unlabeled mannitol in the lumen, the rate of washout into the vascular bed can be described by the sum of two exponential terms (assuming a two-compartmental model applies). The contributing compartments are a fast-releasing (vascular flow rate dependent) and a slow-releasing one (vascular flow rate independent), which represent (1) Q01 mucosal epithelium layer and (2) Q02 deeper submucosal (muscle) layer. Assuming that each pool unloads independently, then each pool will have its own rate constant: the fast, K1, and the slow, K2.

Evaluation

Tissue ion conductance (G) is calculated from PD and Isc according to Ohm's law. For the measurement of basal glucose fluxes, the tissue is clamped at zero voltage by continuously introducing an

appropriate Isc with an automatic voltage clamp every 5 min, except for 5–10 s when PD is measured by removing the voltage clamp. Tissue pairs are matched for conductance and discarded if conductance varied by >20 %. The non-metabolizable glucose analog 3-O-methyl-D-[1-3H]glucose (5 μCi) is added to either the serosal or mucosal side after mounting and the tissue is allowed to equilibrate for 20 min. Net directional flux from mucosal-to-serosal surface is determined for conductance-matched tissues by measuring four consecutive 5 min fluxes before the addition of compound/drug candidate and four 5 min fluxes following the addition of the agent.

Modification of the Method

Epithelial glucose uptake can be measured according to the method described by Walker and coworkers (2005).

Transport Activity of Brush-Border Membrane Vesicles

Preparation of Intestinal Brush-Border Membrane Vesicles

Brush-border membrane vesicles (BBMV) are prepared from mucosal scrapings as described by Corpe and coworkers (1996) on the basis of a standard procedure introduced by Maenz and Cheeseman (1986). Every stage of the preparation is performed at 0–4 °C to prevent changes in trafficking after the intestine had been excised. Briefly, the jejuna of two rats are perfused with compounds/drugs as described above. The timing of the final in vitro perfusion is such as to get as direct a correspondence as possible between the rates of transport and extent of trafficking. Perfusions for vesicle preparations are therefore terminated at a point corresponding to half the time period over which the average rate of transport is determined, as described above. Immediately after perfusion, each jejunum is rinsed with ice-cold buffered mannitol (20 mM imidazole buffer, pH 7.5, containing 250 mM mannitol, and 0.1 mM PMSF), placed on an ice-cold glass plate and slit longitudinally so that the muscle of the jejunum flattened out onto the cold plate. The mucosa is scraped off with a microscope slide and placed in 65 ml of ice-cold mannitol/Tris buffer (300 mM mannitol, 5 mM EGTA, 12 mM Tris/HCl, pH 7.4, and 0.1 mM PMSF). The tissue is homogenized in a Polytron homogenizer (four 30 s bursts using the large probe at setting seven) before the addition of MgCl2 to a final concentration of 12 mM. After stirring the solution on ice for 15 min, the solution is centrifuged (1,600 × g, 15 min) to remove debris. The supernatant is further centrifuged (20,000 × g, 30 min) and the pellet homogenized in half-strength mannitol/Tris buffer (150 mM mannitol, 2.5 mM EGTA, 6 mM Tris/HCl, pH 7.4, and 0.05 mM PMSF) with a glass homogenizer before further addition of MgCl2. After stirring on ice, the centrifugation is repeated as before, and the pellet is then washed (300 mM mannitol/5 mM Tris/HCl, pH 7.4) before repelleting (20,000 × g). This BBMV preparation is diluted to a protein concentration of 8 mg/ml. Enrichment of sucrase activity in these highly purified preparations typically range from 16- to 20-fold. There is no significant enrichment of Na+/K+-ATPase activity.

Preparation of Renal Brush-Border Membrane Vesicles

Both kidneys are removed from anesthetized animals and placed in ice-cold 154 mM NaCl. After removal of the renal capsule, the kidneys are weighed and then sliced into 2 mm thick sections and the cortex dissected away. Cortical fragments from six kidneys are pooled, weighed, and suspended in 30 ml of 300 mM mannitol, 12 mM Tris/HCl (pH 7.4), 5 mM EGTA. The resulting suspension was homogenized for 2 min (Ultra-Turrax homogenizer) at half-speed, followed by the addition of 42 ml of cold distilled water and MgCl2 to a concentration of 12 mM, and then stirred on ice for 15 min. The solution is then centrifuged (2,000 × g, 15 min) and the supernatant then recentrifuged (21,000 × g, 30 min). The pellet is suspended in 20 ml of 150 mM mannitol,

6 mM Tris/HCl (pH 7.4), 2.5 mM EGTA using ten cycles of a hand-operated glass-Teflon homogenizer. MgCl2 is added to a concentration of 12 mM, and after stirring on ice for 15 min, low- and high-speed centrifugations described above are repeated. The pellet is then resuspended in 20 ml of 300 mM mannitol, 12 mM Tris/HCl (pH 7.4), 2.5 mM EGTA, and centrifuged (21,000 × g, 30 min). The purified BBMV pellet is finally resuspended in the same buffer to a protein concentration of 3–6 mg/ml using 5–6 passes through a syringe fitted with a 21-gauge needle. All steps are carried out at 4 °C. The concentration of protein (Bradford 1976) and activity of alkaline phosphatase and Na+/K+-ATPase in the initial homogenate and BBMV preparation are determined in order to derive values for enrichment of these marker enzymes (see Corpe et al. 1996).

Glucose Transport

Transport studies are carried out at 20 °C on freshly prepared BBMV as described (Sharp and Debnam 1994). The transport process is initiated by mixing equal volumes of BBMV suspension and uptake buffer consisting of 200 mM NaSCN, 20 mM HEPES, and 0.1 mM MgSO4 containing D-[3H]glucose (9 Ci/mmol) and such that the final concentration of glucose is 5–500 μM. Due to the wide glucose concentration range used, the osmotic balance is maintained by adding mannitol to give a final glucose plus mannitol concentration of 100 mM. Uptake is terminated after 4 s by the addition of 3 ml of ice-cold 154 mM NaCl containing 0.5 mM phlorizin, followed by vacuum filtration through 0.45 μm nitrocellulose filters (Sartorius, Germany). Three further washes with stop solution are carried out.

Evaluation

Scintillation counting of the filters is used to calculate glucose accumulation and this is expressed as nmol (mg protein)–1 (4 s)–1. The kinetic parameters of Vmax (maximum transport capacity) and Kt (glucose concentration at half Vmax) for phlorizin-sensitive uptake were derived using a nonlinear least squares curve-fitting program.

Parallel uptakes using buffer containing 1 mM phlorizin to block SGLT-mediated transport indicated that uptake is >90 % phlorizin-sensitive. In order to assess GLUT-mediated transport, a higher glucose concentration (20 mM) is used in the presence of 1 mM phlorizin. This value is consistent with the low affinity of GLUT transporters for glucose binding. In addition, uptake of L-glucose at both 100 μM and 20 mM should be performed to establish the contribution of passive glucose transport.

Apical Expression of GLUT2

Biotinylation of Surface Proteins

The method of surface protein biotinylation for analysis of GLUT2 expression at apical membranes has been introduced by Au and coworkers (2002). For the detection of GLUT2 in the apical membrane of enterocytes, i.e., of the translocation of GLUT2 from intracellular vesicles to the apical cell surface, proteins expressed on the apical surface of jejunal enterocytes are labeled with N-hydroxysuccinimide (NHS)-SS-biotin introduced into the intestinal lumen. At the end of the in vivo perfusion, the intestine is maintained in situ, but cooled on ice. The luminal solution containing NHS-SS-biotin, 1.5 mg/ml in 10 mM triethanolamine/2.5 mM CaCl2/250 mM sucrose buffer (pH 9.0), is introduced into the lumen and left for 30 min. The lumen is then flushed with a PBS/100 mM glycine buffer to quench the free biotin before two final washings with PBS. Mucosal scrapings are then used to make protein extracts as described below.

Isolation of Biotinylated Proteins

Proteins are extracted from the homogenate for 1 h at 4 °C using the following buffer: 1 % Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris (pH 7.5). After centrifugation (14,000 × g, 10 min), the supernatant is collected and

incubated overnight with streptavidin beads. After washing twice with the Triton X-100 buffer to remove non-linked protein, the beads are washed with a high-salt buffer (500 mM NaCl) and finally with a no-salt buffer (10 mM Tris, pH 7.5). The isolated biotinylated proteins are then solubilized in Laemmli sample buffer to be run on SDS-PAGE for immunoblotting. Comparisons of total cell GLUT2 with apical GLUT2 are made by running samples of the supernatant after spinning down the streptavidin-coated beads on the same immunoblots as samples of recovered biotinylated protein.

Detection of Apical GLUT2 by Immunoblotting

Membranes (15 μg of protein) are solubilized in Laemmli sample buffer and separated by SDS-PAGE (10 % gel). The proteins are blotted on to nitrocellulose membrane by electrotransfer for 75 min at 4 °C. The membranes are stained for total protein with Ponceau S to ascertain that equivalent amounts of protein are loaded and transferred from each lane. Blocking of the membrane is carried out in 3 % nonfat milk in PBST (0.05 % Tween 20/PBS, pH 7.4) for 1 h and then incubated with 1:1,000 rabbit polyclonal antibody to rat GLUT2 in 3 % nonfat dry milk in PBST overnight at 4 °C. The membrane is washed three times in 3 % nonfat dry milk/PBST for 15 min, 1 h, and 15 min, respectively. The nitrocellulose membrane was then incubated with a secondary antibody, antirabbit IgG coupled to horseradish peroxidase diluted 1:2,000 in 3 % nonfat dry milk/PBST for 1 h. Three subsequent washes followed as described above. Finally, the membrane is treated with the ECL detection solution (Amersham Pharmacia) before autoradiography for 30 s using Kodak XAR-5 film with an intensifying screen. The specificity of the antibody was determined by running parallel lanes of isolated biotinylated proteins and probing them with the native antibody or with antiserum which had been preincubated at room temperature for 1 h with the peptide used to raise the antibody. Two antibodies are employed, one that recognizes an epitope in the large extracellular loop between transmembrane segments 1 and 2, amino acids 40–55 (SHYRHVLGVPLDDRRA; Biogenesis, UK), and a second which recognized a portion of the C-terminal sequence of rat GLUT2 (CVQMEFLGSSETV; Research Diagnostics, USA).

Evaluation of Glucose Absorption In Vivo

Purpose and Rationale

The inhibition of glucose absorption can be determined by measuring blood glucose after administration of starch or disaccharides with and without the inhibitor. In addition, non-absorbed starch or disaccharides can be determined in the intestine.

Procedure

Male Wistar rats are kept on a standard diet with free access to tap water at constant temperature (24 ± 1 °C). Sixteen hours prior to the experiment food but no water is withheld. Groups of rats receive by stomach tube 2.5 g/kg raw starch in a water suspension without or with various doses of the α-amylase inhibitor. After 10, 20, 30, 60, 120, and 240 min, blood is withdrawn for determination of blood glucose and nonesterified fatty acids. The animals are sacrificed after these intervals and the residual starch in the stomach and the intestine determined. Definitely more starch is found in the intestine after simultaneous application of the α-amylase inhibitor. Similar experiments are performed in dogs for determination of serum insulin. The increase of blood glucose and serum insulin and the decrease of NEFA are inhibited.

Evaluation

The values of starch content in the stomach and intestine, as well as the blood glucose, serum

insulin, and NEFA values, are compared between control and treated animals.

Modifications of the Method

In order to test the inhibition of glucosidase in addition, loading tests with sucrose or other disaccharides are performed (Puls and Keup 1973; Puls et al. 1977; Matsuo et al. 1992). Matsuo and coworkers (1992) performed experiments with genetically or experimentally obese rats (female Zucker fatty rats or female ventromedial hypothalamic nuclei-lesioned rats) and male yellow KK mice. LeMarchand-Brustel and coworkers (1990) studied the effect of an α-glycosidase inhibitor on experimentally induced obesity in mice. Male Swiss albino mice were rendered obese by injection of gold thioglucose at the age of 3 weeks. Since gold thioglucose treatment does not produce obesity in all injected mice, pro-obese mice were selected at 8 weeks of age on the basis of their body weight gain. They were divided in a control group receiving chow without drug and a treated group receiving chow containing the glycosidase inhibitor. Weight gain, glycemia, and insulinemia were followed over 120 days. Madar and Omusky (1991) studied the inhibition of intestinal α-glucosidase activity and postprandial hyperglycemia by α-glucosidase inhibitors in fa/fa rats. Various starch sources were used. Blood samples were taken at various intervals after starch load. Takami and coworkers (1991) studied the antidiabetic actions of a disaccharidase inhibitor in spontaneously diabetic (GK) rats. Okada and coworkers (1992) reported antiobesity and antidiabetic actions of a new potent disaccharidase inhibitor in genetically obese-diabetic mice.

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Monitoring of Diabetic Late Complication

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Aldose Reductase Activity

General Considerations

Secondary symptoms of long-lasting diabetes mellitus are diabetic neuropathy with sensory symptoms, motoric disturbances due to reduced nerve conduction velocity, and diabetic cataracts. Both are related to enhanced conversion of glucose to polyols, such as sorbitol, by the enzyme aldose reductase (van Heyningen 1959; Clements 1979). Sorbitol is converted to fructose by sorbitol dehydrogenase. A low activity of this enzyme enhances the accumulation of sorbitol, thus contributing to cellular damage. Inhibitors of aldose reductase have been developed with positive results in diabetic patients (Kador et al. 1985).

There are even some experimental hints for the benefit of aldose reductase inhibitors in diabetic cardiomyopathy (Cameron et al. 1989). Experimental data provide also evidence for possible beneficial effects for diabetic neuropathy and retinopathy, measured by nerve conduction velocity or electroretinography; lens cataract formation; vascular permeability and filtration (Williamson et al. 1987; Tilton et al. 1988, 1989; Pugliese et al. 1990); and nephropathy (Sarges and Oates 1993).

Geisen and coworkers (1994) reported on sorbitol-accumulating pyrimidine derivatives which inhibited sorbitol dehydrogenase, induced a dose-dependent increase of tissue sorbitol, and accelerated cataract development. Surprisingly, an acceleration of motor nerve conduction velocity in normal and diabetic rats and a normalization of glomerular filtration rates in diabetic rats were found.

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Measurement with Normal Lenses

Purpose and Rationale

The enzyme aldose reductase from bovine lens has been characterized by Hayman and Kinoshita (1965). Using this preparation, the in vitro effectiveness of various aldose reductase inhibitors can be determined (Varma and Kinoshita 1976; Billon et al. 1990).

Procedure

Aldose reductase from lenses of calf eyes is isolated from the homogenates by ammonium sulfate precipitation of the concomitant proteins and by column chromatography on DEAE-cellulose. DL-Glyceraldehyde is used as substrate. The activity is expressed as the rate of OD340 [nm] due to the utilization of NADPH in the reaction. The reaction mixture contains 0.1 M phosphate buffer (pH 6.2), NADPH 2.5×10^{-4} M, DL-glyceraldehyde 1.5×10^{-3} M, and the enzyme. The total volume of the reaction mixture is 1 ml. The reference blank consists of all the above compounds except the substrate. The effects of inhibitors on the enzyme activity are determined by including the compound being tested at the desired concentration in the reaction mixture. Appropriate blanks are used to correct for nonspecific reduction of NADPH and absorption by the compounds being tested. The test compounds are added first in 10^{-3} M solution and diluted as desired.

Evaluation

Percentage of inhibition is tested at various concentrations and IC50 values are calculated.

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Measurement with Cataract Lenses

Purpose and Rationale

The effect of aldose reductase inhibitors can be tested by determining the level of aldose reductase, NADPH, and NADP+ in the lenses of galactose treated or streptozotocin-diabetic rats after treatment with the inhibitor compared to controls.

Procedure

Galactose Treatment

Cataract formation can be induced by galactose feeding to young rats. Male Sprague-Dawley rats weighing 40–50 g are randomly divided into two groups. One group is fed a laboratory chow, the other group is fed a galactose diet containing 50 % galactose, 20 % cornstarch, 15 % casein, 9 % hydrogenated oil, 4 % salt mixture, and 2 % cod liver oil. Diabetes is induced in male Sprague-Dawley rats weighing 80–90 g by a single intravenous dose of 100 mg/kg streptozotocin.

Streptozotocin Treatment

Diabetes is induced in 230–260 g male Sprague-Dawley rats by administration of a single tail vein injection of streptozotocin (55 mg kg^{-1}) 2–4 weeks prior to study. Streptozotocin is dissolved in freshly prepared 0.05 M citrate buffer (pH 4.5) and administered under light isoflurane anesthesia. Animals are glycosuric 24 h after streptozotocin treatment. The weight of the control animals is matched to that of the 2–4-week diabetics. Animals were allowed ad libitum access to a standard rat chow (Diet RM1, SDS Ltd, UK) and water until the time of experimentation, with the exception of those subjected to an overnight fast. For all experimental procedures animals are terminally anesthetized with intraperitoneal pentobarbitone sodium (90 mg kg^{-1}) before removal of the eyes. The progression of cataracts is observed in controls and in rats treated with the aldose reductase inhibitor.

Assay

At appropriate times, the rats are sacrificed and the lenses are dissected. They are frozen on solid CO_2 and stored at −70 °C until analysis. Single rat lenses are homogenized in 200 µl of 10 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) for 0.5 min at 0 °C. A cell-free extract is obtained by centrifuging

(17,300 × g, 10 min) the total lens homogenate. The enzyme reaction mixture (final volume 1 ml) contains 0.4 M $(NH_4)_2SO_4$, 0.1 M HEPES/NaOH buffer (pH 7.0), 10 mM DL-glyceraldehyde, and 0.12 mM NADPH. A 20 ml aliquot of supernatant is added to initiate the reaction. Decrease in absorbance at 340 nm is followed spectrophotometrically. Enzymatic activity is expressed as nM of NADPH oxidized per min.

Evaluation

Results are expressed as aldose reductase units/mg lens (wet weight). Means ± SE are calculated and compared using Student's non-paired *t*-test.

Modifications of the Method

Naeser and coworkers (1988) studied sorbitol metabolism in the retina, optic nerve, and sural nerve of diabetic rats treated with an aldose reductase inhibitor. Activities of aldose reductase and sorbitol dehydrogenase and content of sorbitol were assayed in these tissues.

Freeze-dried tissue samples are sonicated for 5–10 s in 215 µl of 0.04 M Tris (pH 6.8). For determination of aldose reductase, the homogenate is centrifuged for 45 min at 37,000 rpm and 100 µl is taken from the supernatant for analysis. The remaining sample is centrifuged further (96,000 rpm, 2 h) and the supernatant used for sorbitol determination. For determination of aldose reductase, 10 µl of the sample is mixed with 15 µl buffer solution containing 0.14 mM Tris, 151 µM NADH, and 0.6 M glucose. After incubation in a water bath for 20 min at 38 °C, the incubation is stopped by transfer to ice. Samples mixed with buffer solution without glucose serve as blanks. Twenty µl of the incubated material is then mixed with 1.25 ml of 0.04 M NaOH solution containing 1.7 mM NaCl. Fluorescence is measured in a Farrand Ratio Fluorometer with primary filters 5,860 and 5,970 (Corning Glass Works) and secondary filters 2A (Turner Optical Co.) 4,308 and 3,387 (Corning Glass Works). The amount of NADPH consumed serves as a measure for aldose reductase activity.

Sorbitol dehydrogenase is measured by adding 5 µl of fresh homogenate to 20 µl of a buffer composed of 0.1 mM Tris (pH 6.8), 1.25 mM NAD, and 50 mM sorbitol. After incubation for 30 min at 38 °C, the reaction is stopped by the addition of 5 µl 0.1 N NaOH and transfer to ice. The amount of NAD+ converted to NADH is determined luminometrically in 10 µl samples with 100 µl of NAD(P)H reagent (Roche Diagnostics) containing 5 mg/L luciferase, 25 mM potassium phosphate, 100 mM dithiothreitol, 38 U/L Triton X-100, and 20 mM myristic aldehyde. The flux of light is instantly registered, using a photomultiplier.

Sorbitol is determined in the centrifugation supernatant. After immersion in boiling water for 5 min, aliquots of 15 µl are added to 10 µl buffer containing 0.1 M Tris (pH 8.6), 3.5 mM NAD, and 2.7 U/ml sorbitol dehydrogenase. Tissue blanks are run without sorbitol dehydrogenase. The incubation is terminated and the amount of NADH formed is determined as for the sorbitol dehydrogenase assay.

Gonzales and coworkers (1986) studied the effect of an aldose reductase inhibitor on integration of polyol pathway, pentose phosphate pathway, and glycolytic route in diabetic rat lens. Diabetes was induced in male Wistar rats weighing about 230 g by subcutaneous injection of 200 mg/kg alloxan monohydrate. After 1 week, in the diabetic rat lens, there was an apparent increase in the flux of glucose through the pentose phosphate pathway, as measured by the difference in the yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose [C1–C6]. Treatment with the aldose reductase inhibitor reduced the values toward normal. With glucose tritiated on carbons 2 or 3, it has been shown that the flux of glucose through the polyol route is increased, whereas the flux through the glycolytic pathway is decreased in the diabetic rat lens. Both parameters were restored to normal in diabetic rats treated with the aldose reductase inhibitor.

Meydani and coworkers (1994) investigated the onset and progression of cataract in weanling Sprague-Dawley rats fed 10, 15, 20, and 30 % dietary galactose for 45–226 days.

Ohta and coworkers (1999) studied cataract development in 12-month-old rats fed a 25 % galactose diet and its relation to osmotic stress and oxidative damage.

Using phosphorus-31 nuclear magnetic resonance spectroscopy, Sakagami and coworkers (1999) investigated the metabolic kinetics of organophosphate compounds in the rat lens during cataract development induced by different doses of galactose added to rat chow.

Mackic and coworkers (1994) developed a model of galactose-induced cataract formation in guinea pigs and studied the morphologic changes and accumulation of galactitol.

Sato and coworkers (1998) demonstrated that the formation and progression sugar cataracts in galactose-fed dogs can be dose-dependently inhibited by the aldose reductase inhibitors.

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Nerve Conduction Velocity

Purpose and Rationale

Aldose reductase inhibitors can be tested in rats with diabetes induced with streptozotocin by measuring nerve conduction velocity (Miyoshi and Goto 1973; Mayer and Tomlinson 1983; Tomlinson et al. 1982, 1984; Yue et al. 1982) and resistance to ischemic conduction block (Price et al. 1988).

Procedure

Male Wistar rats weighing 100–150 g are rendered diabetic with an i.v. injection of 60 mg/kg streptozotocin. Diabetes is confirmed by the presence of glucosuria and elevated blood glucose levels 24 h after injection. The rats are kept on standard diet and water ad libitum for 28 days after streptozotocin treatment. Groups of rats are treated with the test compound (aldose reductase inhibitor) or the vehicle once daily by gavage starting 24 h after streptozotocin injection. One group of age-matched rats serves as control.

For measurement of sciatic nerve conduction velocity, rats are anesthetized with 2 % halothane in oxygen and placed in a Perspex chamber that is maintained at a constant temperature by a copper coil in the base through which warm water is circulated. A fine needle thermocouple is inserted in the lateral aspect of the left hind limb, and the subcutaneous temperature is measured with an electronic thermometer. The conduction velocity of the left sciatic-tibial nerve is measured by a modification of the method of Sharma and Thomas (1974). Two fine platinum-stimulating electrodes are inserted in the sciatic notch, and two more are inserted through the skin of the ankle to lie adjacently to the tibial nerve. The

electrodes are 5 mm apart, the distal end being the cathode. A recording electrode is inserted under the skin of the lateral side of the foot and another between the third and fourth toes. A ground electrode is inserted under the skin on the dorsum of the foot. Both stimulation and recording are carried out with a neurophysiological unit (e.g., model MS92a, Medelec, Old Woking, UK). The nerve is stimulated supramaximally with square-wave pulses of 0.1 ms duration. The conduction velocity is calculated as the distance between the distal electrodes divided by the difference between the latencies at the two stimulation points. The mean of three recordings is taken. The coefficient of variation of nerve conduction velocity is calculated by measuring nerve conduction velocity six times in one rat from each treatment group, withdrawing and reinserting the electrodes each time.

To measure resistance to ischemic conduction block, two fine platinum stimulating electrodes (1 cm apart) are inserted in the lateral aspect of the tail 3 cm from the base. Two recording electrodes are inserted near the tip, and a ground electrode is inserted midway between these and the stimulating electrodes. A thermocouple is attached to the middle of the tail. Both stimulation and recording are carried out as described above. To render the tail ischemic, a small sphygmomanometer cuff is inflated to 240 mmHg around the base of the tail. The nerve is stimulated at 2.5 min intervals until the action potential disappears. The tail temperature is maintained at 32 °C. At the end of the experiment, 5 ml blood is taken by cardiac puncture. The animal is then sacrificed, and the sciatic nerves are removed, cleaned, weighed, snap-frozen in liquid nitrogen, and stored at −20 °C for subsequent estimation of polyol level.

Nerve polyol estimation is performed by the method of Stribling and coworkers (1985). Frozen nerves are thawed, placed in 1 ml water containing 60 μg of α-methylmannoside as an internal standard, and boiled for 20 min. Zinc sulfate (200 μl of a 5 % solution) is added, samples are homogenized with a Polytron homogenizer, 200 μl 0.3 N barium hydroxide solution is added, and the samples rehomogenized. After centrifugation, the supernatant is freeze-dried overnight. The residue is silylated with 250 ml of a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane (10/2/1, Pierce) at room temperature for 24 h. The reaction is stopped by adding 2 ml water, and 200 μl cyclohexane is added. Samples are shaken and 2 μl of the organic phase is injected into a gas chromatograph. Plasma glucose levels are measured with the use of a glucose analyzer. Plasma fructosamine, which reflects plasma protein glycosylation, is measured with the use of a commercial kit. Plasma glucose, plasma fructosamine, and nerve sorbitol are increased and nerve conduction velocity decreased in streptozotocin treated rats.

Evaluation

Data from diabetic rats treated with the aldose reductase inhibitor are compared with the values of untreated diabetic rats and normal controls. Statistical comparisons are made by use of the Mann-Whitney *U* test.

Modifications of the Method

Sima and coworkers (1990) studied motor nerve conduction velocity and neuroanatomical abnormalities in insulin-deficient diabetic bio-breeding rats (BB rats) with and without long-term administration of an aldose reductase inhibitor. Their findings of a significant, but incomplete prevention of neuropathy in these animals by aldose reductase inhibition suggest that additional mechanisms besides polyol pathway activation may be of importance in the pathogenesis of diabetic neuropathy. Carrington and coworkers (1991) studied the effects of impulse conduction after aldose reductase inhibition with imirestat in sciatic nerves of streptozotocin-diabetic rats both in vivo and in vitro. Cameron and coworkers (1991) studied nerve blood flow monitored by microelectrode polarography and hydrogen clearance in experimental diabetes in rats in relation to conduction deficits. Using these methods, Cameron and Cotter (2003) studied the effects of

5-hydroxytryptamine 5-HT2 receptor antagonists on nerve conduction velocity and endoneurial perfusion in diabetic rats.

Schmidt and coworkers (1991) performed ultrastructural studies in mesenteric nerves of streptozotocin-induced diabetic rats.

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Nerve Blood Flow (Doppler Flux)

Purpose and Rationale

In addition to nerve conduction studies, Calcutt and coworkers (1994) described a method to measure nerve blood flow by laser Doppler flowmetry in rats with streptozotocin-induced diabetes treated with aldose reductase inhibitors.

Procedure

Diabetes is induced in female Sprague-Dawley rats weighing 200–280 g by a single i.p. injection of 50 mg/kg streptozotocin after an

overnight fast and is confirmed 2 days later by blood glucose determinations. Aldose reductase inhibitor-treated rats receive the test compound orally by gavage once a day for 2 months. For the experiment, the animals are anesthetized with an intraperitoneal injection (2 ml/kg) consisting of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in 0.9 % NaCl. For estimation of nerve blood flow, a laser Doppler flowmeter (TSI model BPM 403A, MN) with a wavelength of 780 nm is used. Flow determinations are made with a filter frequency of 30 Hz–18 kHz and, depending on the magnitude of the reflected signal, one of four bandwidths (30 Hz–1.3 kHz, 30 Hz–3 kHz, 30 Hz–7.5 kHz, or 30 Hz–18 kHz). The flowmeter continuously displays a moving average for the preceding 5 s in units of Hz × 102. For flow measurements, the sciatic nerve is exposed and the probe tip of the flowmeter placed 1 mm above the mid-thigh region of the nerve using a micromanipulator. After a 10 s stabilization period, the first measurement is recorded. The probe is then advanced 1 mm distally for a second measurement and this process repeated until ten readings are recorded over 1 cm of nerve. The mean of 10 values is taken as average nerve laser Doppler flow. Blood flow velocity, expressed in m/s, is reduced in diabetic rats.

Evaluation

The effects of streptozotocin diabetes and of aldose reductase treatment are assessed by one-factor analysis of variance (ANOVA). Individual post hoc comparisons are made with the Newman-Keuls method when the F ratio is $P < 0.05$.

Electroretinogram

Purpose and Rationale

Diabetic neuropathy is one of the important symptoms of long-lasting diabetes (Engerman 1989). Several animal studies with aldose reductase inhibitors have been performed (Lightman et al. 1987; Hotta et al. 1988; Nagata and Robison 1988). Segawa and coworkers (1988a, b) measured the development of electroretinogram abnormalities and the possible role of polyol pathway activity in diabetic hyperglycemia and galactosemia in the rat.

Procedure

Male Sprague-Dawley rats weighing 310–400 g are dark-adapted for 20 min and anesthetized with i.p injections of ketamine 50–80 mg/kg and atropine sulfate 2 mg/kg. Electroretinography is performed monocularly with the pupil maximally dilated. Photic stimulation is delivered with an intensity of 1 J in 20 s interstimulus intervals. Using a contact lens-type electrode, electroretinograms (ERG) evoked by strong flushes are amplified by a preamplifier, displayed on an oscilloscope and summed using a signal averager which also provides a copy of the averaged ERG. The amplitudes of the a- and b-waves are measured from the baseline to the trough of the a-wave and from the trough of the a-wave to the crest of the b-wave, respectively. The amplitudes of the oscillatory potentials are measured. The peak latencies are measured as the intervals between the stimulus onset and the peak of the corresponding a- and b-waves and oscillatory potentials. The oscillatory potentials (designated O1, O2, and O3 in order of appearance on the ascending limb of the b-wave) are added together. The sum of these amplitudes is expressed as the wavelet index.

For inducing galactosemia, male Sprague-Dawley rats weighing 140–185 g at the beginning of the study receive a diet of 30 % galactose. Test compounds are given as 0.1 % to the diet.

Evaluation

Data are calculated as mean ± SEM and significance levels are estimated using the Wilcoxon rank sum test for unpaired data (two-sided). Linear regression is calculated by the least square method. A p-value of <0.05 is regarded as statistically significant.

Modifications of the Method

De la Cruz et al. (2003) studied the effects of clopidogrel and ticlopidine on experimental ischemic retinopathy in streptozotocin-diabetic rats. The percentage of the retinal surface occupied by horseradish peroxidase-permeable vessels was recorded.

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Streptozotocin-Induced Cataract

Purpose and Rationale

The increased incidence of cataracts, i.e., changes in the transparency or of the refractory index of the lens, in diabetic patients is well known. Evidence has been accumulated for the involvement of polyol metabolism and the enzyme aldose reductase in diabetic cataractogenesis (van Heyningen 1959; Pirie and van Heyningen 1964). The enzyme aldose reductase catalyzes the reduction of aldoses such as glucose and galactose to the corresponding polyols, i.e., sorbitol and dulcitol. Sugar-induced cataractogenesis has been shown to parallel lenticular polyol accumulation (Kinoshita 1965). Since polyols do not readily diffuse through intact cellular membranes, they create a severe osmotic stress within the lenticular cells which leads to cellular swelling and loss of integrity of the cellular membrane (Kinoshita 1974). Aldose reductase inhibitors have been shown to prevent sugar-induced cataracts (Dvornik et al. 1973; Varma and Kinoshita 1976; Kinoshita et al. 1979; Muller et al. 1985).

Procedure

Experimental diabetes is induced in Wistar rats weighing 150–180 g by intravenous injection of 50 mg/kg streptozotocin (Griffin et al. 1984).

Evaluation

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with streptozotocin only.

Modification of the Method

Instead of systemic application, the aldose reductase inhibitor is applied twice daily for 6 weeks in solution as eye drops to female Sprague-Dawley rats with a starting weight of 120–140 g after diabetes induction with 70 m/kg streptozotocin i.v. Body weight and blood sugar are registered twice a week. The progression of cataractous changes is followed by slit lamp and Scheimpflug photography analysis every second week. At the end of the experiment, the animals are sacrificed and the lenses analyzed for their content of ATP, ADP, AMP, glucose, sorbitol, fructose, glucose-6-phosphate, and fructose-6-phosphate.

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Naphthalene-Induced Cataract

Purpose and Rationale

Systemic application of naphthalene induces cataract in Brown-Norway rats. Aldose reductase inhibitors are tested for prevention of the cataract changes by parallel systemic or topical application (as eye drops).

Procedure

Female Brown-Norway rats at an age of 6–7 weeks with a starting weight of 70–100 g are used. They are fed standard lab chow and receive water ad libitum. Naphthalene is dissolved in warm paraffin oil (10 g/100 ml) and is administered orally by gavage every second day in a dose of 1 g/kg. The test compound is administered orally every day or applied topically as suspension every day once, twice, or four times to the right eye. The duration of the treatment is 6 weeks. Slit lamp microscopy (Zeiss photo slit lamp) during mydriasis with 1 % atropine eye drops is performed in weekly intervals, including a baseline examination before the start of the treatment and a final examination prior to sacrifice. Scheimpflug photography of the anterior eye segment (Topcon SL-45 Scheimpflug camera) is carried out at the baseline examination, after 3 weeks of treatment and at the end of the experiment. Prior to sacrifice, blood samples are taken by cardiac puncture under ether anesthesia. After sacrifice, lens fresh weight, concentration of oxidized and reduced glutathione, and the concentration of the aldose reductase inhibitor in the lenses and in blood samples are determined.

Evaluation

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with naphthalene only.

Modifications of the Method

Rathbun and coworkers (1996a) induced rapid-onset cataracts in SPF C57bl/6 mice by i.p. administration of naphthalene following cytochrome P-450 induction with phenobarbital. Rathbun and coworkers (1996b) assessed the activity of a L-cysteine prodrug suitable for glutathione biosynthesis rat lenses in vitro and as an agent for the prevention of acetaminophen- and naphthalene-induced murine cataract in genetically susceptible mice. Holmen and coworkers (1999) compared different methods of photographic evaluation of cataract formation in rats in response to different regimes of naphthalene treatment.

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Determination of Advanced Glycation End Products (AGEs)

General Considerations

Common to both type I and type II diabetes is the development of inflammatory and vascular complications that, over time, might portend significant morbidity and early mortality in affected subjects. Although multiple studies have suggested a direct role for adverse effects of glucose itself in modulating cellular properties, both in the extra- and intracellular milieus (Greene et al. 1987; Inoguchi et al. 1994; Xia et al. 1994; Nishikawa et al. 2000), recent observations suggest an emerging role for the products of nonenzymatic glycoxidation of proteins and/or lipids, the advanced glycation end products (AGEs), in the pathogenesis of diabetic complications.

Formation of AGE

The nonenzymatic glycation and oxidation of proteins and/or lipids is a well-recognized phenomenon that occurs naturally, albeit at a very slow rate, during the course of aging. However, certain conditions within the cellular environment might enhance the formation of these modified structures. Specifically in the context of diabetes, hyperglycemia is a major trigger for the accelerated formation and deposition of AGEs (Brownlee 1995; Ruderman et al. 1992). Multiple specific AGEs, formed by glycation and/or oxidation, such as carboxy(methyl)lysine (CML), pentosidine, and pyralline, have been identified in diabetic tissues (Ikeda et al. 1996; Reddy et al. 1995). It is speculated that, in diabetes, hyperglycemia alone might not be the sole perturbant driving formation of these modified structures, as AGE formation is enhanced in

pro-oxidant states, such as that observed in diabetes itself (Baynes 1991). The observation that products of glycoxidation have been noted in hyperlipidemic atherosclerotic lesions, even in euglycemia, supports this concept. A recent suggestion is that in vitro AGE formation might be driven by the myeloperoxidase-hydrogen peroxide-chloride system, thereby providing a mechanism for conversion of hydroxyamino acids into glycolaldehyde, a precursor in the steps leading to the formation of CML. Thus, these findings could have implications for AGEs in inflammatory settings, once confirmation in vivo has been established. It is important to note that in diverse conditions, such as renal failure and Alzheimer's disease, AGE epitopes have also been identified within affected structures (Miyata et al. 1996), thereby providing a contributory mechanism for the cellular stress observed in these settings.

AGE and Mechanisms of Cellular Perturbation

It is likely that AGEs exert their effects via a number of mechanisms. Accumulation of cross-linking AGEs that tend to become insoluble, such as pentosidine, is thought to contribute to the tissue perturbation and rigidity that typify advanced diabetes, especially in the skin and basement membranes (Sell et al. 1992; Beisswenger et al. 1993). These factors are potentially linked to vascular leakiness and hyperpermeability. Vlassara and coworkers (1995) identified galectin-2 as a high-affinity binding protein for AGE. In addition, work from M. Brownlee's laboratory suggests that intracellular formation of AGE might modify the activity of growth factors, such as basic fibroblast growth factor.

Beyond the direct effects of AGEs on modulating cellular structure and function are those effects postulated to occur during ligation of specific cell surface receptors. In this context, the best-characterized receptor for AGEs (RAGE) is a multi-ligand member of the immunoglobulin (Ig) superfamily of cell surface molecules (Schmidt et al. 1992; Neeper et al. 1992). RAGE is composed of an extracellular region containing one "V"-type Ig domain, followed by two "C"-type Ig domains. This portion of the molecule confers the ligand-binding properties, which are probably specific to the "V"-domain. This portion of the molecule is followed by a hydrophobic transmembrane-spanning domain and, lastly, by a highly charged, short cytosolic domain that is essential for RAGE-mediated cellular effects upon engagement of ligand (Kislinger et al. 1999a, b; Yamamoto et al. 2001; Arancio et al. 2004; Hadding et al. 2004).

Purpose and Rationale

Interactions between AGE and their binding proteins lead to the activation of a range of secondary messenger systems and increase the production of cytokines, including TGF-β, PDGF, and IL-1 (Ikeda et al. 1996). Recently, compounds that can cleave established AGE cross-links (Reddy et al. 1995), such as ALT711, a stable 4,5-dimethylthiazolium derivative of the prototype compound N-phenyl-thiazolium bromide, have been investigated. In vivo efficacy has been demonstrated in animal models, showing that these compounds can result in reduced AGE accumulation and can reverse age- and diabetes-dependent increases in arterial stiffness (Baynes 1991). RAGE as target for the prevention and treatment of the vascular and inflammatory complications of diabetes has been critically reviewed by Schmidt and Stern (2000a, b). The following assays enable the study of the AGE-RAGE interaction including its cellular effects as well as the modulation of both processes by compounds/drug candidates, which may be helpful for future therapy of diabetic late complications.

Procedure

Cell Culture

The well-characterized normal rat kidney epithelial cell line (NRK-52E) can be obtained from the American Tissue Culture Collection. NRK-52E cells are believed to be of a proximal tubular origin on the basis of patterns of collagen secretion, C-type natriuretic peptide secretion, and the

presence of EGF receptors. Cells are maintained in DMEM containing 4.5 g/l glucose with 10 % FCS at 37 °C in a 5 % CO_2 atmosphere and passaged twice a week.

In Vitro Preparation of AGE Ligands

Procedures for the preparation of AGE ligands have been given by Oldfield and coworkers (2001) and Kislinger and coworkers (1999a, b). For preparation of AGE-BSA and AGE-RNase, BSA (10 mg/ml) or RNase (10 mg/ml) is incubated at 37 °C for 6 weeks with D-glucose (90 g/l) in a 0.4 M phosphate buffer containing azide (Vlassara et al. 1985). Control preparations are treated identically except that glucose is omitted. Preparations are extensively dialyzed against phosphate buffer to remove free glucose. The extent of advanced glycation is assessed by characteristic fluorescence (excitation 370, emission 440 nm). Advanced glycation is associated with an approximately tenfold increase in fluorescence compared with controls.

For preparation of human AGE-IgG, human IgG (5 mg/ml in PBS) is subjected to nonenzymatic glycation by incubation in PBS containing 25 mM D-ribose. The solution is sterile-filtered (0.2 μm) and then incubated at 37 °C for 6 weeks under aerobic conditions. At the end of that time, the mixture is extensively dialyzed versus PBS at 4 °C to remove unreacted ribose. This material is then chromatographed onto resin containing Affi-Gel 10 (Bio-Rad) to which had previously been adsorbed to recombinant human soluble RAGE. After incubation, the resin is washed extensively with ten-column volumes of TBS (20 mM Tris, pH 7.4, 100 mM NaCl). Bound components are eluted by 20 mM glycine (pH 2.5). The absorbance at 280 nm of each fraction is determined. Positive fractions are immediately neutralized and dialyzed versus TBS.

For preparation of carboxymethyl lysine-modified BSA (CML-BSA) (Kislinger et al. 1999a, b), 50 mg/ml aliquots of BSA are incubated with increasing concentrations of glyoxylic acid (5–90 mM) in the presence of approximately fivefold molar excess of sodium cyanoborohydride. Control proteins are prepared under the same conditions, except that glyoxylic acid was omitted. The extent of chemical modification of lysine residues is determined as described previously using 2,4,6-trinitrobenzenesulfonic acid (Cayot and Tainturier 1997). The extent of lysine modification was up to 34 % for CML-BSA preparations.

Iodination of AGE-BSA

AGE-BSA was iodinated by incubating AGE-BSA with chloramine-T (Greenwood et al. 1963). Bound 125I was separated from free 125I using a Bio-Gel P-6DG desalting gel (Bio-Rad Laboratories Inc.). Specific activity of the tracer is typically 400 Ci/mM.

Membrane Preparation

Membranes are prepared based on the method of Skolnik and coworkers (1991). NRK-52E cells are grown to confluence in 150 cm^2 tissue-culture flasks. Cells are washed twice with PBS and then detached from plates using a HEPES (100 mM) solution containing BSA (0.1 %) and Triton X-100 (0.1 %) with EDTA (5 mM), leupeptin (1 μM), and PMSF (2 mM). Cells are centrifuged (2,000 × g, 5 min) and resuspended in the above buffer before disruption by ultrasound. Cell debris is removed by further centrifugation (2,000 × g, 10 min) and the supernatant centrifuged (100,000 × g, 1 h, 4 °C). The supernatant is discarded, and the cell membranes in the precipitate are resolubilized in the above buffer. Protein concentrations are determined by the method of Bradford. The membrane preparation is used in binding studies and for ligand and Western blot analysis.

Binding Assay with Radiolabeled AGE

For binding studies according to the procedure of Oldfield and coworkers (2001), cell membrane extracts are incubated with 125I-AGE-BSA (0.5 nM) and increasing concentrations of unlabeled AGE-BSA (0.015–7.46 μmol) in the absence or presence of compounds/drug candidates for 3 h at 4 °C in HEPES (100 mM) binding buffer with BSA (0.1 %), Triton X-100 (0.1 %), leupeptin (1 μM), and PMSF (2 mM). A Brandel cell filter (Biomedical Research and Development

Laboratories) is used to separate bound and free radioligand. A Tris-HCl (10 mM) polyethylene glycol (6.6 %) buffer is used to wash components through the apparatus onto glass filter papers, which are counted in a γ-counter for 1 min. Binding experiments are performed in duplicate in five separate experiments, and the specificity of binding is assessed in further experiments using unlabeled, unmodified BSA (1 μmol) as the competitor. Binding data were analyzed using a specific binding program (LIGAND).

Binding Assay with Radiolabeled RAGE

Human soluble recombinant RAGE (sRAGE) prepared as described above is radiolabeled with 125I by incubation with IODO-BEADS (Pierce) to a specific activity of ~4,000–5,000 cpm/ng protein. In all cases, after radioiodination, precipitation of the radiolabeled material in TCA exceeded 90 %. CML modifications of proteins or native, unmodified proteins are loaded onto the wells of plastic dishes (5 ng/well) in bicarbonate/carbonate buffer (pH 9.6) and incubated for 16 h at 4 °C. Material in the wells is aspirated, and unoccupied sites are blocked by incubation with PBS containing 1 % BSA for 2 h at 37 °C. Wells are washed twice with PBS containing 0.005 % octyl β-glucoside. Radioligand binding assay is performed in PBS containing 0.2 % BSA with increasing concentrations of 125I-human sRAGE alone or in the presence of a 50-fold molar excess of unlabeled human sRAGE or compounds/drug candidates for 2 h at 37 °C. At the end of that time, wells are washed rapidly five times with washing buffer. Elution of bound material is performed in a solution containing 1 mg/ml heparin. Solution is aspirated from the wells and counted in a γ-counter.

Evaluation

Equilibrium binding data are analyzed according to the equation of Klotz and Hunston (1984): B = nKA/1+ KA, where B indicates specifically bound ligand (total binding, wells incubated with tracer alone, minus nonspecific binding, wells incubated with tracer in the presence of excess unlabeled material), n indicates sites/cell, K indicates the dissociation constant, and A indicates free ligand concentration using nonlinear least squares analysis. Specific binding of CML-BSA to radiolabeled RAGE is further determined by subtraction of nonspecific binding (counts obtained upon binding of radiolabeled sRAGE to immobilized BSA) from that obtained upon binding of radiolabeled sRAGE to immobilized CML-BSA. In case of binding to immobilized BSA, counts are negligible and less than 10 % that observed in the presence of CML-BSA. Pretreatment with either antibodies, soluble RAGE, or the potential competitors and competitors/drug candidates is performed for 2 h prior to binding assay. Sometimes, it may be helpful to use materials eluted from the RAGE-Affi-Gel 10 columns (after passage of AGE-IgG) other than isolated RAGE domains for testing as unlabeled competitor in the binding assay. For this, human RAGE cDNA encoding the V, C1, or C2 domain is inserted into the EcoRI site of pGEX4T vector containing GST. Fusion proteins, V-GST, C1-GST, and C2-GST, are expressed in *E. coli*, purified on a glutathione-Sepharose column, and cleaved with thrombin (Amersham Pharmacia). RAGE domains are then purified to homogeneity using glutathione-Sepharose and characterized by SDS-PAGE and amino-terminal sequencing prior to testing in the radioligand binding assay.

Ligand and Immunoblotting Analysis

Cell membrane extracts (20 μg of membrane protein per lane) are subjected to nonreducing SDS (12–15 %) PAGE and electroblotted onto nitrocellulose membranes (Hybond, Amersham Pharmacia). For ligand blotting the membranes are blocked overnight at 4 °C in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.1 % Tween buffer containing 2.5 % BSA, before incubation with 125I-AGE-BSA (110 ng/ml) for 2 h at room temperature. After washing with 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.1 % Tween, the membrane is exposed to Kodak BioMax MS film for 1–4 h. Receptor-ligand-binding specificity is studied in competitive experiments where unlabeled AGE-BSA, AGE-RNase, BSA, and RNase (all 100 μg/ml) are added as competing ligands.

In immunoblotting experiments, the nitrocellulose membrane is incubated at room temperature for 1 h with a polyclonal goat Ab against human RAGE 1:2,000 (Soulis et al. 1997; Brett et al. 1993). Membranes are washed before a 15 min incubation with a biotinylated secondary antibody and a streptavidin horseradish peroxidase conjugate. Immunoreactivity is detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Primary antibodies are omitted in experiments as negative controls and RAGE antibody specificity is confirmed by preincubation of the membrane with recombinant RAGE before the addition of RAGE antibody.

Isolation of Human Serum Albumin and Immunoblotting for AGE

Plasma samples are obtained from human donors with diabetes, renal failure, or age-matched healthy controls in accordance with the laws of the respective national ethics committees. Samples (2 ml) are dialyzed for 16 h at 4 °C in 20 mM phosphate buffer (pH 7.1) in a total volume of 4 ml. After dialysis, samples are subjected to filtration (0.8 μm) and then chromatographed onto columns containing Affi-Gel blue resin (Bio-Rad) previously equilibrated in 20 mM phosphate buffer (pH 7.1). 5 ml of resin is employed per ml of plasma. The resin is washed in 2.5 column volumes of phosphate buffer as above, and human serum albumin is eluted by application of phosphate buffer containing 1.4 M NaCl. The absorbance at 280 nm for each fraction is determined, and positive fractions are determined by SDS-PAGE followed by staining with silver. For immunoblotting, the materials (30 μg of protein, each) are employed to SDS-PAGE gels (8 %). Simultaneously, marker proteins are added. After electrophoretic separation and transfer of the proteins to nitrocellulose membranes, the unoccupied sites on the membranes are blocked in the presence of non-fat dry milk (13.5 %) in TBS for 4 h at room temperature. Immunoblotting is performed using affinity-purified anti-CML IgG as above (10–20 μg/ml) in milk buffer (5 %) for 1.5 h at 37 °C. Membranes are washed extensively in TBS containing 0.1 % Tween 20. The membranes are incubated with goat anti-rabbit IgG labeled with horseradish peroxidase for 1 h at 37 °C, washed extensively in the above buffer. Visualization of antibody binding was performed employing the ECL detection system (Amersham Pharmacia). Quantitative evaluation of band intensity is performed using Molecular Dynamics/ImageQuant.

Measurement of Reactive Oxygen Species (ROS) Production

General Considerations

All forms of diabetes, both inherited and acquired, are typified by hyperglycemia, a relative or absolute lack of insulin, and the development of diabetes-specific microvascular pathology in the retina (retinopathy), renal glomerulus (nephropathy), and peripheral nerve (neuropathy). At the molecular cell biology level, three major hypotheses regarding the mechanisms by which hyperglycemia causes diabetic complications, i.e., the aldose reductase pathway (see above), formation of AGE (see above), and activation of PKC, have each generated a great deal of supporting data (Brownlee 1995; Koya and King 1998; Schmidt and Stern 2000b). Seminal work from Michael Brownlee's laboratory has shed new light on the mechanisms by which elevated concentrations of glucose perturb cellular properties in a fundamental way, thereby joining these three pathways into a unifying hypothesis for hyperglycemia-induced late complications (Nishikawa et al. 2000). In endothelial cells subjected to physiologically relevant glucose concentrations as a model system, the non-insulin-dependent uptake of glucose via GLUT1 leads to oxidative stress (OS) with concomitant generation of reactive oxygen species (ROS), the increase of both is positively correlated to the glucose concentration. Several pathways are considered as likely candidates for the emergence of OS and the formation of oxygen free radicals in cells, including the accelerated flux of glucose through glycolysis and feeding of pyruvate, thus formed, to the tricarboxylic acid cycle, which overloads mitochondria and thereby

causes excessive generation of ROS. In turn, OS and ROS have recently been shown to cause upregulation of the mitochondrial uncoupling protein 2 (UCP-2), leading to a vicious cycle of increased electron flux via the respiratory chain and concomitant elevated production of ROS. Most importantly, it has been demonstrated recently that suppression of intracellular ROS, using low molecular weight inhibitors or by expression of the antioxidant enzyme, manganese-dependent superoxide dismutase (MnSOD), prevents each of the three molecular mechanisms mentioned above (Nishikawa et al. 2000). Consequently, glucose-induced formation of oxidants is a proximal step in cellular perturbation prevalent during diabetes. Thus, assays which monitor the generation of OS and ROS in response to compounds/drug candidates may be helpful for the finding and characterization of future antidiabetic drugs, which may reduce the risk for the development of diabetic late complications.

Purpose and Rationale

Although there are various methods to assess oxidative damage of cells, such as measuring lipid peroxidation products and DNA adducts (Holley and Cheeseman 1993), none of them evaluate the OS directly. With the first description of using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorometric assay for hydrogen peroxide (Keston and Brandt 1965), it became popular to use dichlorofluorescin (DCFH) as a probe to evaluate intracellular hydrogen peroxide formation by flow cytometry. The theory behind using DCFH-DA is that nonfluorescent fluorescein derivatives will emit fluorescence after being oxidized by hydrogen peroxide (LeBel et al. 1992). The emitted fluorescence is directly proportional to the concentration of hydrogen peroxide. When applied to intact cells, the nonionic, nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH (LeBel et al. 1992; Bass et al. 1982). In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) (LeBel et al. 1992). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall OS in cells.

Many studies have used fluorescent microscopy to quantify OS in cells using DCFH-DA. This method has the problem of inducing photooxidation of DCFH to DCF intracellularly and emitting fluorescence, because it is difficult to control the time of light exposure when trying to locate and focus cells under the microscope. In addition to the problem of photooxidation, there is no standard way to quantify the OS using a microscope. In order to prevent the overestimation of the OS due to photooxidation, an instrument equipped with fast light excitation and fast fluorescence capturing, which will not induce excessive photooxidation, is needed. Wang and Joseph (1999) have introduced a fluorescent microplate reader-based method to evaluate OS in cultured cells, induced by applying various free radical generators extracellularly, using DCFH as the probe.

Procedure

Measurement of OS

Cells (e.g., PC12) are grown in growth medium containing 85 % RPMI-1640 with L-glutamine, 10 % heat-inactivated horse serum, 5 % FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cells are maintained in collagen-coated plates in 5 % CO_2/95 % air at 37 °C. The culture medium is changed twice every week and the cells are split 1:4 or 1:8 every week. Cells were split and counted by trypan blue exclusion. Viable cells (104/well) are plated into 96-well collagen-coated plates 1 day before the experiments. On the day of the experiments, after removing the medium, the cells in the plates are washed with KRH buffer and then incubated with 100 μM 6-carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes Inc., USA) in the loading medium in 5 % CO_2/95 % air at 37 °C for 30 min. For loading the cells with DCFH, DCFH-DA from a 10 mM stock solution in DMSO is mixed with loading medium

(99 % RPMI-1640 and 1 % FBS) to a final concentration of 100 μM. After DCFH-DA is removed, the cells are washed and incubated with KRH buffer (with different concentrations of one of the free radical generators, e.g., H_2O_2, dopamine) in the absence or presence of high glucose concentration. The fluorescence of the cells from each well is measured and recorded.

For OS measurement, DCFH-DA-loaded cells are placed in a CytoFluor Series 4000 multiwell fluorescence plate reader with temperature maintained at 37 °C. The excitation filter is set at 485 nm and the emission filter is set at 530 nm. The fluorescence from each well is captured, digitized, and stored on a computer using CytoFluor (Version 4.0) (PerSeptive Biosystems Inc., USA). Data points are taken every 5 min for 30 min and the data are exported to Excel (Microsoft, USA) spreadsheet software for analysis.

Measurement of ROS

For measurement of ROS production according to the procedure of Freeman and coworkers (2006), a 10 mM stock solution of H2DCFDA-SE (2′,7-′-dichlorofluorescein diacetate with a succinimidyl ester group, DCF, Molecular Probes) is prepared in DMSO, stored at −70 °C, and diluted with KRB (pH 7.0) just before use to a final concentration of 10 μM. The cells are plated in 24-well cell culture plates overnight. They are then washed twice with PBS, incubated in PBS plus DCF for 30 min at 37 °C, washed with PBS, and incubated in KRB containing 5.5 or 20 mM glucose for a further 30 min. They are imaged at room temperature using an IonOptix fluorescence system, with 495 nm excitation and 520 nm emission. The background fluorescence is subtracted. 0.5 mM menadione is used as a generator of ROS as a control. The ROS concentrations are determined from a standard curve of H_2O_2 (5–50 μM). To confirm that glucose generates ROS, dihydroethidium (DHE) conversion to ethidium by oxidation can be measured according the procedure of Russell and coworkers (2002). The ratio of DHE/ethidium is determined using a fluorimeter and standardized against cellular protein. DHE is measured using 355 nm excitation and 430 nm emission, and ethidium is measured using 518 nm excitation and 605 nm emission.

Measurement of Mitochondrial ROS Production

To evaluate the direct production of mitochondrial ROS in cells, the DCFDA measurements have to be combined with ROS-specific staining by using the reduced MitoTracker Red probe (CM-H2XRos; Molecular Probes Inc., USA) according to the procedure described by Degli Esposti and coworkers (1999). For this, the cells suspended in growth medium at 1 × 106/ml are incubated for 15 min at room temperature with freshly prepared CM-H2XRos (0.5 μM), then washed twice with PBS, and collected on a slide using a cytospin apparatus (Wolvetang et al. 1994). The cells are fixed with 3.7 % formaldehyde in PBS, followed by washing first with PBS containing 30 mM NH4Cl and then with distilled water. In some experiments, the fixed cells are counterstained with Hoechst 33342 before mounting with anti-fade medium, and the slides are stored at 4 °C in the dark. In parallel experiments, mitochondrial staining to analyze membrane potential can be performed using 100 nM oxidized MitoTracker Red (CM-XRos) following the same protocol as outlined above for CM-H2XRos. Confocal microscopy is undertaken with a krypton/argon instrument, usually with a 40 × oil objective and intermediate laser and photomultiplier voltage. The 590 nm bandpass filter is used to detect the red fluorescence of CM-XRos staining (Wolter et al. 1997). Epifluorescence is evaluated with a microscope using a Texas Red filter and a CCD camera attached to a microcomputer image analyzer.

Evaluation

The percentage increase in fluorescence per well is calculated by the formula [(Ft30 – Ft0)/Ft0 ∗ 100],where Ft30 = fluorescence at time 30 min and Ft0 = fluorescence at time 0 min. This method of analysis has advantages over analyzing just the net change in fluorescence in that not

only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for variability among wells. This method also cancels out the background fluorescence in each well, and therefore, a "no cell" control is not needed.

Various free radical generators produce concentration-dependent changes in DCF fluorescence, indicating the indiscriminate nature of DCF. Due to the indiscriminate nature of DCFH, which can be oxidized by various ROS and not just H_2O_2, the increase of intracellular DCF fluorescence does not necessarily reflect the levels of ROS directly, but rather an overall OS index in cells. Quantifying cellular OS by the DCF assay using a fluorescent microplate reader is an easy and efficient method with low variability which can be used to quantify the potency of pro-oxidants or can be adapted to evaluate the efficacy of antioxidants against ROS in various cell lines. The use of a 96-well microplate reader enables the generation of a large amount of data with low variability.

Measurement of Mitochondrial Size

Since glucose-induced ROS production and swelling of mitochondria seem to be correlated (Russell et al. 2002), determination of alterations in the size of mitochondria exposed to OS may be indicative for the generation of mitochondrial ROS. For measurement of the mitochondrial size, cells are loaded with MitoTracker orange (CMTMRos; Molecular Probes Inc., USA) using confocal microscopy with 540 nm excitation. Each mitochondrion is a complex fractal-like network, making accurate analysis of volume difficult. Therefore, the maximum cross-sectional area is determined from identification of each individual mitochondrion throughout the z-series. All distinguishable mitochondria at each step level in the z-series are measured at the largest cross-sectional area of the mitochondrion. The size of the mitochondria is determined using the perimeter of visible CMTMRos and reflects the mitochondrial area at the measured level. Identical methods of identification and measurement are applied to each mitochondrion in each cell and in each condition, thus reducing the effect of technical artifact. The mitochondrial areas are then averaged for each neuron, and, where necessary, results are first converted to a log normal distribution before obtaining descriptive statistics or performing tests of inference about the data set. Results are obtained for >100 mitochondria in at least 10 randomly measured neurons per condition.

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Communication Between Metabolically Relevant Cells

Purpose and Rationale

Most eucaryotic cell types manage to store energy in lipid droplets (LD) in case of nutritional abundance (van den Bosch 1974; Coleman et al. 2000; Wang et al. 2010a). However, intracellular energy stores need to be restricted in size, since overloading with lipids could impair cellular functions (= lipotoxicity)(DeFronzo 2004; Chavez and Summers 2010). In mammals, adipocytes and adipose tissues have evolved to specifically store and handle lipids without being damaged by lipotoxic cell injury. White adipocytes are characterized by a unique morphology containing one or a few large LD that occupy almost the entire cell volume and thereby determine the cell size. The currently prevalent idea argues that as long as nutrient excess can be efficiently sequestered in LD of white adipocytes, non-adipose tissues are prevented from lipid "overspill" and lipotoxic damages (Danforth 2000; Unger 2003; Le Lay and Dugail 2009). The regulation of LD and adipocyte growth in the course of ongoing in and out trafficking of fatty acids, in particular within adipose tissue depots with adipocytes of different size, and its potential as a target for pharmacological interference (Langin 2006; Pilch and Bergenheim 2006; Pfeiffer 2007) remain to be elucidated.

Metabolic overload is a paramount problem for people in industrial countries in which the caloric intake exceeds the dietary needs, due to associated risks of secondary disorders including obesity, hepatic steatosis, chronic inflammation, hypertension, type 2 diabetes (T2D), atherosclerosis, atherothrombosis, and neurodegeneration. It is estimated that more than 50 % of the population in developed countries can be regarded as obese according to WHO standards. Moreover, obesity is the entry gate of T2D and most of the abovementioned secondary disorders. Despite many of the secondary disorders are cell type and organ specific, there is overwhelming information that the blood plasma compartment and

other body fluids contain many components that orchestrate distant and paracellular events in disease initiation and progression. Among these, metabolites of energy metabolism (e.g., carbohydrates, fatty acids), cytokines, chemokines, adipokines, and hepatokines (Lehr et al. 2011; Fasshauer and Blüher 2015; Famulla et al. 2011; Trayhurn et al. 2011) are well-established culprits for vascular and metabolic disease and associated low-level chronic inflammation and autoimmunity in the elderly ultimately leading to myocardial infarction, stroke, dementia, and organ failure of the kidney, heart, liver and the neural system.

Organization of Adipocytes into Tissue Structures

During embryonic development, adipose tissue depots (= fat pads) arise from vascularized and innervated spherical clusters of adipocytes and differentiating preadipocytes (i.e., as opposed to being randomly distributed throughout the body), which are derived from the so-called stromal vascular cells (SVC), in the mesenchyme (Brown et al. 1997). The organization of these "primitive organs" may be actively maintained and stabilized through cell-to-cell and cell-matrix interactions. This idea is supported by the observation that adhesion molecules and matrix components expressed by preadipocytes change during differentiation into adipocytes (Torri et al. 2010; Wang et al. 2010b). During subsequent adipose tissue morphogenesis, newly differentiated or differentiating adipocytes give rise to cellular extensions, migrate (from the stroma), form cell-to-cell junctions, and organize themselves into these spherical clusters with sufficient extracellular, i.e., interstitial, space to organize a surrounding basal lamina. At an early stage the formation of these "primitive organs" must also involve the release of "information," i.e., soluble or vesicular factors, needed for the initiation of vascularization, angiogenesis, inflammatory responses, and innervation (Hazen et al. 1995), which lead to the formation of endothelial cells, blood vessels, macrophages, and nerve cell extensions.

From an experimental point of view, adipose tissue depots represent a complex mixture of SVC, (pre)adipocytes, fibroblasts, endothelial cells, and macrophages, which typically are dissociated by collagenase digestion and subsequent low-speed centrifugation (flotation). Thereby mature adipocytes floating at the top of the centrifugation tube are separated from the sedimenting non-adipose tissue cells, such as SVC, fibroblasts, endothelial cells, and macrophages. Importantly, on the basis of this method, the recovery and identification of immature and differentiating preadipocytes as well as of (very) small adipocytes will depend on their specific (buoyant) densities relative to those of the non-adipose tissue cells, which is critically affected by the type and degree of LD biogenesis in these (pre)adipocytes. In consequence, early precursor cells of adipocytes as well as very small adipocytes within adipose tissue depots, which per definition lack any LD, should be recovered together with the fraction of non-adipose tissue cells.

Heterogeneity of Adipocytes in Size and Function

During adipose tissue morphogenesis, the upregulated LD biogenesis triggers increase in adipocyte size, as is manifested in the positive correlation between lipid mass and adipocyte volume in both subcutaneous adipose tissue, which represents about 80 % of total body lipid, and visceral adipose tissue, which has a strong link to metabolic diseases (Björntorp 1974; Hirsch and Batchelor 1976). Furthermore, upon major weight loss or gain, adipocyte volume but not number is found to be significantly reduced and elevated, respectively (Sims et al. 1968; Björntop et al. 1975; Häger et al. 1978; Spalding et al. 2008).

Very recently, distinct responses of different adipose tissue depots to diet-induced increases in total body fat in lean healthy adults are reported on the basis of the analysis of authentic adipocytes (Tchoukalova et al. 2010). Depending on the fat depot (abdominal vs. femoral), an increase in total fat mass is attributed to adipocyte hypertrophy (abdominal) or hyperplasia (femoral). Interestingly, the observed decrease in the average size of adipocytes (to less than before the overfeeding started) from females who have large adipocytes

to start with, in the abdominal depot, was proposed to rely on hyperplasia. Thus when controlled for body fat mass, in a subset of the population adipogenesis (or at least the occurrence of small adipocytes) seems to be fostered. No depot differences in preadipocyte replication or apoptosis that would explain lower-body adipocyte hyperplasia and abdominal subcutaneous adipocyte hypertrophy is found. However, basal levels of a subset of typical transcription factors for adipocyte differentiation turn out to be significantly elevated in abdominal compared to femoral subcutaneous preadipocytes, consistent with the ability of abdominal subcutaneous adipocytes to achieve a larger size. Consequently, it is argued that inherent differences in preadipocyte cell dynamics may contribute to the distinct responses of different adipose tissue depots to overfeeding, and adipocyte number increases in certain depots in adults after only a relatively short period of increased food intake (Tchoukalova et al. 2010).

These findings are also consistent with earlier data in rats (Faust et al. 1978) and humans (Kashiwagi et al. 1985) showing that adipocytes manage only to enlarge to a finite degree in the setting of short-term overfeeding, which, however, results in the disappearance of small adipocytes. Consequently, it is concluded that attainment of an upper mean size triggers differentiation, growth, and lipid storage of a pool of very small and normally undetectable immature (precursor) adipocytes (Faust et al. 1978). As such, in the setting of increased nutrient uptake and lipid storage demands, if certain individuals are unable to mature a new population of adipocytes owing to an impairment in their maturation and LD biogenesis, lipids would be forced to accumulate and exert lipotoxic injuries in non-adipose tissues, as has been reported in insulin-resistant subjects (Savage et al. 2005). Importantly, the adipocytes do not increase in size in a synchronized fashion, even within the same adipose tissue depot, in both rodents and humans. Instead, they can be divided into small (diameter <50 μm) and large (diameter >100 μm) cells accompanied by profound differences in lipid storage, expression of major genes of lipid and glucose metabolism, and secretory function (Holm et al. 1975; Cushman and Salans 1978; Blüher et al. 2002). Moreover, the well-known physiological differences between adipocytes from different white, i.e., visceral vs. subcutaneous, adipose tissue depots (Rebuffe-Scrive et al. 1989) may result from differential compositions of small and large adipocytes.

Recently, the size distribution of cells constituting the adipose tissue depot (see above) and in the following called adipose tissue cells, which had been derived from equally obese, otherwise healthy subjects exhibiting the full range of physiological insulin sensitivity/resistance, is re-investigated using a method avoiding collagenase digestion of the adipose tissue and counting of the cells with a Coulter counter (McLaughlin et al. 2007). A population of very small adipose tissue cells becomes detectable. Surprisingly, 20–26 % of the total adipose tissue cells are of very small size (diameter 20–30 μm) in lean as well as obese donor subjects and remain constant in size and proportion irrespective of the body mass index with the ratio of very small vs. large cells being higher for insulin-resistant compared to insulin-sensitive subjects (McLaughlin et al. 2010). It is tempting to speculate that intrinsic limitations in the number of adipose tissue cells could account for why some individuals have a different ratio between this minor subclass of very small cells, which could actually represent the precursor of adipocytes, albeit this remains to clarified, and the major class of large adipocytes. According to recent experimental evidence, the cellular morphology of adipose tissue, which should be critically affected by this ratio, is determined by the generation rate of the constituting adipocytes (Spalding et al. 2008; Arner 2010). A low generation rate of adipocytes results in hypertrophy and a high generation rate in hyperplasia as has been elucidated by retrospective radiocarbon analysis.

These findings can also be reconciled with interesting data on preadipocyte availability. The proportion of SVC constituting the subcutaneous adipose tissue that are committed preadipocytes according to the body fat distribution phenotype with preadipocytes of upper-body-obese women

seem to exhibit reduced differentiation and are more prone to apoptosis than preadipocytes isolated from adipose tissue depots of lower-body-obese or lean women (Tchoukalova et al. 2007). The resulting concept that body fat distribution may be regulated partly through differences in early adipogenesis has recently been extended to take into consideration the fact that dimensions of adipocytes during obesity may result in their disruption due to physical and geometrical restraints as has been suggested on the basis of the strong positive correlation between adipocyte size and rupture upon exposure to common physical forces (Monteiro et al. 2006). The commonly observed appearance of macrophages around dead adipocytes (Cinti et al. 2005) probably initiates low-grade chronic inflammation. Consequently, it is suggested that the increase in the ratio of adipocytes hyperplasia and hypertrophy may lower the inflammatory complications of obesity (Monteiro et al. 2007).

However, in contrast to the above study, which led to the apparent identification of very small adipose tissue cells proposed, but not demonstrated, to represent preadipocytes and was based on the analysis of all adipose tissue cells with diameter >20 μm that did not float upon low-speed centrifugation (McLaughlin et al. 2010), the investigation of pure authentic human adipocytes did not reveal a bimodal distribution of cell size, which should have resulted in an exponential tail of very small adipocytes (from 20 to 55 μm) despite the use of a smallest cutoff size of 35 μm (Tchoukalova et al. 2010). Thus, it will be of tremendous importance to unambiguously determine the nature of these very small human adipose tissue cells, i.e., whether they really represent very small adipocytes. Irrespective of the outcome, i.e., whether they represent adipocytes or non-adipocyte adipose tissue cells, the recent results obtained with the analysis of all small adipose tissue cells (McLaughlin et al. 2007, 2010) seem to indicate that insulin resistance in obese individuals is associated with an expanding population of (very) small adipose tissue cells which differentiates them from equally obese subjects who are insulin sensitive (McLaughlin et al. 2004; Hauner 2010). This view does not necessarily contradict the previously introduced paradigm of insulin resistance in obese subjects relying on drastically increased numbers of large adipocytes which is in accordance with the majority of studies published during the last four decades (e.g., Salans et al. 1968; Stern et al. 1972). Moreover, very recently, this paradigm was reevaluated with women of the same body fat mass but characterized by either (relative) adipocyte hypertrophy or hyperplasia (Arner 2010). Not unexpectedly, the former exhibited higher plasma insulin levels and lower insulin sensitivity compared to the latter. It is thus conceivable that progression of insulin resistance is the consequence of either the failure of a subset of very small adipocytes to mature into fully differentiated lipid-storing adipocytes or the inability of very small non-adipocyte adipose tissue cells to store lipids, even after eventual further differentiation and maturation. Actually, this sequence of events may accompany or follow the accumulation of large adipocytes with exhausted lipid storage capacity. The simultaneous existence of large adipocytes and (very) small (adipocyte or non-adipocyte) adipose tissue cells may explain the correlation between insulin resistance and adipocyte hypertrophy as delineated in most studies (Arner 2010). Furthermore, the apparent unsynchronized growth of large and small adipocytes, even within the same adipose tissue depot, argues against simple filling-up of nascent adipocytes with lipids (Le Lay et al. 2008). Consequently, it is tempting to speculate about signals sent by large adipocytes to order small adipocytes, but apparently not non-adipocyte adipose tissue cells, the takeover of the burden of lipid loading. Three types of signals seem to control the (para- and/or endocrine) communication between adipocytes.

Communication Between Adipocytes via Cell-to-Cell Contacts

Adhesion molecules are apparently involved in adipocyte-matrix and adipocyte-to-adipocyte contacts. Basement membrane components stimulate the migration and organization of isolated primary adipocytes in culture into three-dimensional multicellular spherical clusters

(Hazen et al. 1995). This organization is associated with the formation of adipocyte-to-adipocyte contacts as well as migration through and remodeling of the original extracellular matrix, which is presumably provoked by secretion of MMP-2, a metalloendoprotease of the matrixin family, from the adipocytes (Brown et al. 1997). Extensive junctions between the newly differentiated adipocytes are observed in the early stages of cluster formation before large unilocular LD occluded their cytoplasm (Wasserman 1965). The formation of gap junctions (at a functional syncytium) at these adipocyte-to-adipocyte contacts cannot be excluded. Furthermore, spherical clusters in vitro and adipose tissue depots in vivo are characterized by elaborate intercellular tubular extensions, molding of restricted plasma membrane areas, as well as filopodia- and lamellipodia-like structures (Benelli et al. 1994; Cornelius et al. 1994). The directed nature of these cell-to-cell contacts (i.e., random formation is not observed, in general) and the pattern of migration of the adipocytes (i.e., directed toward preexisting or forming clusters) strongly imply that chemoattraction (Han et al. 2010) and/or pathway guidance is involved in adipose tissue morphogenesis as well as for the coordination of lipid storage between mature, i.e., large, and nascent, i.e., small, adipocytes.

Communication Between Adipocytes via Soluble Factors and Adipokines

Collectively, adipose tissue depots and adipocytes constitute the body's largest endocrine organ, producing an array of peptide hormones, called adipokines of pro- and anti-inflammatory nature, and soluble factors of different nature and activity (Kershaw and Flier 2004; Hauner 2005; Wang et al. 2005; Kiess et al. 2008; Klimcakova et al. 2010). Initially, the possibility for involvement of soluble factors and adipokines in inter-adipocyte communication has been raised by a number of studies that revealed association of substantial changes in their secretion and increases in cell size (Abbasi et al. 2004). Apparently, the adipocyte size, i.e., the mean adipocyte volume, even after correction for differences in cell surface area, is a critical parameter for the differential expression and secretion of adipokines (Guo et al. 2004; Skurk et al. 2007).

Among the very recent examples for a soluble factor is cyclic phosphatidic acid (cPA) which has been shown to bind to PPARγ with nanomolar affinity and antagonizes its activation by synthetic or naturally occurring agonists (Tsukahara et al. 2010). Under physiological conditions, PPARγ bound to agonists, such as 15-keto-PGE2, positively regulates gene expression by binding as a heterodimer with RXRs to PPAR response elements in the vicinity of adipogenesis target genes. cPA has been detected in human serum, but the pathway leading to its production in mammalian cells, in particular adipocytes, remains poorly defined. It is assumed that cPA is produced from lyso-phosphatidylcholine by action of phospholipase D2 (Tsukahara et al. 2010). Interestingly, lyso-phosphatidylcholine is generated by phospholipase A2 from phosphatidylcholine, which simultaneously liberates a polyunsaturated fatty acid which serves as substrate for the production of 15-keto-PGE2. It will be important to determine whether these putative PPARγ agonists/antagonists are released by adipocytes into the interstitial space of adipose tissue and/or into the circulation and whether the release is dependent on the cell size. Apparently, cPA and 15-keto-PGE2 manage to pass the adipocyte plasma membrane and could operate as signals between adipocytes with the functional outcome depending on their relative production and thus balance between agonist and antagonist under the control of the cell size.

A number of adipokines have been shown to affect lipid metabolism, in particular lipid synthesis, lipolysis, and LD biogenesis, upon direct treatment of adipocytes. Acylation-stimulating protein (ASP) is a potent stimulator of lipid synthesis in adipocytes in vitro (Baldo et al. 1993) and is released by adipose tissue in vivo in the postprandial period, when net fatty acid uptake by the tissue occurs (Saleh et al. 1998). Tumor necrosis factor-α (TNF-α) blocks free fatty acid uptake by adipocytes (Cawthorn and Sethi 2008) and stimulates lipolysis through downregulation of the LD-associated protein perilipin, leading to exposure of LD to lipases, and suppression of

the anti-lipolytic G-protein Gαi (Ryden and Arner 2007). Interleukin-6 (IL-6) increases basal as well as isoproterenol-stimulated lipolysis in 3T3-L1 (Petersen et al. 2005) and in human mammary adipocytes (Päth et al. 2001). Chemerin acts as an inhibitor of both basal and stimulated lipolysis in 3T3-L1 adipocytes, possibly via a reduction in intracellular cAMP levels (Goralski et al. 2007), and enhances lipogenesis in 3T3-L1 adipocytes, possibly by upregulation of insulin-mediated insulin receptor substrate-1 phosphorylation (Takahashi et al. 2008). Vaspin and omentin activate lipogenesis in rat adipocytes, presumably via induction of insulin-stimulated glucose uptake and Akt phosphorylation (Yang et al. 2006; Rabe et al. 2008). Adiponectin (full-length and globular) inhibits insulin-stimulated lipogenesis in pig adipocytes (Jacobi et al. 2004). Leptin induces lipolysis and loss of lipid stores and interferes with insulin inhibition of lipolysis in mouse and rat adipocytes (Frühbeck et al. 1997; Müller et al. 1997). In human adipocytes, however, from either lean or obese healthy subjects, leptin treatment fails to affect (basal, isoproterenol-stimulated or insulin-inhibited) lipolysis (Elimam et al. 2002). It is known that high-circulating leptin levels in obesity are not effective in body weight regulation due to the accompanying leptin resistance. However, induction of hyperleptinemia in leptin-sensitive lean rats results in rapid depletion of lipid stores. In addition, constitutive overexpression of the leptin receptor in adipose tissue, which overcomes leptin resistance, prevents high-fat-induced obesity. At a molecular level, this is accompanied by induction of the typical leptin signaling pathways (STAT-3), activation of AMPK and PGC-1 α, and upregulation of UCP-1 and UCP-2 in white adipose tissue (Wang et al. 2005). Data from a transgenic model suggest that leptin impairs fatty acid re-esterification in the adipose tissue and thus leads to elevated fatty acid release (Pravenec et al. 2008).

Very recently, the involvement of soluble glycoproteins of the Wnt family in the control of insulin-driven adipogenesis has been proposed (Ouchi et al. 2010). Wnt5a is expressed in adipose tissue and presumably secreted from adipocytes. It signals through the cell surface receptor "frizzled" (Fz) to stimulate the noncanonical signaling pathway leading to activation of the guanosine triphosphatases, RhoA and Rac1, and in the course of the serine-threonine kinase, c-Jun amino-terminal kinase 1 (JNK1)(Staal et al. 2008). In adipocytes JNK1 mediates inflammation and blockade of insulin-stimulated lipogenesis and adipogenesis (Kawano and Kypta 2003). Importantly, expression of secreted frizzled-related protein five (Sfrp5) that is released from adipocytes and sequesters Wnt5a in the extracellular environment, thus preventing activation of Fz and attenuating noncanonical Wnt signaling, is found to be decreased in various rodent models of obesity (Ouchi et al. 2010). When put on a high-fat diet, mice genetically engineered to lack Sfrp5 showed greater adipose tissue inflammation and insulin resistance, presumably due to impaired adipogenesis, which all relies on unrestrained Wnt5a activity. This phenotype is prevented by overexpression of Sfrp5 in adipocytes of these animals or systemic administration of Sfrp5. Apparently, the balance between secretion of the adipokines, Wnt5a and Sfrp5, determines noncanonical Wnt signaling in adipocytes and thereby controls the efficacy of (insulin-stimulated) LD biogenesis and adipogenesis. Thus, Wnt5a and Sfrp5 secreted in concert into the interstitial adipose tissue spaces may mediate communication between adipocytes via paracrine and/or autocrine mechanisms (Christodoulides et al. 2008).

Adipocyte hypertrophy per se has been reported to be related to insulin resistance in T2D patients independent of total body fat (Lundgren et al. 2007) via, in part, the decreased secretion of a subset of adipokines, such as adiponectin (Hotta et al. 2001). Dietary weight loss combined with exercise is found to improve insulin sensitivity, decrease adipocyte size, and increase plasma adiponectin concentrations in obese individuals (Shadid and Jensen 2003). Furthermore, large and small adipocytes prepared from the same subjects/animals differ in terms of adipokine secretion (Skurk et al. 2007) and basal lipolysis (Wueest et al. 2007). Most interestingly, 13% weight loss in T2D patients during a 1-year

lifestyle intervention study is accompanied by 46% increase in insulin sensitivity in parallel with 36% elevation in plasma adiponectin levels (but no alterations in the proinflammatory cytokines, TNF-α and IL-6). Since the mean adipocyte size decreases mainly due to changes in the large subcutaneous abdominal adipocyte subfractions (size 0.75–0.44 μl, relative number 19–26 %), it is proposed that large adipocytes may contribute to improvement in insulin sensitivity via upregulated adiponectin secretion (Pasarica et al. 2009). Importantly, this correlation, which does not necessarily imply causal relationship, could not be obtained by measurement of the mean adipocyte size, only. Thus, serum adiponectin concentration increases in parallel with a decrease in size of large adipocytes, suggesting that the decreased size may contribute to improved insulin sensitivity via adiponectin (Pasarica et al. 2009). It has been shown that lipolysis is upregulated with exercise (Despres et al. 1984) and that larger adipocytes respond to lipolytic stimuli more so than smaller ones (Wueest et al. 2007). This may explain the reduction in size predominantly of the large adipocytes during weight loss.

The negative correlation between cell size and adiponectin secretion as elucidated recently (Pasarica et al. 2009) appears to contradict previous data (Skurk et al. 2007) that adiponectin secretion from adipocytes is directly proportional to the cell size (i.e., larger adipocytes release more adiponectin than small ones). It is, however, possible that the increased number of the large adipocytes (the relative percentage of which is elevated, whereas the total adipocyte number does not seem to have been altered during weight loss (Hirsch and Knittle 1970)), even if smaller in size, may explain the higher adiponectin levels. Thus, large adipocytes reduced in size may keep a higher capacity for adiponectin secretion. This hypothesis remains to be tested in vitro and in overfeeding studies. In murine models, overfeeding causes a remodeling of adipose tissue with increased macrophage content, the key source of proinflammatory cytokines in adipose tissue, which gathers mostly around hypertrophic adipocytes (Cinti et al. 2005). Future studies of weight gain by overfeeding rather than weight loss will indicate whether the expansion of adipose tissue is associated with increased macrophage infiltration and increased circulating cytokines.

In conclusion, it is widely assumed that the increase in cell size leads to a shift in the pattern of secreted adipokines in both rodent and humans predominantly as a result of dysregulation of hypertrophic large adipocytes. On the basis of the opposing effects of these adipokines on lipid synthesis, lipolysis, and insulin action, their final overall effect on adipogenesis, adipose tissue morphogenesis, inter-adipocyte communication, and size regulation critically depends on the fine-tuning of their individual activities, which is difficult to predict from the in vitro data alone. Possibly, systems biology may facilitate the recognition of adipokine patterns associated with adipocytes of different size and degree of maturation which could be derived from adipose tissue depots of obese humans before and after weight loss. Moreover, studying the adipocyte size distribution offers some advantages over the mean adipocyte size in understanding potential mechanisms that link adipocyte size with insulin sensitivity via adipokine secretion and lipolysis.

Communication Between Adipocytes via Exosomes and Microvesicles (EMVs)

There is accumulation evidence that EMV levels and components in plasma may relate to vascular and metabolic diseases and other diseases of the elderly. EMV components orchestrate immunomodulation, autoimmunity, tissue remodeling, the interplay of cells in angiogenesis, cell proliferation, and apoptosis/survival. EMVs are heterogeneous organelles with a different size and are composed of an array of macromolecules derived from the cells of origin including proteins, lipids, nucleic, acids, and other metabolites. EMV components influence cell signaling and transport and drive epigenetic modulation, metabolic memory, and immunometabolism in cells and organs. The size heterogeneity of EMVs directly relates to their release mechanisms. At least three mechanisms have been proposed for the constitutive and regulated release of EMVs from cells: (i) exocytic fusion of multivesicular bodies resulting in

exosomes, (ii) budding of vesicles directly from plasma membrane microdomains (lipid rafts) resulting in shed microvesicles (ectosomes), and (iii) release of apoptotic bodies from plasma membrane blebs during cell death. While the first two mechanisms are energy-requiring properties of viable cells, apoptotic body formation does not require ATP.

EMVs exhibit overlapping similarities in size (dynamic light scattering, flow cytometry, atomic force microscopy, impedance microscopy), morphology (electron microscopy), density (sucrose gradient centrifugation), protein markers (immunoblotting and mass spectrometry), lipid species (mass spectrometry), nucleic acids (gene arrays, TaqMan, deep sequencing), and mechanism of secretion (single-molecule tracking, fluorescence imaging). EMVs float within a density between 1,13 and 1,17 g/ml forming up to five distinct density fractions with a diameter ranging between 50 and 200 nm (exosomes), 0.1–1 μm (microvesicles), and 1–5 μm (apoptotic bodies). The increased release of EMVs and their accumulation in body fluids including plasma, liquor, urine, and interstitial fluids (e.g., ascites, pleural fluids) is directly related to cell activation and multiple diseases. While the exact mechanisms of EMV release remain still obscure, their release is modulated by receptor-dependent extracellular signals. Exocytosis can be either constitutive (Ca^{2+} independent) or regulated (Ca++ dependent).

Recently, the release of EMVs (by plasma membrane shedding or exocytosis of multivesicular bodies) from primary rat and cultured mouse adipocytes has been reported (Aoki et al. 2007). These EMVs harbor specific subsets of luminal proteins, transmembrane proteins, and GPI-anchored proteins (Müller et al. 2009a). GPI-anchored proteins with their protein moiety typically facing the outer face of the plasma membrane become co-translationally modified at their carboxy-terminus with an amide-linked glycolipid structure consisting of phosphatidylinositol, a complex carbohydrate glycan core and a phosphodiester-ethanolamine bridge (Nosjean et al. 1997). EMV release from rat adipocytes is found to become upregulated upon challenge with certain stimuli, such as hormones (e.g., insulin); fatty acids (e.g., palmitate); the antidiabetic sulfonylurea drug, glimepiride; or reactive oxygen species, e.g., hydrogen peroxide (Aoki et al. 2007). Curiously, prior to their release the GPI-anchored proteins, Gce1 and CD73, which cooperate in the degradation of cyclic adenosine monophosphate (cAMP) via AMP to adenosine, are translocated from the plasma membrane to cytoplasmic LD (Müller et al. 2008a, b, c, d) and only then incorporated into the EMVs (Müller et al. 2009b). LD consist of a central core of neutral lipids, such as triacylglycerol and cholesterylesters, and are surrounded by a phospholipid monolayer with inserted GPI-anchored proteins, such as Gce1 and CD73, and a coat consisting of structural and regulatory so-called PAT (perilipin, adipophilin, TIP47) and non-PAT proteins (Martin and Parton 2006; Le Lay and Dugail 2009). Importantly, the release of CD73 and Gce1 into EMVs critically depends on the cell size with large rat adipocytes being significantly more efficient than small ones (Müller et al. 2010a).

Surprisingly, it was found that EMVs released from rat adipocytes transfer their constituent GPI-anchored proteins, Gce1 and CD73, to plasma membranes of acceptor adipocytes which following challenge with palmitate, glimepiride, or hydrogen peroxide are translocated to LD resulting in inhibition of lipolysis and stimulation of esterification (Müller et al. 2010a). Small adipocytes are more efficient than large ones in acting as acceptor cells for translocation of EMV-derived GPI-anchored proteins from plasma membranes to LD (Müller et al. 2010b) as well as in being responsive to EMV-regulated lipid metabolism (Müller et al. 2010a). Esterification stimulation and lipolysis inhibition by palmitate, glimepiride, and hydrogen peroxide turned out to be more prominent in mixed populations of small and large rat adipocytes compared to those of uniformly small or large adipocytes (Müller 2010; Müller et al. 2010c) and to be completely abrogated by depletion of Gce1- and CD73-harboring EMVs from the incubation mixtures.

Epigenetic Control of EMV Release

Importantly, the release of those EMVs from large (but not small) primary and differentiated rat

adipocytes as well as differentiated human adipocytes in response to physiological (palmitate, H_2O_2) and pharmacological (antidiabetic sulfonylurea drug glimepiride) stimuli is shown to be significantly reduced in the presence of 5-aza-2′-deoxycytidine (5-Aza-CdR) known for its ability to inhibit cytosine methylation and induce substantial remodeling of heterochromatic domains as well as the specific inhibitor of histone lysine methyltransferases, BIX01294. The inhibition of EMV release by 5-Aza-CdR and BIX01294 does not correlate with altered apoptotic rate but is accompanied by impairment of the H2O2 (but not insulin)-induced stimulation of lipid synthesis and inhibition of lipolysis in large (but not small) primary and differentiated rat adipocytes and differentiated human adipocytes. In contrast, the simultaneous presence of 5-Aza-CdR and BIX01294 has almost no effect on the palmitate-, glimepiride-, and H_2O_2-induced release of those EMVs and lipid metabolism. These findings argue for regulation of the induced release of EMVs harboring GPI-anchored proteins, mRNAs, and microRNAs that are specific for the control of lipid metabolism, from rat and human adipocytes by DNA and histone methylation in interdependent fashion and suggest that the EMV-mediated transfer of lipogenic and anti-lipolytic information between large and small adipocytes induced by certain physiological and pharmacological stimuli may be inherited by epigenetic mechanisms. Consequently, it is tempting to speculate that interference with the epigenetic control of the information transfer between adipocytes and adipocytes and other relevant cells (e.g., monocytes, macrophages, pericytes) could modulate the complex molecular mechanisms through which environmental conditions especially in early life (e.g., diet, hormones, stress) lead to a biochemical memory effect that influences the susceptibility to metabolic syndrome.

The idea that epigenetic modifications could be manipulated by small molecules to modulate the expression of multiple genes and effectively reverse pre-programmed disease susceptibility is highly attractive as exemplified by the growing evidence for successful pharmacological interference with epigenetic mechanisms: (i) The selective p300 histone acetyltransferase inhibitor, C646, is identified through virtual library screening and showed inhibition of cell growth and histone acetylation. (ii) Entinostat is the most advanced example of the 2-aminobenzamide chemotype, aiming to bypass pharmacokinetic and toxicological issues associated with hydroxamic acids, and is currently in Phase II trials for several malignancies. (iii) Tubastatin and PCI-34051 represent recent advances in targeting HDAC subtypes 6 and 8, respectively. (iv) Recently identified BET bromodomain inhibitors exemplified by JQ1/SGCB01 and I-BET have demonstrated the tractability of this protein family for small-molecule modulation and show promising antitumor and anti-inflammatory properties. (v) Advances in structure-based design have led to the discovery of two series of JMJD2 demethylase inhibitors represented by substituted pyridine-2,4-dicarboxylates (SID85736331) and compound 8, which showed effects on cellular H3K9Me3 levels and anti-proliferative activities, respectively. (vi) The availability of a protein crystal structure for the methyltransferase G9a allowed the design of highly potent and selective inhibitors represented by UNC224.

Recent progress in the discovery of drug-like small-molecule inhibitors for the majority of the classes of histone modifiers, including writers, readers, and erasers of acetyl and methyl marks, demonstrates the tractability of these protein families for drug development. The availability of selective inhibitors/modulators for use as chemical probes as well as of test systems for analyzing the microvesicular communication between adipocytes and relevant immune cells in vitro will be essential in unravelling the involvement and function of individual players in the epigenetic control of the metabolic syndrome and the understanding of its underlying "histone code." This will be prerequisite for the initiation of future drug discovery projects aimed to modulate the relevant "writers," "readers," and "erasers" as critical targets for the therapy of metabolic syndrome.

Integration of the Multiple Communication Pathways Between Adipocytes

On the basis of those findings, the following hypothetical model for the regulation of lipid storage and cell size between adipocytes by intercellular communication within and eventually between adipose tissue depots has been proposed: It relies on a polarized structure of adipose tissue depots with large mature adipocytes harboring one or a few LD only, capable of very pronounced esterification in parallel with high lipolysis and being located in the immediate neighborhood to blood vessels and from there a gradient of nascent adipocytes of small and very small size. (A) Prolonged delivery of free fatty acids by the blood vessels and their resulting efficient esterification into LD cause size increase of the adipocytes and exhaustion of their lipid storage capability with accompanying upregulated lipolytic release of fatty acids into the interstitial space. This triggers the maturation of (very) small precursor adipocytes lacking visible LD in the deeper adipose tissue layers that are capable of esterification and lipolysis to a very moderate degree, only, by one or several types of intercellular signaling. (D) Upon induction of novel specific cell-to-cell contacts, as have already been formed between the large adipocytes, or (B) the (vesicular) secretion of adipokines, such as ASP, or soluble factors, such as 15-keto-PGE2, the targeted cell adhesion molecules or adipokine receptors at the cell surface or nuclear hormone receptors, respectively, induce signaling to enhanced esterification stimulation and/or lipolysis inhibition, which finally lead to upregulated LD biogenesis in the growing small adipocytes. (C) Alternatively or in addition, upon challenge with high concentrations of fatty acids originating directly from the blood vessels or from lipolytic release (or possibly with other physiological or pharmacological stimuli, such as hydrogen peroxide or the antidiabetic sulfonylurea drug glimepiride), the large adipocytes release the GPI-anchored proteins, Gce1 and CD73, into EMVs. Following passage through interstitial tissue spaces and eventually the circulation, the EMVs interact with plasma membranes of (very) small adipocytes for subsequent transfer of Gce1 and CD73 to the surface of LD where the enzymes degrade (c)AMP. Again, the resulting parallel upregulation of esterification and downregulation of lipolysis lead to enlargement of the LD and size of the adipocytes (Müller 2010). The value of this hypothetical model critically depends on the demonstration of the organized/polarized maturation of the very small precursor adipocytes into large adipocytes occurring in "waves" along a gradient in localized and sequential rather than randomly distributed fashion.

After having successfully ordered "takeover" of lipid loading and LD biogenesis to small (precursor) adipocytes, the large mature adipocytes discontinue all their efforts in lipid storing and consequent growing, which occurs in parallel to approaching their well-known upper cell size limit. This is manifested in apparent resistance of large "old" rodent and human adipocytes toward insulin stimulation of glucose transport, fatty acid synthesis and esterification, as well as inhibition of lipolysis, which together drive insulin-dependent adipogenesis, and can be demonstrated in vivo and in vitro (Faust et al. 1978; Salans et al. 1968; Björntop and Karlsson 1970; Björntop et al. 1975; Olefsky 1977; Julien et al. 1989; McLaughlin et al. 2007; Müller et al. 2010a). The molecular mechanisms underlying this physiological insulin resistance of large adipocytes are ill-defined so far, but may guarantee prevention of their pathological "ballooning" finally leading to increased cell death, infiltration of macrophages, and low-grade chronic inflammation in the adipose tissue depots.

A few years ago, cholesterol was proposed to act as a key regulatory molecule in the feedback control of LD and cell size in adipocytes (Dugail et al. 2003; Martin and Pol 2005). Plasma membranes and LD are generally assumed to represent the major compartments of cholesterol storage in mammalian adipocytes. Numerous studies have highlighted a strong correlation between adipocyte cholesterol content and LD content, i.e., the higher the LD content, the greater the need for cholesterol transport from plasma membranes to LD. In plasma membranes cholesterol accumulates in special areas, the so-called lipid rafts, together with glyco(sphingo)lipids, GPI-anchored

proteins, and a subset of transmembrane proteins, such as the cholesterol-binding protein, caveolin-1, and the insulin receptor (Brown and London 1998; Varma and Mayor 1998; Johannes and Mayor 2010). During adipocyte differentiation, caveolin-1, commonly assumed to be important for homeostasis of the LD cholesterol pool (Dugail et al. 2003; Dugail and Hajduch 2007), is translocated from the plasma membrane lipid rafts to the LD in response to cholesterol, fatty acids, or heavy lipid loading (Pol et al. 2005; Blouin et al. 2008; Le Lay et al. 2006). Furthermore, disruption of lipid rafts by cholesterol depletion has been demonstrated to abrogate signaling from the insulin receptor to downstream metabolic effector systems, such as glucose transport and fatty acid esterification in rat adipocytes (Müller et al. 2002; Aboulaich et al. 2006). Together with recent pulse-chase metabolic labeling studies (Müller et al. 2010b), it is tempting to speculate that the translocation of Gce1 and CD73 from plasma membrane lipid rafts to LD in concert with cholesterol complexed to caveolin-1 serves as an intracellular signal for ongoing LD biogenesis, i.e., increase in LD size and number, during adipocyte differentiation and enlargement. The translocation of caveolin-1 to mature LD has recently been shown to involve activation of Src family tyrosine kinases (by insulin) and presumably its phosphorylation (Le Lay et al. 2006). Thus, tyrosine phosphorylation of caveolin may serve as the initial trigger for its translocation from lipid rafts to LD in conjunction with cholesterol, which is a prerequisite for LD biogenesis. However, with increasing time this continuous flux of cholesterol will increasingly counteract the induction of adipogenesis by insulin. It will be interesting to study whether the apparent involvement of Src family kinases and caveolin-1 in the feedback desensitization of large adipocytes toward upregulation of LD and cell size by insulin is also true for other adipogenic signals, such as fatty acids, glimepiride, and reactive oxygen species. Interestingly, glimepiride has been shown to cause activation of the Src family kinase, Lyn, as well as phosphorylation of caveolin-1 in primary rat adipocytes (Müller et al. 2005).

Communication Between Adipocytes as a Pharmacological Target

It is tempting to speculate that the molecular mechanism underlying the insufficient triacylglycerol accumulation and LD biogenesis with the resulting defective conversion from the very small into large adipocytes is based on the missing information transfer from already existing large donor to the very small putative acceptor cells by one or the other of the three types of signaling mechanisms (Müller et al. 2010a). Thus, they may all may provide novel pharmacological targets for proper control of LD biogenesis in the same or distinct adipose tissue depots of obese insulin-resistant subjects.

With regard to communication via EMVs, multiple sites of interference are conceivable. Among them are the secretion of EMVs from the large adipocytes, EMV transport, and distribution within the same or between distinct adipose tissue depots, GPI-anchored protein transfer from EMVs to the small adipocytes, GPI-anchored protein translocation from plasma membranes to LD of the small adipocytes, or lowering of the cAMP concentration at the LD surface zone in the small adipocytes by Gce1 and CD73. Thus, defects in the coordinated upregulation of esterification and downregulation of lipolysis in small adipocytes by Gce1- and CD73-harboring EMVs released from large adipocytes could be responsible for the considerable increase and maintenance in the number of small adipocytes with proinflammatory and non-lipogenic/adipogenic phenotype in obese insulin-resistant subjects.

Interestingly, on the basis of recent studies addressing the molecular mode of action of insulin sensitizers of the glitazone class, the benefit of storage of free fatty acids in LD to bypass lipotoxic mechanisms does not seem to be restricted to adipocytes of primary adipose tissue depots, but may also encompass adipocytes in secondary depots, in particular those surrounding skeletal muscle cells (Sears et al. 2009). It is tempting to speculate that perimyocellular adipocytes correspond in their phenotype to small adipocytes of typical adipose tissue depots with their parallel pronounced upregulation of esterification of fatty acids into lipids and downregulation of the

release of the fatty acids from lipids. Small adipocytes support LD biogenesis and thereby would remove detrimental free fatty acids from the surrounding skeletal muscle cells, which prevents them or their derivatives, such as phosphatidic acid, diacylglycerol, or ceramides, from inducing lipotoxic mechanisms. Thus, it will be interesting to study whether large and small (if any of the) perimyocellular adipocytes function preferably as donors and acceptors, respectively, of EMVs. Consequently, putative approaches to limit lipotoxicity could be to transform large adipocytes of secondary adipose tissue (e.g., muscle) depots to small ones capable of pronounced esterification and retention of fatty acids by stimulating the expression of specific LD-associated proteins, such as PAT proteins. Application of this strategy will require extensive knowledge about the transcriptional program leading to LD biogenesis in adipocytes and non-adipose tissue cells.

In conclusion, the identification of the molecular mechanisms underlying and of molecules modulating the adipocyte-to-adipocyte contacts, the secretion of adipokines and other soluble factors and the release/translocation of Gce1/CD73-harboring EMVs, which are involved in paracrine and/or endocrine signaling of lipogenic and antilipolytic information, could be helpful for a pharmacological therapy of T2D and obesity based on shifting the burden of lipid loading and storage from large to small adipocytes within the same or distinct adipose tissue depots.

EMVs in Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH) Diseases

Both NAFLD and NASH are often associated with obesity, T2D, and asymptomatic (slight) elevations of serum liver enzymes. The progression of these hepatological diseases is accompanied by inflammation development which involves several actors, such as chemokines (e.g., MCP-1), cytokines (e.g., TNFa), and metalloproteinases. In comparison with NAFLD patients, NASH patients are older, are more obese, and more often have high serum liver enzymes, T2D, and metabolic syndrome. To date the diagnostic/prognostic of NAFLD/NASH is performed routinely using ultrasound of the liver for detection of fatty infiltration, but does not allow assessment of the presence or degree of inflammation and fibrosis. Therefore, diagnosis of fat in the liver is easily made by ultrasound but diagnosis of NAFLD or NASH cannot be performed without a liver histology.

Several biomarker-based approaches have been proposed to improve liver fibrosis diagnosis. These include serum marker panels, genetic markers of disease progression, plasma lipidomic signature, newer imaging methods, and breath tests. For example, a patented algorithm was evaluated and showed improved diagnostic performance for stages F2–F4 compared with the fibrosis score and AST-to-platelet ratio index. This marker panel index is based on relatively simple and readily accessible parameters such as age, glucose, AST, ALT, ferritin, platelets, and body weight but still requires further validation. Plasma lipidomic signature is also used for the characterization of NAFLD/NASH patients with increased lipogenesis, desaturase, and lipoxygenase activities compared to healthy patients. Interestingly, impaired peroxisomal polyunsaturated fatty acid metabolism and nonenzymatic oxidation are associated with progression to NASH. This strategy provides information that can serve as the basis for future hypothesis-driven studies of the role of specific metabolic pathways in the pathogenesis and progression of NASH. Albeit the combinations of various serum markers for liver fibrosis and the results from transient elastography measured by the fibroscan have been suggested to predict the presence of NASH and fibrosis, liver biopsy remains the accepted gold standard to differentiate NASH from NAFLD. There is a still an unmet medical need for the discovery of noninvasive and accurate diagnostic tools for this population of patients bearing these liver diseases and their stratification.

Tissue remodeling occurring during NAFLD/NASH may induce formation of EMVs. EMVs are present at relatively low concentrations in the circulation of healthy individuals. Numerous clinical studies have reported increased plasma EMV levels associated with cardiovascular risk factors (hypertension, smoking, obesity, and T2D) and cardiovascular diseases. Concerning liver

diseases, an increase of plasma EMVs has been reported in patients with hepatitis C and hepatocellular carcinoma, but it is unknown whether liver EMVs are detectable in patients with NASH. It is commonly accepted that it is a progressive process involving steatosis and inflammation. Accordingly, it is reasonable to expect that these conditions may result in EMV generation by liver cells, such as the hepatocytes, Kupffer cells, and/or recruited macrophages. So far, there are no clinical data available linking EMVs with fatty liver diseases. Nevertheless, in apolipoprotein E2 knock-in (apoE2-KI) mouse, which is an experimental model of atherosclerosis and NASH, early diet-induced NASH and its prevention by fibrates as well as the presence of EMVs was observed both in the atherosclerotic lesions and in the liver.

Communication Between Adipocytes and Enterocytes via EMVs

The journey of GPI-anchored proteins having successfully crossed the intestinal barrier and remaining associated with the small secreted vesicles via their intact GPI anchor eventually does not terminate in the circulation. It has recently been reported that upon incubation of small vesicles secreted from "donor" adipocytes with intact "acceptor" adipocytes, the constituent GPI-anchored proteins, such as Gce1 and CD73, are being transferred from the small secreted vesicles to plasma membrane lipid rafts of the "acceptor" adipocytes, presumably in the course of their direct interaction (Müller et al. 2010b, c). Subsequently, the journey of the transferred GPI-anchored proteins is continued within the "acceptor" adipocytes by their transport from the outer leaflet of the plasma membrane lipid rafts to cytoplasmic lipid droplets. This again was found to be stimulated by palmitate, glimepiride, and hydrogen peroxide (Müller et al. 2010a). Thus, the small secreted vesicles with the inserted GPI-anchored proteins do not only operate as "passive" vehicles for their distribution to target tissues via the circulation. They also play a more "active" role in tissue distribution of GPI-anchored proteins by mediating their passage across the plasma membrane into the cytoplasm of the "acceptor," i.e., target cell. This is compatible with recent findings that the type of the fatty moieties of the GPI anchor (i.e., length, saturation) critically contributes to the distribution of the GPI-anchored protein between plasma and tissues, between distinct tissues, and between subcellular compartments (Müller et al. unpublished data). This is due to, at least in part, mediation of the interaction of the GPI-anchored protein with lipid raft-like membrane microdomains (Brown 1992) of small secreted vesicles as well as of cytoplasmic lipid droplets by the GPI anchor fatty acyl chains.

It will be an important and challenging task for the future to elucidate in detail all the structural features of the GPI anchor which determine the pharmacokinetic and pharmacodynamic profiles as well as putative immunological and toxic properties of GPI-anchored proteins for the realization and optimization of their oral application in humans. Part of the desired information may be derived from in vitro investigations using a modified version of the multiplex assay system described above. For this, the filter plate with the adherent sealed endothelial monolayer at the top is placed above an additional sealed monolayer of putative target tissue cells, such as hepatocytes, myocytes, and adipocytes. The filter plate prevents direct cell-to-cell contacts between the two monolayers, but allows the exchange of small molecules, such as nutrients and ions. In the bottom of the culture dish to which the target tissue cells adhere, liquid scintillation cocktail is incorporated. The transport of vesicle-, liposome-, or micelle-associated radiolabeled GPI-anchored proteins applied to the upper apical area across the enterocytes via the fluid space formed by the pores of the filter plate and representing the basolateral area into the underlying target tissue cells is monitored as accumulation of radioactivity in the monolayer cells adherent to the bottom of the culture dishes by liquid scintillation counting.

Evaluation

Cells both in vivo and in vitro release heterogeneous membrane structures currently defined as

microparticles, microvesicles, endosomes, multivesicular bodies, exosomes, ectosomes, or apoptotic bodies and herein summarized for simplicity as EMVs. The use of EMVs as diagnostic markers in clinical medicine is becoming increasingly attractive for many (common) diseases. The future aims are the establishment of omics-based EMV analysis as novel biomarkers in metabolic- and age-related diseases for their prediction, diagnosis, prognosis, and therapy monitoring. The improvement and validation of high-throughput analytical platforms will enable the quantitation and discrimination of EMV heterogeneity as related to their physicochemical properties (size, density, refractive index) and to multi-omic analysis of their proteomic, lipidomic, metabolomic, and nucleic acid (DNA, RNA, small RNA/miRNA) composition. For this, a number of points have to be fulfilled:

(i) Improvement and minimization of preanalytical procedures and calibration and standardization of flow cytometric platforms suitable for EMV analysis: (ii) establishment and validation of multiparameter combinations of fluorescently labeled monoclonal antibodies and nucleic acid staining and preanalytical protocols of fixation/permeation for simultaneous multiparameter flow cytometric analysis of cell-type-specific EMVs in body fluids, (iii) use of explorative and quantitative mass-spectrometry-based proteomics as reference method and as bridging technology for the analysis of EMV protein composition and content in EMVs derived from different cell types and diseases to decide on the final selection of antibody targets for single and multiplex ELISA platforms or multiparameter flow cytometry, (iv) definition and validation of quantitative mass-spectrometry-based lipid species analysis in EMVs from different cell types relevant for metabolic- and age-related diseases using the high-throughput/content lipid species profiler (>800 lipid species) with special emphasis on regulatory and high-affinity lipid ligands (e.g., fatty acids, lysophospholipids, sphingolipid/phosphates) for G-protein receptors, annexins, nuclear receptors (e.g., PPARγ LXR/RXR), and other transmembrane receptors (e.g., CD36, integrins, selections, GPI-anchored proteins), (v) establishment and validation of novel highly membrane-permeable dequencher dyes to increase specificity, sensitivity, and accuracy of fluorescent nucleic acid staining (DNA, RNA, small RNA, miRNA) in EMVs with excitation/emission profiles suitable for multiparameter flow cytometry, (vi) improvement and validation of preanalytics (extraction, inhibitor elimination) and analytics (primer design, assay formate) of array-based and quantitative RT-PCR Taqman analysis of miRNA content in EMVs and plasma, (vii) establishment and validation of multiomics-based chemometric and biometric software tools and graphic data presentation for EMV analysis to generate actionable health information, and (viii) performance of cell-type-specific in vitro analysis of EMV levels and composition and to use specific EMV preparations for spiking and assay validation in body fluids. No doubt, these methodologies will contribute to the generation of information for evidence-based medicine establishing the value of EMV analysis as novel biomarkers in metabolic- and age-related diseases for risk assessment, diagnosis outcome prediction, prognosis, and therapy monitoring.

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Insulin Analogs: Assessment of Insulin Mitogenicity and IGF-I Activity

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Introduction and Application to Insulin Analogs

The metabolic activity of insulin has been studied extensively in vitro and in vivo, based on the initial assessment of insulin receptor affinity, followed by methods to estimate the metabolic activity in vitro. These estimates provide some guidance about the biological activity which will be found in vivo; they need to be confirmed and supplemented by testing the glucose-lowering activity in animals (mice, rats, dogs, pigs). The biological effects (hypoglycemic activity) are related to the direct activation of the insulin receptor and subsequent signaling through intracellular mechanisms. The second group of biological effects is related to cell proliferation (mitogenic activity), which may be mediated by the insulin receptor, by the IGF-I receptor, and by hybrids of the two receptors. The evaluation of the relevance of mitogenicity estimates may be performed in in vitro and in vivo. One approach is cell proliferation in benign and malignant cell lines, for example, on mammary epithelial cell lines MCF-10 and MCF-7 (Milazzo et al. 1997).

In the in vivo evaluation, the metabolic activity is determined in animals (see chapter "▶ Measurement of Blood Glucose-Lowering and Antidiabetic Activity") using the rat or rabbit bioassay (Lin et al. 1999) by intravenous injection, to confirm the blood glucose-lowering activity after direct receptor activation. This initial test confirms intrinsic activity at the receptor level. However, it does not indicate depot activity and is applicable in the same manner to fast-acting insulin analogs (lispro, aspart, glulisine) and to long-acting analogs (glargine, detemir). The rat bioassay by intravenous injection that is preferred is the initial test for in vivo biological activity. In order to evaluate depot activity, the time-action profile after subcutaneous injection may be determined subsequently, in order to distinguish between the rapid-acting insulin analogs and long-acting insulin analogs. This has been done in clinical studies by measuring the glucose lowering and the residence of 125-I-labeled insulin analogs at the subcutaneous injection site (Kang et al. 1991a).

In a practical approach, testing for depot activity may be performed by the pharmacodynamics of glucose lowering, preferably in intact dogs. Such studies may be later be followed up by euglycemic clamp studies. The pharmacokinetics can be based on nonspecific measuring of immunoreactive insulin and concentration changes of C-peptides. More specific determination of the insulin analog concentrations requires advanced methods, for example, there is the specific radioimmunoassay for lispro insulin (Bowsher et al. 1999; von Mach et al. 2002).

General Considerations

A general approach may be suggested for the in vitro evaluation of new insulins. This approach is based on the consideration that insulin has metabolic as well as mitogenic activity which is mediated predominantly by signaling via the insulin receptor and at high concentrations – as used in vitro – may also be mediated by signaling via the IGF-I receptor. The primary step is determination of insulin receptor affinity, followed by IGF-I receptor affinity (Baehr et al. 1997). There is one important reference compound, [B10-Asp] insulin, which should be used in these evaluations as comparator (Berti et al. 1998), because of the toxicological findings with [B10-Asp] insulin which have raised concern about enhanced mitogenic activity of insulins with prolonged residence time at the insulin receptor (Jorgensen et al. 1992). [B10-Asp] insulin was evaluated as fast-acting insulin analog in early clinical trials (Kang et al. 1991b; Nielsen et al. 1995) and was subsequently found to induce benign and malignant mammary gland tumors in rats, during 12-month toxicology studies. This has been attributed to enhanced mitogenic signaling via the insulin receptor and alternatively to increased affinity for the IGF-I receptor with subsequent growth-promoting effects on mammary gland tissue. A guideline regarding the evaluation of new insulin analogs for carcinogenicity was issued by the European Agency for the Evaluation of Medicinal Products (EMEA 2001), with specific guidance about the in vitro and in vivo evaluation to be

performed. In this guideline, the specific reference compounds mentioned a human insulin, [B10-Asp] insulin and IGF-I (somatomedin C).

Purpose and Rationale

The structure and approach to the evaluation of new insulins are directed toward efficacy in terms of glucose lowering, in the predictive manner to differentiate between monomeric insulins with fast absorption kinetics, and long-acting insulins with delayed absorption from the subcutaneous injection site.

Procedure

The initial steps are performed in vitro and focused on receptor interaction. Subsequent steps are aimed at elucidating the receptor-mediated signaling in vitro, which is however at the present time difficult to attribute precisely to biological and clinical effects. This is due to the wide concentration range permissible during in vitro studies, whereas the narrowly limited clinical concentration range needs to be kept in mind during any interpretation of clinical relevance (Zib and Raskin 2006).

The main aim of the in vitro evaluation is to establish the relation of metabolic activity to mitogenic activity. Corresponding efforts were already successful for structure activity studies on insulin analogs, which subsequently lead to predicting the clinical relevance of these observations, when comparing new compounds with the established biochemical and toxicological profile of [B10-Asp] insulin, and the clinically used fast-acting and long-acting insulin analogs.

Modifications of the Method

The primary step of measuring insulin receptor affinity may be performed in several modifications, depending on the receptor preparation and on the source of insulin receptors, e.g., from fibroblasts, placental membranes, skeletal muscle cells, and other sources. The second step of measuring IGF-I receptor affinity also has several options; for research on the IGF-I receptor, a human osteosarcoma cell line (SAOS B10) was in several instances applied due to the high number of IGF receptors and lower number of insulin receptors on the cells (Kurtzhals et al. 2000).

The biological profile in animals is directed at predicting clinical utility either as a fast-acting (mealtime) insulin or at establishing depot activity in a suitable animal species. Blood glucose-lowering (hypoglycemic) activity is also important for the design of safety studies depending on the preclinical metabolic to mitogenic ratio. In the chronic toxicity studies, and where applicable in carcinogenicity studies, the dose range from biologically effective dose to maximum tolerated dose may be 50–100-fold; the results then need to be interpreted carefully for their relevance in relation to the therapeutic concentrations found in clinical therapy.

Critical Assessment of the Method

When considering the extensive knowledge on three fast-acting insulins and two long-acting insulins currently in clinical use, the predictive relevance of the methods described here can be assessed in each case, and selection of the appropriate study design is based on existing clinical experience.

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Insulin Receptor Affinity

General Considerations

An initial step in the evaluation of insulin analogs is determination of their affinity for the insulin receptor (Lee and Pilch 1994). The studies may include receptor association and dissociation as well as the signaling via the insulin receptor (insulin receptor tyrosine kinase activity and tyrosine phosphorylation of substrates). These methods provide some guidance about the biological potency

to be expected, although they provide only limited information about the time-action profile.

Receptor affinity data have to be extended by evaluation of postreceptor events (signaling). The time-action profile in animals and humans may differ considerably from the predictions derived from the receptor affinity alone, but may correlate with the kinetics of the relevant insulin signaling processes.

Purpose and Rationale

These methods described the initial binding of insulin analogs to the receptor preparation obtained from different sources. Tissues differ in their receptor content, the early methods used membrane receptors isolated from different organs, and more recent methods use solubilized human insulin receptor protein.

Procedure

There are several important modifications of the assay principle. This depends on the selection of the initial source of receptor (tissue), the preparation of the receptor protein, and the procedure to determine receptor affinity using a radioactive-labeled insulin preparation. Some details of the methods are described in chapter "► Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes".

Slieker et al. 1997 described the method using placental membranes and mammary epithelial cells as the receptor preparations for [125I]-Insulin and [125I]-IGF-I binding assays. The placental assay employed incubating 30–40 mg of membrane protein with approximately 10 fmol of iodinated ligand in a final volume of 500 ml of 100 mmol/l HEPES, pH 7.8, 120 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 8 mmol/l glucose, and 0.25 % BSA for 16–18 h at 4 °C. Membranes were collected on glass fiber filters pretreated with 0.1 % polyethyleneimine by using a cell harvester (Skatron, Lier, Norway). Binding assays with mammary epithelial cells were performed under similar conditions using confluent cells on either P12 (IGF-I receptor) or P6 (insulin receptor) plates (Costar, Cambridge, Mass., USA). Monolayers were incubated in insulin-free medium for 24 h prior to initiating the binding assay. Cells were incubated in the above buffer containing iodinated IGF-I and cold competing ligands for 16–18 h at 4 °C. After washing the monolayers with cold assay buffer, cells were solubilized in 0.1 N NaOH and counted for 125I. EC50 values were determined by fitting displacement data to a four-parameter model by nonlinear regression. Dissociation constants for the iodinated (TyrA14-125I) insulin analogs – LysB28ProB29, and AspB10 were obtained according to the method of Drejer et al. (1991).

For the quantification of receptor numbers of insulin and IGF-I on Chinese hamster ovary (CHO) cells using the [125-I] labeled ligands, Hansen et al. (1996) applied the method which was also modified for time course studies of binding to these receptors.

CHO-hIR and CHO-K1 cells were seeded on 24-well Nunclon plates at a density of 5 × 10 % cells/well in DMEM containing 20 mM Hepes, 1 ug/l human albumin, 10 % fetal calf serum, 1 % nonessential amino acids, and 0.1 % bacitracin (pH 7.4); cells were used at confluence. The numbers of insulin and IGF-1 receptors present at the surface of the CHO-K1 and CHO-hIR cells were determined by saturation of the respective receptors with unlabeled ligand. Cells were incubated overnight at 4 °C with 50 pM 125I-labeled insulin or [125I]IGF-1 in the presence of increasing amounts of unlabeled insulin or IGF-1, respectively. The number of receptors was estimated with the aid of the LIGAND program fitting a two-site model (Munson and Rodbard 1980). The native CHO-K1 cells were estimated to express approx. 3.000 high-affinity binding sites for insulin and 50.000 IGF-1 receptors, whereas CHO-hIR cells were found to express 60.000 IRs and 50.000 IGF-1 receptors.

For the time course of receptor association, the procedure was as follows (Hansen et al. 1996): CHO-hIR cells were cultivated in DMEM containing 2 mM glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin, 10 % fetal calf serum, 1 % nonessential amino acids, and 1 lM

methotrexate. Cells were grown to 80 % confluence in 60 mm dishes, 6-well plates (IRTK activity assay) or 12-well plates before the experiments were carried out. Before stimulation with insulin or analog, the cells were starved for 18 h in a serum-free medium supplemented with 0.5 % insulin-free BSA. The cells were stimulated with 1 ml of 0.1 lM insulin or analog for 30 min and then washed thoroughly three to five times (2–5 ml each wash) and subsequently incubated in serum- and insulin-free medium. Because of the large variations in receptor kinetics and potencies of the analogs, the high concentration of 0.1 μM and the 30 min stimulation time were used to ensure maximal stimulation. At various times poststimulation (0–180 min), the medium was removed and the cells processed further (insulin receptor tyrosine kinase (IRTK) activity and tyrosine phosphorylation of IR, IRS-1, and Shc).

Kurtzhals et al. (2000) used a preparation of human solubilized insulin receptor protein. Human insulin receptor (hIR) (isoform without exon 11) was isolated from transfected baby hamster kidney (BHK) cells by solubilization and partial purification on a wheat germ agglutinin column. For binding experiments, human insulin receptor (hIR) was incubated with 3 pmol/l TyrA14[125I] human insulin and various concentrations of unlabeled human insulin or insulin analog in a binding buffer containing 0.1 mol/l HEPES, 0.1 mol/l NaCl, 0.01 mol/l MgSO4, 0.5 % HSA, 0.2 % g-globulin, and 0.025 % Triton-X-100, pH 7.8, for 42 h at 4 °C. Bound tracer was isolated by precipitation with 400 μl 25 % PEG 8000 and washing with 1 ml 15 % PEG 8000. The data were fitted to a 4-parameter logistic function where fimax and firmin were fixed and slope and concentration required for half-maximal effect (EC50) varied. The relative affinity of an insulin analog was calculated as the ratio between the EC50 value for human insulin and that of the insulin analog.

Dissociation from the insulin receptor was determined by Kurtzhals et al. (2000) using CHO cells. Dissociation constants for A14Tyr [125I]-insulins were determined as previously described using Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor (CHO-hIR cells) (9). The cells were cultured at 37 °C in a 5 % CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (vol/vol) fetal calf serum (FCS), 1 % nonessential amino acids, 50 μg/ml streptomycin, 50 U/ml penicillin, and 1 μmol/l methotrexate. Cells were subcultured at a 1:5 split ratio every 3–4 days for maintenance. For dissociation studies, cells were seeded on 24-well plates at a density of 5 3 104 cells/well in DMEM containing 10 % FCS, 1 % nonessential amino acids, 20 mmol/l HEPES, 0.1 % HSA, and 0.1 % bacitracin (pH 7.4). Cells were used at confluence and were incubated with 50 pmol/l A14Tyr[125I]-insulin or analog for 3 h at 4 °C. Cells were washed quickly twice with ice-cold HEPES-buffered DMEM (pH 7.4), and the dissociation of radioactivity was measured after the addition of HEPES-buffered DMEM containing 0.1 μmol/l unlabeled human insulin to measure the maximal accelerated dissociation rate. Cell-associated radioactivity was measured as a function of time, and the dissociation rate constant (kd) was calculated from the fitted monoexponential dissociation profiles using the following equation: B = Bmin + (Bmax – Bmin) 3 exp(−kd 3t), where B represents cell-associated radioactivity.

Modifications of the Method

The main modifications are related to the selection of receptor protein (membranes or solubilized receptor protein, cells transfected with the human insulin receptor – CHO-hIR cells).

Critical Assessment of the Method

It is important to use the same system when comparing the insulin receptor affinity of several insulins. To some extent, this can be used as the selection tool in structure activity studies. There are however considerable limitations when comparing affinities with in vitro potency and ultimately with in vivo hypoglycemic activity. Therefore, results of the insulin receptor affinity need to be supplemented by data on receptor

association and dissociation, as well as postreceptor signaling mechanisms.

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Signaling via Insulin Receptor

General Considerations

The postreceptor events after stimulation of the insulin receptor are of considerable interest for understanding the effects on different tissues, changes associated with insulin resistance and mitogenic activation of normal tissue and tumor tissues. This is a very complex area of research, because the mitogenic signaling is concentration dependent and many effects may be obtained in vitro which have no equivalent within the therapeutic range of insulin application in patients. Due to the complexities of the signaling mechanisms and their redundancy with regard to cell proliferation, apoptosis, and tissue growth, these investigations are necessary for better understanding, but not sufficient at the present time to fully explain the antiapoptotic and mitogenic activities in vivo and the clinical relevance.

Purpose and Rationale

Studies on signaling of insulin analogs via the insulin receptor over a wide range of concentrations in vitro are performed to elucidate the intracellular mechanisms and the components of the signaling chain.

Procedure

Among the many procedures and protocols used for the investigation of signaling in cells stimulated with insulin analogs, Hennige et al. (2005) provide

an example for the assessment of the fast-acting insulin glulisine. Their tests were performed in 10-week-old male C57BL/6 mice from Charles River acclimatized for 2 weeks before entering the study. The mice were maintained on a normal light/dark cycle and kept on a regular diet. Glucose levels were sampled from mouse tail bleeds using a Glucometer Elite (Bayer, Elkhart, IN).

The procedures for in vivo insulin stimulation and Western blot analysis were described for a short-term experiment, by single-dose stimulation with regular human insulin or insulin glulisine (1 IU/kg body wt) injected intraperitoneally. For short-term stimulation, 2 IU of insulin was injected into the inferior vena cava. Control animals received a comparable amount of diluent. Tissues (liver, muscle, and hypothalamus) were removed at the indicated time points and homogenized at 4 °C, as previously described (8). Homogenates were allowed to solubilize for 30 min on ice and were clarified by centrifugation at 12,000 g for 20 min. To detect insulin-stimulated tyrosine phosphorylation, supernatants containing 0.5 mg of total protein were immunoprecipitated with antibodies directed against the COOH-terminal sequences of the insulin receptor and of the insulin receptor substrates IRS-1 and IRS-2. Visualization of immunocomplexes after gel electrophoresis and Western blotting with the antiphosphotyrosine antibody 4G10 was performed with the nonradioactive enhanced chemiluminescence system. Blots were subsequently stripped and reprobed to reveal expression of total protein. To assess activation of downstream signaling elements, tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with anti-phospho-AKT and anti-phospho-mitogen-activated protein (MAP) kinase antibodies (Cell Signaling, Beverly, MA), respectively.

Assay of PI 3-kinase activity: Tissue lysates were immunopurified with anti-PY20 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, and immunocomplexes were absorbed to protein a-sepharose for 12 h. Immunoprecipitates were washed three times and pellets were directly incubated with 0.1 mg/ml L-PI (Sigma) and 50 mol/l [32P]-ATP (Perkin Elmer) at room temperature for 10 min. After 1501 of 1 mol/l HCl was added, lipids were extracted twice with 4501 chloroform/methanol (1:1 by vol). Products were separated by thin-layer chromatography, as previously described (9). ^{32}P-labeled phospholipids were detected by autoradiography.

[^{3}H]thymidine incorporation into C2C12 myoblasts: To measure [^{3}H]thymidine incorporation, C2C12 cells were grown in Dulbecco's modified Eagle's medium (4.5 g/l glucose and 10 % FCS) in 6-well culture plates and subsequently starved for 24 h in serum-free medium. After cells were stimulated with insulin for 16 h, [^{3}H]thymidine (0.5 Ci/ml) was added for 4 h. The dishes were then rinsed twice with ice-cold phosphate-buffered saline (PBS) and once with 10 % trichloroacetic acid. After 20 min, dishes were washed once with ice-cold 10 % trichloroacetic acid. Cells were then lysed with 5001 of 0.2 N NaOH/1 % SDS and neutralized with 0.5 ml of 0.2 N HCl. Radioactivity was determined by liquid scintillation counting.

Modifications of the Method

There are many options to analyze the postreceptor events to characterize signaling in insulin-stimulated cells (Gronborg et al. 1993; Chang et al. 2004), with considerable overlap toward the IGF-I stimulated signaling (Valentinis and Baserga 2001). For the practical approach of risk assessment for insulin analogs, these methods are not suitable, due to the complexity and the as yet only partially defined relation to proliferative activity in normal tissues and in preexisting tumors. The research potential of these methods in defined cell lines in culture is very important; the application to insulin-stimulated animals and their tissues is also of considerable heuristic relevance.

Critical Assessment of the Method

Exploration of the insulin receptor-related signaling mechanisms is at present an active research

area with many well-defined methods (see chapter "▶ Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes").

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IGF-I Receptor Affinity

General Considerations

IGF-I receptor affinity is characterized by binding of IGF-I to receptors on cell membranes or to the IGF-I receptor protein. The cell lines which have been selected for their high density of IGF-I receptors provide estimates of affinity which may vary considerably and need to be supplemented by methods for the interaction of insulin analogs with the receptor (dissociation, residence time) and receptor-mediated effects on proliferation in normal tissues and in tumor cell lines.

For the IGF-1 receptor affinity of insulin analogs, cell lines have been used with a favorable relation of IGF-I receptors to insulin receptors. Clearly, results differ with the test system used; therefore, the determination of IGF-I receptor affinity is considered a preselection step for the further investigation of signaling via the IGF-I receptor and the insulin receptor. Placental membranes or mammary epithelial cell lines (Slieker et al. 1997) and human skeletal muscle cells (Ciaraldi et al. 2001) have been used for structure activity studies and full investigations on signaling (Ciaraldi et al. 2005).

Purpose and Rationale

Studies are performed to obtain an estimate of the proliferative and antiapoptotic activity to be expected based on the affinity for the IGF-I receptor (De-Meyts et al. 1994; Rubin and Baserga 1995) and to explore the mitogenic/metabolic ratio in preparation for detailed investigation of the effects on cells (e.g., thymidine incorporation) and on postreceptor signaling (LeRoith et al. 1994).

Procedure

Human skeletal muscle cells (Ciaraldi et al. 2001) are used for the assessment of insulin analog interaction with insulin receptors and IGF-I receptors.

Cell Culture

Biopsy of the vastus lateralis muscle was performed according to published procedures (19). Human skeletal muscle cells were isolated and grown in culture as described in detail previously (Henry et al. 1995). When myoblasts attained 80–90 % confluence, the growth medium was changed to MEM supplemented with 2 % FBS and antibiotics to induce fusion to multinucleated myotubes. The medium was changed every other day during cell fusion. All studies were performed on cells after one passage.

Insulin and IGF-I Binding Assays

Hormone binding assays were performed by the modification of a method described previously (Henry et al. 1995). Fully differentiated human skeletal muscle cells from nondiabetic subjects and patients with type 2 diabetes were washed four times with reaction buffer and then incubated with reaction buffer and [125I-Tyr A14]insulin (final concentration, 67 pm) or [125I-Tyr A14] IGF-I (final concentration, 39 pm) for 4 h at 12 C in the absence or presence of varying concentrations of unlabeled hormone. Results were calculated based on the displacement of specific insulin/IGF-I binding normalized to protein concentrations in cells from both nondiabetic subjects and patients with type 2 diabetes.

Modifications of the Method

One particular assay based on IGF receptor affinity in an osteosarcoma cell line SAOS B10 was used extensively in the preclinical evaluation of mitogenic activity (Kurtzhals et al. 2000). This cell line was initially selected for research on IGF-I and subsequently adapted to structure activity studies. The finding of slightly increased IGF-1 receptor affinity in the presence of a rate of receptor dissociation similar to human insulin is not associated with any biological consequences, as shown by the subsequent extensive studies on absence of toxicity and carcinogenicity mice and rats (Stammberger et al. 2002).

Critical Assessment of the Method

It remains the highly active and controversial area of research to define which mechanisms are involved and contribute to a significant extent to cell proliferation and apoptosis via the insulin receptor and/or the IGF-I receptor (Blakesley et al. 1996; De Meyts 1994; De-Meyts et al. 1995a, b). Evidence for these mechanisms relies to significant extent on the time factor of interaction with the insulin receptor; prolonged residence time as concluded from the incidence of [B10-Asp] insulin would be dissociated with stimulation of tissue proliferation and could have an effect on tumor progression (Prager and Melmed 1993; LeRoith 1994; De Meyts et al. 1995b; Strobl et al. 1995).

The assessment of IGF-I receptor affinity and its physiological role becomes even more complicated with the unresolved role of insulin/IGF-I hybrid receptors (Soos et al. 1993; Takata and Kobayashi 1994), which may also have a role in the microvascular and macrovascular complications of diabetes (Li et al. 2005). At present time, there is no evidence for a pathophysiological role of slightly increased IGF-I receptor affinity in the presence of physiological residence time at the receptor, with dissociation rates similar to human insulin. Investigation of insulin glargine has shown that there is no effect on proliferation of benign and malignant mammary cell lines (Staiger et al. 2005a, and no effect on tumor formation in rodent carcinogenicity studies (Stammberger et al. 2002). There is also no evidence for any proliferative effect of the clinically used fast-acting insulin analogs in animal toxicology studies (EPARs for insulin lispro, insulin aspart, and insulin glulisine). Investigation of mammary gland tissue for proliferation markers after a 6-month and 12-month treatment in rats has shown no proliferative effect (Stammberger et al. 2006).

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Signaling via IGF-1 Receptor

The insulin receptor (IR) and the insulin-like growth factor I receptor (IGF-IR) belong to the same subfamily of receptor tyrosine kinases with two hormone-binding alpha-subunits and two transmembrane beta-subunits with tyrosine kinase activity. They share a high similarity of structure and intracellular signaling events (Dupont and Leroith 2001). However, the IR and the IGF-IR mediate different effects on metabolism, cell proliferation, apoptosis, and differentiation. Although some of the variation can be attributed to a different tissue distribution or subcellular localization, it can also be explained by structural differences in the β-subunit, which may result in activation of specific substrates and signal pathways.

The presence and formation of hybrid receptors, heterodimers composed of the subunits of the insulin receptor, and IGF-I receptor has been documented both in normal tissue and in tumor tissue samples (Salzman et al. 1984; Schumacher et al. 1991; Soos et al. 1993; Siddle et al. 1994). It has been proposed that signaling in tumor tissue may proceed via such hybrid receptors.

In cells expressing the insulin receptor isoform A (IRA) and the insulin-like growth factor-1 receptor (IGF1R), the presence of hybrid receptors, constituted of an alpha-beta-IRA chain associated with an alpha-beta-IGF1R chain, has been demonstrated. These heterodimers are found in normal cells and also appear to play crucial roles in a number of cancers (Belfiore et al. 1999; Frasca et al. 2003; Pandini et al. 1999). However, they remain difficult to study, due to the concomitant presence of IRA and IGF1R homodimers. Using bioluminescence resonance energy transfer (BRET), Blanquart et al. (2005, 2006) have developed assays to specifically monitor the activation state of IRA/IGF1R hybrids, both In vitro and in living cells. In the first assay, only hybrid receptors were BRET competent. In the second assay, the activation state of IRA/IGF1R hybrids was monitored in real time, in living cells. In hybrid receptors, trans-phosphorylation of the kinase-dead alpha/beta-Rluc moiety by the wild-type alpha/beta moiety induced the recruitment of YFP-PTP 1B-D181A-Cter, resulting in a hybrid-specific ligand-induced BRET signal. Both methods allow monitoring of the activity of IRA/IGF1R hybrid receptor and could be used to detect molecules of therapeutic interest for the treatment of cancer (Blanquart et al. 2008, Issad et al. 2007).

Critical Assessment of the Method

There are presently a number of methods applicable for the detection of insulin/IGF-I hybrid receptors and their state of activation. It will be important to investigate insulin analogs with reference to their effects on hybrid receptors and signaling in normal tissue (Lamphere and Lienhard 1992) as well as in tumor tissue. There are observations of adaptive changes in the formation of insulin/IGF-I hybrid receptors in type 2 diabetic patients, attributed to chronic hyperinsulinemia (Mosthaf et al. 1991; Federici et al. 1998a, b), as well as alterations of the hybrid receptor ratio in tumor such as thyroid cancer (Belfiore et al. 1999) and breast cancer (Pandini et al. 1999; Frasca et al. 2003).

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Mitogenic Activity

General Considerations

The determination of mitogenic activity on benign and malignant cell lines is a preliminary approach which has been proposed in the points-to-consider document of the EMEA safety working party. Enhanced cell proliferation may be the result of signaling via the insulin receptor at near physiological concentrations, via the IGF-I receptor at supraphysiological concentrations, and presumably also related to the presence of hybrid receptors which are preferentially stimulated by IGF-I (Li et al. 2005; Slaaby et al. 2006)

Purpose and Rationale

The tests for mitogenic activity are considered to be in early toxicology procedure which provides guidance about the subsequent toxicological evaluation to be performed. In case of enhanced mitogenicity found with the particular cell line, the 6 months of 12-month toxicology studies may include histological investigation of specific organ tissues, together with proliferation markers in these tissues (as an example, refer to Stammberger et al. 2006 for investigation of mammary gland tissue in rats).

Procedure

The procedure by Slieker et al. (1997) is based on stimulation of human mammary epithelial cells (HMEC). Mitogenicity was assessed by measuring insulin analog-stimulated growth of human mammary epithelial cells (HMEC) in culture. HMEC were obtained from Clonetics Corporation (San Diego, Calif., USA) at passage 7 and were expanded and frozen at passage 8. A fresh ampule was used for each experiment so that cells were not grown beyond passage 9. Cells were maintained in MCDB 170 medium containing bovine insulin (5 mg/ml), recombinant human epidermal growth factor (10 ng/ml), hydrocortisone (0.5 mg/ml), bovine pituitary extract (50 mg/ml), and gentamicin/amphotericin B. For a growth experiment, cells were plated in 96-well trays at a density of 12,500 cells/cm2 in the above medium modified as follows: 0.1 % BSA was added and 5 mg/ml bovine insulin was substituted by a graded dose of human insulin or analog from 0 to 1,000 nmol/1 final concentration. Trays were incubated for 72 h and the cells were counted by Coulter counter (Coulter Electronics, Hialeah, Fla., USA) after trypsinization. Typically, the maximal growth response was between three- and fourfold stimulation over basal and did not differ between analogs. Response data were normalized to between 0 % and 100 % response equal to 100 ′ (response at dose X – response at zero dose) divided by (response at maximal dose – response at zero dose). Dose-response data were fit by nonlinear regression employing JMP (SAS Institute, Inc., Cary, N.C., USA).

The method by Trueb and Froesch in human osteosarcoma cells (SAOS-B10) for the determination of the binding of insulin analogs to the IGF receptor and proliferative activity was developed in 1995 and subsequently applied by Kurtzhals et al. (2000). Human osteosarcoma cells (B10, a subline of Saos-2, supplied by S. B. and G. A. Rodan, West Point, Pennsylvania) are used because they exhibit a high rate of proliferation and high number of IGF-1 receptors on the cell surface. From competition of 125-I-insulin by unlabeled human insulin, a number of only 740 insulin binding sites per cell have been calculated, whereas 21,000 or 39,000 IGF-1 binding sites per cell were determined in two separate experiments. IGF-1 receptor binding is measured (competition with 125-I IGF-I). The dilution of

the stock solutions of the various analogs and natural insulins is performed with HBB/1 % HSA (HBB: 118 mM NaCI, 5 mM KCI, 1.2 mM MgSO4, 8.8 mM D-glucose, 100 mM Hepes, pH 8.0). In the studies by Froesch, possible concentration of the stock dilution was 5.5–10″ M for insulin glargine and the related Arg(B31)Arg (B32) insulin due to a lower solubility of both substances at neutral pH.

Therefore, the maximum final concentration in the competition experiment was limited to 5.10~ M for these analogs (10-M for human insulin and the animal insulins). The limitations of preparing stock solutions for insulin analogs with a marked change in isoelectric point should be considered in the experimental design. In another series, initial stock solutions with a standard concentration of 5.5 + 10″ M were used, again limiting the final maximum concentration to 5.1 0″ M for the insulin analogs in the competition experiment.

Osteosarcoma cells (BlO, passage 27, 125,000 or 150,000 per well) were seeded in 24-well tissue culture plates and kept overnight at 37 °C in DMEM/F12 (1:1) with the addition of 10 % fetal calf serum. For deinduction, the medium was changed to 500 µl serum-free medium for 4 h, and then the cells were incubated with 200 µl HBB/1 % HSA-[125-I]-IGF-1 tracer (0.03 pick/well = 52,000 cam, 8.1 × 10–13 M final concentration) + 20 µl insulin analog or reference in the respective dilution overnight at 4 °C. Plates are washed three times with cold PBS and dried at room temperature. Then 200 µl 2 N NaOH is added to each well for 1 h at room temperature. The lysed cells are transferred to scintillation vials (each well washed with additional 200 µl NaOH) counted for bound radioactivity with a gamma-counter (triplicate measurements).

Mitogenic activity was tested by thymidine incorporation. The dilution of the stock solutions of the various analogs and natural insulins was performed with the basic medium (F12/1 g BSA). The concentration range used was between 10 (-11) and 10(-6) M. Cells (50,000) were seeded in 24-well tissue culture plates and kept overnight in DMEM/F12 (1:l) with the addition of 10 % fetal calf serum at 37 °C. The medium was changed to DMEM/F12 1:l with 5 % fetal calf serum for a second overnight incubation period, followed by F12/1 g BSA/L for 30 min. The medium was then changed to 400 µl (200 µl in another study) F12/1 BSA + 40 µl (20 µl in another study) insulin analog or reference compound in the respective dilution. Cells were kept at 37 °C for 17 h; then 30 µl (20 µl in another study) 3H-methyl-thymidine was added (0.3 µCi/well). After 3–4 h at 37 °C, the medium was aspirated, plates were washed three times in cold PBS, and 500 µl 10 % TCA was added to each well for 30 min at 4 °C. After aspiration, wells are washed twice with 10 % TCA and dried at room temperature (30 min). Then 500 µl 1 N KOH is added to each well for 1 h at room temperature. Two hundred fifty microliters is removed from each well for liquid scintillation counting (gamma-counter, triplicate counting) after mixing the sample first with 250 µl 1N-HCI and second with 5 ml scintillation fluid.

The method for mitogenic potency described by Kurtzhals et al. (2000) is based on thymidine incorporation of the SAOS-B10 line with predominant IGF-I receptors and very few insulin receptors, previously used as SAOS cells extensively for research on chondrocytes and bone formation. The human osteosarcoma cell line Saos/B10 (20), a subclone of Saos-2 (ATCC HTB-85), was used by Kurtzhals. B10 cells express ~30,000 IGF-I receptors and <1,000 insulin receptors per cell. Cells were cultured in DMEM/F12 supplemented with 10 % FCS, L-glutamine, and penicillin/streptomycin. During the entire experiment, cells were grown and kept at 37 °C in a humidified atmosphere with 5 % CO_2. For experiments, cells were trypsinized, plated in 24-well dishes at ~75–100 3 103 cells per well, and cultured for 16 h. Growth medium was then removed and replaced with test buffer (DMEM/F12 without FCS but with 0.5 % BSA), and the cells were starved for 4 h. Then, the medium was withdrawn, and cells were incubated with test buffer containing 0.8 pmol/l to 0.8 mmol/l insulin analog for 16 h at 37 °C before addition of [^{3}H]methyl-thymidine. [^{3}H]thymidine incorporation was allowed to take place for 4 h at 37 °C. The cells were then washed three times with ice-cold phosphate-buffered saline and lysed in

10 % trichloroacetic acid for 15 min under constant gentle rocking at 4 °C. The soluble free [^{3}H]-thymidine was removed by three consecutive washes in ice-cold 10 % trichloroacetic acid. The high molecular weight part of the cell lysates was resolubilized in 1 mol/l KOH and neutralized with an equal volume of 1 mol/l HCl, and DNA-incorporated [^{3}H]thymidine was measured in a b-counter. Each concentration was assayed in triplicate for each experiment. The experiments were repeated three times. To compare the mitogenic potential of the insulin analogs with that of human insulin, log-dose response profiles were generated, and EC50 values were estimated using a 4-parameter logistic function (19). Relative mitogenic potency was calculated as the ratio between the estimated EC50 values (EC50,HI/EC50,analog), assuming parallelism of the log-dose response profiles.

The relevance of these mitogenicity estimates in a special cell line with predominant IGF-I receptors has been challenged, in a particular because insulin glargine, which showed slightly enhanced mitogenic activity in this test dissociate is rapidly from the insulin receptor (similar to human insulin), and does not stimulate the IGF-I receptor at therapeutic concentrations in humans, because it's affinity for the IGF receptor over the therapeutic dose range is much lower than that of endogenous IGF-I at physiological concentrations.

Modifications of the Method

These tests may be performed on cell lines in vitro or on tissue specimens ex vivo.

Critical Assessment of the Method

Mitogenicity estimates our guidance for subsequent evaluation of tissues in animal toxicology studies.

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Insulin and IGF-1 Assays

General Considerations

Methods for the quantitation and the determination of plasma concentrations of insulin, IGF-I, and the IGF binding proteins (not considered here in detail) have been developed based on diverse analytical principles such as radioimmunoassay, enzyme-linked assays, chemiluminescence assays, and other principles. Their particular application is for the measurement of concentration changes of insulin, IGF-I, and binding proteins during repeated dose treatment of animals, and of course the main application in clinical chemistry is to the evaluation of type 2 diabetes and growth disorders in children.

Purpose and Rationale

The main purpose is to establish a relation of pharmacokinetics and pharmacodynamics, namely, in states of insulin resistance (increased insulin concentrations) and during the treatment of diabetes (from decompensation before treatment to established glycemic control).

Modifications of the Method

Specific methods for determination have been established for insulin analogs, for example, aspart insulin (Andresen et al. 2000) and lispro insulin (Bowsher et al. 1999; Cao et al. 2001), and some of these assays are available commercially (e.g., Linco assay for lispro insulin). For other insulin analogs, methods have been adapted for pharmacokinetic determination (e.g., Mudaliar et al. 2000; Kuerzel et al. 2003 for insulin glargine). There are many sensitive assays for animal insulins and for human insulins, which can be adapted for animal experimentation (Temple et al. 1992; Clark and Hales 1994); the insulin assays have been particularly useful in establishing physiological and pathological secretory patterns (Polonsky et al. 1988a, b). Many of

the studies have been extended and supplemented by the measurement of human or animal C-peptide in order to assess endogenous insulin production and suppression of endogenous insulin during treatment (Ashby and Frier 1981; Bonser and Garcia-Webb 1981; Tillil et al. 1988). Measurement of excretion of C-peptide in the urine is also a helpful method of evaluating secretion rates.

Concerning the methods for the determination of IGF-1, many methods were developed for pediatric applications and subsequently their use has been extended to other questions (Teale and Marks 1986; Quarmby et al. 1998; Tchao et al. 1995; Moses et al. 1996; Frystyk et al. 1999; Juul 2003; Elmlinger et al. 2004, 2005; Khosravi et al. 2005; Yakar et al. 2005). Methods for the determination of IGF binding proteins have been reviewed (Rutanen and Pekonen 1991), and several options are now available. In this context, the classical assays for IGF-I activity also need to be mentioned but no longer have practical relevance for the evaluation of the effects of insulin analogs (Zapf 1998).

In the context of measuring circulating concentrations, it may also be helpful to measure of the adaptation of receptor concentrations in tissues by using the appropriate insulin and IGF receptor assays ex vivo (Pedersen 1983; Taylor 1984; Johansson and Arnquist 2006).

Critical Assessment of the Method

The selection of the appropriate methods for the measurement of insulin, C-peptide, IGF-I, and IGF binding proteins is facilitated by a number of options, based on laboratory developed methods and an increasing number of commercially available assays which have been extensively validated.

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Assessment of Metabolic-Mitogenic Ratio In Vitro

General Considerations

When structure activity studies are performed with insulin analogs, it is useful to determine the relative contributions of the hypoglycemic activity (metabolic) and the proliferative activity (mitogenic). Both activities are intrinsic to the insulin molecule; it is important to realize that

they are activated at vastly different concentrations in vitro and that the wide concentration range for testing is permissible in such studies. The metabolic-mitogenic ratio needs to be supplemented by data on the receptor association/dissociation, because experimental evidence for the [B10-Asp] insulin with enhanced mitogenic activity and causing tumor stimulation in rodent toxicology studies indicates that prolonged residence time and decreased rate of dissociation from the insulin receptor may be the critical factor. As a consequence, this information is critical a required for a new insulin analogs. When additional information about enhanced affinity for the IGF-I receptor is obtained, mitogenicity may be tested by methods described here in "Mitogenic Activity" and needs to be followed up by studies on mammary epithelial cells, when available also on other benign and malignant tissue-derived cell lines, and ultimately by assessment of carcinogenic potential in animals.

Purpose and Rationale

Insulin glargine (Lantus®) is a long-acting basal insulin analog that demonstrates effective day-long glycemic control and a lower incidence of hypoglycemia than NPH insulin. After subcutaneous injection, insulin glargine is partly converted into the two main metabolites M1 ([GlyA21]insulin) and M2 ([GlyA21,des-ThrB30]insulin). The aim of these methods is to characterize the glargine metabolites in vitro with regard to their insulin receptor (IR) and IGF-1 receptor (IGF1R) binding and signaling properties as well as their metabolic and mitogenic activities.

Insulin glargine (Lantus®, [GlyA21,ArgB31, ArgB32]insulin) is a long-acting human insulin analog with an almost peakless activity profile very closely mimicking the natural physiological profile of basal endogenous insulin secretion (Rosskamp and Park 1999; Lepore et al. 2000; Home and Ashwell 2002; Levien et al. 2002; Goykhman et al. 2009). Insulin glargine differs from human insulin by substitution of asparagine by glycine in position 21 of the A chain and by carboxy-terminal extension of the B-chain by two arginine residues. These alterations cause a shift in the isoelectric point from pH 5.4–6.7. Following subcutaneous administration as a clear solution of pH 4, insulin glargine precipitates at the injection site because of its low solubility at physiological pH. The prolonged blood glucose-lowering activity of insulin glargine may be a consequence of the subsequent slow dissolution of the microprecipitate on the basis of a low dissociation rate (Brange et al. 1990). However, the extended action profile of insulin glargine may be dependent on more than just a shift in its isoelectric point (Hilgenfeld et al. 1992; Berchtold and Hilgenfeld 1999).

Insulin glargine may be released from the microprecipitate by proteolytic degradation leading to soluble yet fully metabolically active metabolites. In vivo metabolism studies in rats and dogs have demonstrated significant plasma levels of two main metabolites of insulin glargine, M1 ([GlyA21]insulin) and M2 ([GlyA21,des-ThrB30]insulin), formed by the sequential removal of the two arginines from the carboxy-terminus of the B-chain and additional deamination of the threonine in position B30. The intermediate IM ([GlyA21,ArgB31]insulin) can be detected as minor species only (Kuerzel et al. 2001). This pattern of insulin glargine metabolism was also observed following its subcutaneous administration in healthy humans (Kuerzel et al. 2003). Interestingly, degradation to both M1 and M2 occurred at the site of injection and continued within the circulatory system, although with considerable variation in efficacy and time course between the individuals tested. Analysis of tissue samples from the site of injection revealed an average ratio of 50:50 for parent compound and M1/M2 (Kuerzel et al. 2003). In vitro incubation of insulin glargine in 69 sera for 30 min at 37 °C resulted in metabolizing of insulin glargine into M1 from 46 % to 98 % (Agin et al. 2007). The processing enzymes have not been identified so far, but metallocarboxypeptidases such as carboxypeptidases E, H, N, and U represent candidates for the rapid and efficient metabolism of insulin glargine (Agin et al. 2007). These enzymes are found in plasma and are likely to occur also in subcutaneous tissues (Agin et al. 2007).

The efficient generation of IM, M1, and M2 at the subcutaneous injection site and in plasma carries the notion that the proteolytic degradation products of insulin glargine contribute to the long-lasting systemic metabolic activity at least in part. In addition, it may shed new light on the current intense and controversial debate concerning the growth-promoting activity of human insulin and insulin analogs as determined in vitro (Kurtzhals et al. 2000; Le Roith 2007; Staiger et al. 2007; Shukla et al. 2009) and its in vivo relevance for insulin therapy (Eckardt and Eckel 2008). The growth-promoting effects of human insulin or insulin analogs are usually studied in cell lines under in vitro experimental conditions in the absence of IGFs and other physiological serum growth factors. However, several such studies have yielded conflicting results that may be due to experiment-to-experiment variability in specific cell lines and/or to the precise experimental conditions used (Sandow 2009; Smith and Gale 2009).

In addition, modification of human insulin by amino acid substitutions is known to change the signaling properties of the hormone. One prominent example is the aspartate modification in the B-chain of insulin, AspB10, resulting in a higher affinity toward both IR and IGF1R and a prolonged occupancy time for the IR (Kurtzhals et al. 2000). The fact that [AspB10]insulin has induced a higher incidence of breast cancer in a 1-year toxicity study in Sprague-Dawley rats (Dideriksen et al. 1992) and a higher proliferation rate of the osteosarcoma cell line Saos-2 (Kurtzhals et al. 2000) has led to the contention that insulins with a profile in vitro similar to [AspB10]insulin might also have a potential tumor-promoting activity.

The human insulin receptor is expressed as two isoforms as a result of alternative splicing. The two mature proteins IR-A (short form) and IR-B (long form) differ by the presence or absence of 12 amino acids encoded by exon 11 at the carboxy-terminus of the extracellular α-subunit and downstream of the carboxy-terminal sequence that is essential for ligand binding (De and Whittaker 2002; Kristensen et al. 1999). IR-A appears to be expressed predominantly in fetal tissues and cancer, whereas IR-B is expressed in normal adult tissues (Serrano et al. 2005). Importantly, there is considerable evidence that IR-A, which has the peculiar characteristic to bind not only insulin but also IGF-2, may play a critical role in the development of breast cancer (Sciacca et al. 1999), thyroid cancer (Vella et al. 2002), and leiomyosarcoma (Sciacca et al. 2002). In addition, a role of the isoform IR-A has been discussed for hybrid receptors, as increased expression of IR-A/IGF1R hybrids has been found in tumors (Frasca et al. 2008). Therefore, it is important to determine the metabolic and mitogenic as well as receptor binding activities of insulin glargine metabolites, which so far have only been reported in part for M1 (Kurtzhals et al. 2000). The methods described here are appropriate for the creation of IR- and IGF1R-mediated metabolic and mitogenic signaling profiles for human insulin, insulin glargine, IM, M1, and M2, including the differentiation between IR-A and IR-B.

For this, the affinity of human insulin, insulin glargine, and its metabolites to the IR isoforms A and B or IGF1R is analyzed in a competitive binding assay using SPA technology. Receptor autophosphorylation activities are studied via In-Cell Western in CHO and MEF cells overexpressing human IR-A and IR-B or IGF1R, respectively. The metabolic response of the insulins is studied as stimulation of lipid synthesis using primary rat adipocytes. Thymidine incorporation in Saos-2 cells is used to characterize the mitogenic activity.

Procedure

Receptor Binding Assays

The binding of the different insulins to the human IR-A and IR-B is analyzed in a competitive binding assay using the scintillation proximity assay or classical Scatchard plot analysis on the basis of radiolabeled human insulin as previously described (Rissler and Engelmann 1996; Cook et al. 2002; Kohn et al. 2007). Plasma membranes are enriched from CHO cells overexpressing either IR-A or IR-B (~1.3 × 105 IR-A per cell and ~2.2 × 105 IR-B per cell as

determined by FACS analysis) by a series of differential centrifugations including a single flotation through a one-step sucrose gradient. Briefly, cells are grown to confluence and gently detached, transferred to a centrifugation tube followed by centrifugation for 10 min at 600 × g at 4 °C. The pellet is resuspended in ice-cold 2.25 STM buffer (2.25 mM sucrose, 5 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1× Complete Protease Inhibitor) and disrupted using a hand-held Dounce homogenizer followed by sonication. This homogenate is transferred to a centrifugation tube, overlaid with 0.8 STM buffer (0.8 M sucrose, 5 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 1× Complete Protease Inhibitor), and centrifuged for 90 min at 100,000× g at 4 °C. The emerging pellicle at the interface is collected, transferred to a new tube, and washed two times with PBS by centrifugation for 10 min at 1,500× g. The final pellet is resuspended in a small volume of dilution buffer (50 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1× Complete Protease Inhibitor) and homogenized with a Dounce homogenizer. These plasma membrane preparations are stored until usage at −80 °C. Binding experiments are conducted in 96-well microplates. Per well 2 µg of membranes are incubated with 0.25 mg of wheat germ agglutinin polyvinyltoluene polyethylenimine SPA beads, 100 pM A14[125I]-insulin, and various concentrations of unlabeled insulins in a binding buffer containing 0.05 M Tris/HCl, pH 7.8, 0.15 M NaCl, 0.1 % BSA, and Complete Protease Inhibitor for 12 h at room temperature (23 °C). The radioactivity is measured at equilibrium in a microplate scintillation counter (Wallac MicroBeta, Freiburg, Germany).

Receptor Autophosphorylation Assays

CHO cells expressing either the IR-A or the IR-B isoform are used for IR autophosphorylation assays using In-Cell Western (Baus et al. 2008). For the analysis of IGF1R autophosphorylation, the receptor is overexpressed in a mouse embryo fibroblast 3T3 Tet-Off cell line (BD Bioscience, Heidelberg, Germany) that is stably transfected with IGF1R tetracycline-regulatable expression plasmid resulting in the expression of ~2.6 × 105 IGF1R per cell (as determined by FACS analysis, unpublished data). In order to determine the receptor tyrosine phosphorylation level, cells are seeded into 96-well plates and grown for 48 h. Cells are serum starved with serum-free medium αMEM (PAN-Biotech GmbH, Aidenbach, Germany) for 3–4 h. The cells are subsequently treated with increasing concentrations of either human insulin, IGF-1, IGF-2, or the indicated insulin analog for 15 min at 37 °C. After incubation, the medium is discarded and the cells are fixed in 3.75 % freshly prepared paraformaldehyde for 20 min. Cells are permeabilized with 0.1 % Triton-X-100 in PBS for 20 min. Blocking is performed with Odyssey blocking buffer (LI-COR, Bad Homburg, Germany) overnight at 4 °C. Anti-pTyr 4G10 (Millipore, Schwalbach, Germany) is incubated for 2 h at room temperature. After incubation of the primary antibody, cells are washed with PBS containing 0.1 % Tween20. The secondary anti-mouse-IgG-800-CW antibody (Rockland, Gilbertsville, PA, USA) is incubated for 1 h. Results are normalized by the quantification of DNA with TO-PRO3 dye (Invitrogen, Karlsruhe, Germany). Data are obtained as relative units (RU) and calculated as fold over basal.

Metabolic Activity Assay

The metabolic activity of the different insulins is compared using insulin-stimulated lipid synthesis in primary rat adipocytes. Primary rat adipocytes are prepared according to published procedures (Müller and Wied 1993; Frick et al. 1998). Briefly, rats (Sprague-Dawley, male, 140–160 g, fed ad libitum) are provided from Charles River (Sulzfeld, Germany) and killed by cervical dislocation in accordance with the locally relevant animal protection law. The animals are used for the preparation of primary adipocytes, exclusively, and are not subjected to any pretreatment, such as drug administration or starvation. Epididymal fat pads are rapidly removed, stripped of blood vessels, and placed in PBS. Subsequently, the pads are washed with Krebs–Ringer bicarbonate buffer (KRBB) (1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.8 mM KCl, 25 mM NaHCO3, 120 mM NaCl, 2.4 mM $CaCl_2$, containing 1 % BSA, defatted, fraction V, Sigma, Deisenhofen,

Germany, containing 37 μM glucose and adjusted to pH 7.4) and bubbled with 5 % O_2/95 % CO_2. Each pad is then cut into two or three pieces. Two pieces of each are incubated with 1.5 ml of digestion buffer (10 mg collagenase, Worthington Inc., CLS, type I, 190 units/mg, in 10 ml of KRBB containing 9 mg glucose) for 15–30 min at 37 °C in a shaking water bath (240 cycles/min). Released adipocytes are separated from residual undigested tissue by passage through a nylon web (mesh size 150 μm) and washed by flotation in a plastic tube two or three times with KRBB and adjusted to the appropriate titer of 3.5 × 105 cells/ml. For the distribution of the adipocytes, 200 μl of the suspension is removed under continuous gentle stirring with a plastic agitator.

Lipid synthesis is measured as the incorporation of [3-^{3}H]glucose into toluene-extractable lipids at a final cell titer of 0.7 × 104 cells/ml in KRBB according to published procedures (Frick et al. 1998) with the following modifications: 680 μl of KRBB is filled into 10-ml scintillation vials and supplemented with 100 μl of D-[3-^{3}H] glucose (2 μCi/ml KRBB, 1 mM, ARC) and 20 μl of insulin solution in KRBB. Lipid synthesis is started by the addition of 200 μl of adipocyte suspension in KRBB. After incubation for 90 min at 37 °C under an atmosphere of 5 % O_2/95 % CO_2 and gentle shaking, 10 ml of toluene-based scintillation cocktail (Quickszint 501, Zinsser, Germany) is added. The samples are vigorously mixed and, after phase separation (2–4 h), measured for radioactivity by liquid scintillation counting. The determination of the radioactivity contained in the toluene phase and corrected for nonspecific spillover of the radioactivity of unincorporated radiolabeled glucose contained in the water phase predominantly reflects the de novo lipid synthesis.

Mitogenic Potency

The human osteosarcoma cell line Saos-2 is obtained in frozen aliquots from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells are grown in McCoy's 5a medium (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (PAN Biotech GmbH, Aidenbach, Germany) and 2 mM (final) L-glutamine (Sigma Aldrich, Irvine, UK). Subconfluent cultures (9–15 × 106 cells per 225 cm^2 flask) are used to determine the mitogenic activity of the test compounds.

For measuring thymidine incorporation, 40,000 cells are seeded per well of a 96-well Cytostar-T scintillation microplate (GE Healthcare, Amersham, UK), and the plates are incubated overnight at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % O_2. The serum-containing medium is removed and replaced by 200 μl of serum-free McCoy's 5a medium supplemented with 0.5 % (w/v) BSA (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, and antibiotics (penicillin 100 units, streptomycin 100 units, amphotericin B 0.25 μg/ml final, Gibco, Grand Island, NY, USA). The plates are incubated for 4 h at 37 °C in a humidified atmosphere containing 5 % CO_2. One hundred fifty microliters of the medium is removed and substituted by 150 μl of serum-free medium containing the different insulin analogs or IGF-1 (Cell Sciences, Canton, MA, USA) at the indicated concentrations. Thereafter, the plates are incubated at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % O_2. After 19 h of incubation, 10 μl of [2-^{14}C]-thymidine solution (>50 mM, 3.7 MBq/ml) diluted in serum-free McCoy's 5a medium is added per well to yield a final concentration of 500 nCi/ml, and the plates are incubated for additional 6 h at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % O_2. ^{14}C-thymidine incorporation is measured in a Wallac 1450 MicroBeta Trilux Scintillation counter. Dose-response curves are obtained by testing ten different concentrations of the ligands with every concentration tested by octuplicate samples.

Evaluation

The binding of insulin glargine and its metabolites M1 and M2 to the IR using these methods are similar and correlated well with their corresponding autophosphorylation and metabolic activities in vitro. No differences are found toward the two IR isoforms A and B. Insulin glargine shows a higher affinity for IGF1R than

insulin, resulting in a lower EC50 value for autophosphorylation of the receptor and a more potent stimulation of thymidine incorporation in Saos-2 cells. In contrast, the metabolites M1 and M2 are significantly less active in binding to and activation of the IGF1R, and their mitogenicity in Saos-2 cells is equal to human insulin. These findings strongly support the idea that insulin glargine metabolites contribute with the same potency as insulin glargine to blood glucose control but lead to significantly reduced growth-promoting activity. The data unequivocally demonstrate similar metabolic signaling in vitro and activity in rat adipocytes of insulin glargine and its major metabolites with comparable engagement of IR-A and IR-B. In contrast, the insulin glargine metabolites are found to be less potent in mitogenic signaling and less active in Saos-2 proliferation assay compared to insulin glargine. The formation of insulin glargine metabolites may help to explain the established efficacy and safety of insulin glargine as observed in clinical practice and particularly in long-term clinical trials.

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Assessment of Hypoglycemic Activity In Vivo

General Considerations

Methods for the assay of hypoglycemic activity in animals are reviewed in chapter ▶ Measurement of Blood Glucose-Lowering and Antidiabetic Activity and will be discussed here only with reference to the application and structure activity studies with insulin analogs. For the two classes of insulin analogs of clinical interest, a different experimental design is needed.

Tests by intravenous injection indicate the intrinsic activity; human insulin may be used as the comparator (Lin et al. 1999).

The time-action profiles of fast-acting insulins are tested by subcutaneous injection in rats, rabbits, or dogs, with regular human insulin as the reference preparation. The drug concentration of test insulin and reference human insulin needs to be identical, for meaningful comparisons. When dilution is a compared, they should have the same insulin concentration. The preferred test is by using the under eluted insulin analog at the concentration equivalent to U-100. The test interval depends on the insulin dose selected; for fast-acting insulins, test periods of 4–6 h are considered sufficient.

The time-action profiles of long-acting insulin analogs are tested by subcutaneous injection preferably in dogs or pigs; as a preliminary procedure, they may also be tested in rabbits. Human NPH insulin is used as the comparator. The preferred test concentrations are those equivalent to U-100 insulin. The zinc content of formulations is often of interest and will be tested at increasing zinc content, keeping the insulin drug concentration constant.

Purpose and Rationale

The biological activity predicted by in vitro studies needs to be confirmed in animals; there may be considerable differences between the in vitro result and the in vivo activity due to the pharmacokinetics of the selected drug candidate. The first stage is always testing by intravenous injection to enable immediate contact with the insulin receptor.

Depot Activity of Insulin Analogs in Rabbits

Procedure

This test by subcutaneous injection is suitable for the evaluation of depot activity of long-acting insulins and insulin depot preparations. Adult male rabbits body weight 2,5–3,5 kg is suitable for the test; the animals may be used repeatedly when treatment-free intervals of at least 2 weeks (recovery periods) are inserted after each treatment day. In each group, eight to ten rabbits are treated on the test day. Insulin analogs and insulin depot preparations are compared for reference. The insulin analogs (long acting) or depot preparations of human insulin as reference compounds (NPH insulin) are injected subcutaneously, for example, at a dose 0.3 IU/kg by microliter syringes, using the undiluted original concentration (straight concentrations) equivalent to U-100 insulin.

Blood samples for glucose determination are obtained from an ear vein immediately before treatment and then hourly up to 8 h after subcutaneous injection. The blood glucose may be determined by enzymatic assay, for example, in 10 µl of native blood using a commercial test kit.

Evaluation of blood glucose data was performed descriptively and by explorative statistics. For descriptive evaluation of time-action blood glucose profiles, time point and extent of maximum decrease, centroid time, duration/maximal decrease ratio, and total area of blood glucose decrease are determined.

In this experimental design, the concentration of the insulins and the composition may be varied, for example, by changing the zinc content. To test statistically for dependency on insulin concentration or zinc content of the formulation, the drug concentration dependency and/or the zinc concentration dependency of two or more groups is compared using exact one-sided Wilcoxon tests (Hollander and Wolfe 1973; Streitberg and Röhmel 1987).

The test parameters are total peak area and ratio of duration to maximal decrease, accepting an increase within each of these parameters as the relevant test direction for increasing concentrations of drug (insulin) or zinc content (formulation). Each test is performed separately at a significance level of 5 %.

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Modifications of the Method

The principal modifications of this method are the selection of insulin concentrations to be tested. It is established that depot activity depends on local concentration at the injection site; therefore, it is advisable to inject smaller volumes by microsyringe, rather than using dilution of the original concentration to be evaluated.

Critical Assessment of the Method

Testing of depot activity in rabbits is an earlier option, but not as trustworthy as extended testing for depot activity in dogs or pigs.

Depot Activity of Insulin Analogs in Fasted Dogs

General Considerations

The time-action profile of long-acting insulin analogs after subcutaneous injection varies considerably depending on the species used for testing. In general, proof of depot activity cannot be obtained in mice and rats, is difficult to obtain in rabbits unless high dose is tested, and is readily obtained by testing in dogs or pigs.

Purpose and Rationale

The test procedure in dogs is based on experience with human NPH insulin and with insulin zinc suspensions (intermediate-acting insulins). It has shown to be very reliable in the preclinical evaluation of insulin glargine (Seipke et al. 2000).

Procedure

Adult male beagle dogs are used for this study; feed is withheld for 17 h. Groups of dogs (six to ten animals) are treated with the insulin to be tested for depot activity. Human regular insulin (soluble) and human NPH insulin are used as the reference compounds. Tests may be performed sequentially, in parallel, or by crossover. Insulin preparations are injected subcutaneously at the flank of the animal, alternating between the right and left flank. Blood samples for glucose determination are obtained by leg vein puncture, immediately before treatment and hourly up to 10 h after treatment; additional samples may be obtained 12, 14, and 16 h after administration. Blood glucose is determined enzymatically, for example, in 10 µl native blood using the Glucoquant test kit (Boehringer Mannheim) with the EPOS Analyzer 5060 (Eppendorf). For evaluation of the glucose data, means and standard error of the mean at each measuring point of the raw data [or using data converted to percent of the initial values] were used for tabular and/or graphical representation of the time response curves.

For statistical evaluation of the depot activity, centroid times are calculated, and for the evaluation of the blood glucose-lowering activity, the area of blood glucose decrease for each group is calculated. In both cases, a two-way ANOVA is performed at a significance level of 5 %.

The mean blood glucose decrease was calculated by the trapezoidal rule and the centroid time (Ct) by:

ti = times of measurement (i = 1,. . .,n)
c0 = base line glucose concentration
ci = glucose concentration at measurement time ti

Calculation of the centroid time and of the areas of blood glucose decrease is performed with the raw data. The point of onset and endpoint of the blood glucose decrease are defined by intersection of the blood glucose curve with the baseline in the descending or ascending phase, respectively.

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Mitogenic Risk and Safety Evaluation In vivo

General Considerations

An intense discussion was raised by the findings that one fast-acting insulin analog [B10-Asp] insulin showed unexpected toxicity on proliferation and tumor development of rat mammary glands. This finding was attributed tentatively to mitogenic signaling via the insulin receptor, and/or mitogenic signaling via the IGF-I receptor, based on the finding that on the insulin receptor residence time was prolonged and the rate of dissociation was much lower than found with human insulin, and that affinity for the IGF-I receptor was enhanced. This compound has therefore become the reference standard for the assessment of enhanced mitogenicity and its effect in toxicology. The preclinical evaluation of insulin analogs for the risk of enhanced mitogenicity therefore includes determination of the metabolic and mitogenic In vitro characteristics, by affinity for the insulin receptor and rate of association/dissociation, affinity for the IGF-I receptor, evaluation of enhanced mitogenic signaling e.g., by thymidine incorporation into proliferating cells, and details of signaling via the insulin receptor and IGF-I receptor (e.g., tyrosin phosphorylation, phosphorylation of receptor substrates).

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Oral Drug Delivery

Purpose and Rationale

So far the long path from intake through the mouth to the drug target within the body across the intestinal and cellular barriers has remained the major hurdle for the application of protein therapeutics in humans. The intestine is lined by a monolayer of epithelial cells, so-called enterocytes, which are surrounded by a bipartite plasma membrane. The apical plasma membrane area is directed toward the intestinal lumen and forms microvilli; the basolateral area is directed toward the blood vessels and faces underlying tissues. The enterocytes are interconnected to each other by specific protein complexes, so-called tight junctions, which seal the monolayer in a rigid fashion. Any nutrient, chemical, or drug taken up orally has two options for passage from the intestinal lumen into the circulation and the underlying tissues. It can either use the paracellular route thereby crossing the tight junctions or the transcellular route thereby consecutively crossing the apical plasma membrane, cytoplasm, and basolateral plasma membrane. Small-molecule drugs, molecular weight less than 500 Da, can engage both pathways. In contrast, macromolecules, such as proteins and nucleic acids, fail to use either pathway. In fact, the so-called oral bioavailability of even very small proteins, such as insulin, molecular weight about 5,000 Da, typically accounts for less than 1 %. During the past 60 years, biotechnologists and pharmacologists have undertaken enormous efforts to overcome these intestinal and cellular barriers for the application of putative protein drugs in humans (for reviews, see Uhrich et al 1999; Yokoyama 2005; Ebbesen and Jensen 2006; Kirpotin et al. 2006; Yang et al. 2007; Matsumara 2008; Soussan et al. 2009; Gullotti and Yeo 2009; Mok et al. 2009). In the following, two technologies and the corresponding pharmacological assays are presented which hopefully will fulfill the dream of needle-free administration of protein drugs. These are the encapsulation of protein drugs into microspheres or nanoparticles, which take the paracellular route, and their covalent modification with the so-called glycosylphosphatidylinositol (GPI) anchor, which acts as signal for transcellular transport.

Procedure

For in vitro testing of and discrimination between transcellular and paracellular transport of proteins, a cell culture-based so-called multiplex assay system was developed. For this, cultured enterocytes (e.g., CaCo-2) are grown in sealed monolayers with correctly formed tight junctions and adherent to a filter plate, which enables exchange of small-molecule nutrients and ions, only. This assembly separates the apical area above the filter plate from the basolateral area below the filter plate. The protein drug candidates to be tested are continuously applied to the apical area, and their transport across the cell layer is continuously monitored, either simply by conventional analytics of the incubation medium in the basolateral area or in case of using fluorescently labeled proteins by confocal laser scanning microscopy and subsequent virtual reconstruction of three-dimensional pictures from the original two-dimensional images. Thereby the transcellular passage of a fluorescent protein in the X-Z direction from the apical surface into the depth of the monolayer can be visualized directly in real time. Simultaneously, the electrical resistance between the apical and basolateral areas across the monolayer is measured which represents a parameter for the transcellular vs. the paracellular route (see below). In addition, putative effects of the protein drug during transcellular transport on glucose/lipid metabolism as well as protein/DNA synthesis can be detected in parallel. For this, scintillation cocktail is incorporated in the filter plate which monitors accumulation of radiolabeled glycogen, lipid, protein, and DNA formed in the adherent monolayer enterocytes from the corresponding radiolabeled building blocks. This multiplex assays system has meanwhile been adapted to an automated low-throughput scale.

Paracellular Transport of Protein Drugs by Encapsulation

As a representative example for paracellular transport of protein drugs by encapsulation, nanoparticles assembled from the biomaterials chitosan, alginate, mannans, and γ-polyglutamic acid at specific ratios are briefly discussed (Mathiowitz et al. 1997; Lin et al. 2007; Mathiowitz 2008). Along their paracellular route, these nanoparticles are faced with several challenges. First they have to adhere to the negatively charged mucus and glycocalyx layers of the small intestine, which is achieved by their positive net surface charge. Following infiltration of these layers, the nanoparticles have to trigger opening of the tight junctions, presumably by causing redistribution of some of their constituent proteins, e.g., F-actin and ZO-1, for their subsequent passage between two neighboring enterocytes. However, immediately thereafter, the tight junctions have to reseal in order to maintain the intestinal permeability barrier. Otherwise, the uncontrolled flux of nutrients and infectious components from the intestinal lumen into the circulation could lead to a catastrophe.

The opening and closure of the tight junctions during paracellular transport can be followed by measurement of the electrical resistance with the multiplex assay system introduced above. Immediately following addition of correctly assembled nanoparticles, the electrical resistance dramatically drops (Mathiowitz et al. 1997). After their removal by several washing cycles of the filter plate, the electrical resistance does not decline further and instead gradually increases with time to about the initial values within the next 30–60 min. This procedure can be repeated several times with similar results showing the transient and reversible nature of the opening and closure of tight junctions by these nanoparticles. Finally, after the arrival of the nanoparticles at the blood stream, they have to release their protein load, which is achieved by their dissociation. The assembly of this type of nanoparticles is strongly dependent on the pH. They are stable at pH 6.6 as adjusted during their production and dissociate at pH 7.4 as prevalent in the blood. Here the released protein drugs may exert their physiological action, or eventually they have to be further transported across the plasma membrane into the cytoplasm of the target cells (Mathiowitz et al. 1997). Importantly, there are many critical requirements for nanoparticles for (chronic) use in humans, predominantly concerning safety, non-immunogenicity, and non-accumulation in the body due to their proper degradation and excretion. So far, materials fulfilling all these criteria are not available, and consequently there is urgent need for the discovery and development of novel biomaterials.

Transcellular Transport of Protein Drugs by Covalent Modification

As a representative example for transcellular transport of protein drugs by covalent modification, the concept of glycosylphosphatidylinositol-anchored proteins, abbreviated GPI-anchored proteins in the following, is introduced here. In contrast to typical transmembrane proteins, GPI-anchored proteins are embedded in the outer leaflet of the plasma membrane by a specific GPI glycolipid which consists of phosphatidylinositol followed by a glycan core and a phosphodiester-ethanolamine bridge (Brewis et al. 1995; Nosjean et al. 1997; Ikezawa 2002; Orlean and Menon 2007). The protein moiety is coupled via its carboxy-terminus and an amide bond to this ethanolamine residue. In consequence, the protein moieties of GPI-anchored proteins are typically located at the surface of eukaryotic cells from yeast to man and can be released as soluble versions by cleavage with certain hydrolases, such as (G)PI-specific phospholipases C and D (Küng et al. 1997; Hoener et al. 1990; Müller et al. 1994a). Although the overall structure of GPI anchors is highly conserved from yeast to man, they differ considerably with regard to their fatty acid and glycan compositions. The glycan variants from humans, fungi, and protozoa differ with regard to their sugar constituents, side chains, and glycosidic linkages (Nosjean et al. 1997; Ikezawa 2002).

During the past 3 years, it became apparent that the GPI modification can operate as a signal for transcellular transport. These findings were initially obtained with adipocytes from young and old mice or rats. As is typical for all eukaryotic

cells, also adipocytes express GPI-anchored proteins at their cell surface inserted into plasma membrane lipid rafts (Müller et al. 1994b; Varma and Major 1998), i.e., detergent-insoluble glycolipid-enriched membrane microdomains (Brown and London 1998). However unexpectedly, it is found that in primary and cultured rat and mouse adipocytes, certain GPI-anchored proteins, among them the (c)AMP-binding and degrading proteins, Gce1, and CD73, are associated with both plasma membrane lipid rafts (major portion) and lipid droplets (minor portion) (Müller et al. 2008a, b). These observations represent the first examples for intracellular residence and function of GPI-anchored proteins. Importantly, this subcellular distribution of Gce1 and CD73 is detected in basal, i.e., unstimulated, adipocytes. However, upon their challenge with certain seemingly unrelated stimuli, such as the antidiabetic sulfonylurea drug glimepiride (Müller 2005), hydrogen peroxide, or palmitic acid, which all regulate glucose and lipid metabolism in adipocytes, the two GPI-anchored proteins completely disappear from the plasma membranes and simultaneously appear at the lipid droplets in time-dependent fashion (Müller et al. 2008c, d). This unequivocally demonstrates the stimulus-induced transport of Gce1 and CD73 from the cell surface onto the surface of cytoplasmic lipid droplets. As a prerequisite, the GPI-proteins have to undergo redistribution between distinct subspecies of the heterogeneous plasma membrane lipid rafts, which are characterized by different flotation and solubilization behavior (Müller and Frick 1999). Earlier observations with rat adipocytes have revealed the translocation of Gce1 from "typical" lipid rafts of high cholesterol content and low buoyant density to "atypical" ones of lower cholesterol content and higher buoyant density in response to glimepiride (Müller et al. 2001a, b), which apparently is required for its insulin-mimetic activities (Frick et al. 1998; Müller 2005). Gce1 and CD73 cooperate in the degradation of cAMP through phosphodiesteratic cleavage by Gce1 to AMP and further to adenosine through nucleolytic cleavage by CD73. Thus, the coordinated transport of Gce1 and CD73 from plasma membrane lipid rafts to cytoplasmic lipid droplets in response to palmitate, glimepiride, and hydrogen peroxide results in lowering of the cAMP levels at the lipid droplet surface zone and consequently in the coordinated upregulation of esterification and downregulation of lipolysis in adipocytes (Müller et al. 2008e). This apparently represents (one of) the physiological role(s) of this unique transport pathway of Gce1 and CD73 in adipocytes.

Subsequently and unexpectedly, the transport of these GPI-proteins with major but not exclusive localization at the adipocyte cell surface was recognized not to stop at the surface of cytoplasmic lipid droplets. It is known since decades that almost each mammalian cell type is capable of releasing small membrane vesicles either by exocytosis (then they are called exosomes) (Stoorvogel et al. 2002; Thery et al. 2002; Fevrier and Raposo 2004; Keller et al. 2006) or by plasma membrane shedding or blebbing (then they are called microvesicles) (Black 1980; Poste and Nicolson 1980; Piccin et al. 2007; Cocucci et al. 2009). Both exosomes and microvesicles are thought to reflect the physiological or pathological state of the releasing cell and may be used as novel and putatively valuable biomarkers for the personalized diagnosis of common complex diseases (see also above). In addition, Aoki and coworkers (Aoki et al. 2007) and Müller and coworkers (Müller et al. 2009a, b) have recently found that also primary and cultured rat and mouse adipocytes release microvesicles and exosomes. Surprisingly, the GPI-anchored proteins, Gce1 and CD73, can be identified as constituent components of those secreted vesicles, already in the basal state (Müller et al. 2009a, b). However, their incorporation into the secreted vesicles is strongly upregulated in response to glimepiride, hydrogen peroxide, and palmitate. Thus, the same stimuli that induce the transport of these GPI-anchored proteins from plasma membrane lipid rafts to cytoplasmic lipid droplets trigger their subsequent transport from the lipid droplets into the membranes of small vesicles, which then leave the adipocytes. In fact, these two transport steps in concert appear to mediate the transcellular transport of Gce1 and CD73 from the cell surface via cytoplasmic lipid droplets into the secreted vesicles (Müller et al. 2010c, d).

Transcellular Transport of GPI-Anchored Proteins Across Enterocytes

Is this transcellular transport of GPI-anchored proteins specific for adipocytes, only, or also operative in other mammalian cells, such as enterocytes? Preliminary data based on animal experiments argue for the last possibility (Müller et al. unpublished data). For this, human insulin covalently modified at its carboxy-terminus with the GPI anchor and produced by recombinant technologies is used as model protein. This GPI-anchored insulin is labeled with immunogold to enable visualization by electron microscopy. Following oral administration of the GPI-anchored insulin embedded in either micelles or liposomes to normal rats by gavage, the intestine is removed and sectioned. Subsequent analysis of the sections for immunogold-labeled GPI-anchored insulin by electron microscopy reveals rapid association of the GPI-anchored insulin with microvilli of the apical plasma membrane immediately upon its oral administration. The apparently spontaneous insertion of the GPI-anchored insulin into plasma membrane lipid rafts can be further demonstrated by immunoblotting of intestinal subfractions of the plasma membrane with antibodies against human insulin and raft and non-raft proteins. Both GPI-anchored insulin and Gce1 are found to be highly enriched with lipid rafts compared to "non-lipid rafts" of the fractionated intestinal apical plasma membrane along with the corresponding marker proteins, the glucose transporter GLUT2 and the fatty acid transporter CD36. Presumably, the GPI-anchored insulin becomes first inserted into lipid rafts of high cholesterol content and low buoyant density, which are expressed in large amounts in enterocytes (Danielsen and Hansen 2006, 2008). Thereafter, it is translocated within the outer plasma membrane leaflet to lipid rafts of lower cholesterol content and higher buoyant density prior to transport from these "atypical" lipid rafts to cytoplasmic lipid droplets (Müller et al. 2001a, b). Both spontaneous and protein-mediated incorporation of GPI-anchored proteins presented in micelles or liposomes or at the surface of donor cells into the surface of acceptor cells has been amply documented in vitro and in vivo (Zhang et al. 1992; McHugh et al. 1995; Ilangumaran et al. 1996; Medof et al. 1996; Civenni et al. 1998; Kooyman et al. 1998; Nosjean and Roux 1999; Suzuki and Okumura 2000; Premkumar et al. 2001; Milhiet et al. 2002; Morandat et al. 2002; Ronzon et al. 2004). However, so far no therapeutic applications have been derived from this specific characteristic of GPI-anchored proteins.

Following the association with lipid rafts, the immunogold-labeled GPI-anchored insulin can be observed inside the enterocytes in the lumen of small closed membrane vesicles, presumably so-called caveolae (Lisanti et al. 1994; Mayor et al. 1994; Parton et al. 1994; Fivaz et al. 2002), in the immediate neighborhood to the inner leaflet of the apical plasma membrane. Shortly thereafter the immunogold-labeled GPI-anchored insulin appears at the surface of cytoplasmic lipid droplets, which are known to be expressed in enterocytes in large amounts (Zhu et al. 2009). Finally, almost no immunogold-labeled GPI-anchored insulin can be found left inside the enterocytes, which may be taken as indication for its subsequent transport across the basolateral plasma membrane into the blood stream, possibly inserted into the membrane of secreted small vesicles. Eventually after being released from the vesicle surface through cleavage of the GPI anchor by serum phospholipases, free insulin is available for distribution via the circulation to relevant target tissues, such as the liver, muscle, and fat, and exert its physiological action in course of binding to the corresponding insulin receptors.

Since the physiological role of insulin is lowering of blood glucose, this putative effect of orally administered GPI-anchored insulin can be tested in vivo with insulin-free diabetic rats (Müller et al. unpublished results). As expected, oral administration of normal, i.e., unmodified, human insulin by gavage does not result in detectable plasma insulin levels as well as lowering of the high blood glucose levels characteristic for these diabetic animals. In contrast, oral administration of GPI-anchored insulin produced in mammalian cell culture provokes dramatic increases in plasma insulin and in parallel pronounced

decreases in blood glucose. As a control for the role of the GPI modification, the oral administration of GPI-anchored insulin from which the GPI anchor had been removed by bacterial phospholipase C cleavage is completely ineffective. In conclusion, GPI-anchored insulin is apparently transported from the intestinal lumen across the enterocytes into the circulation, then distributed to the liver, muscle, and adipose tissues, subsequently released from the GPI anchor and finally triggers insulin signaling which results in blood glucose decrease.

These proof-of-concept experiments were then repeated with GPI-anchored insulin produced by two different yeast strains rather than in mammalian cell culture (Müller et al. unpublished data). Unexpectedly, the time-action profiles of the three GPI-anchored insulins, which differ only with regard to the structure of the glycan cores of their GPI anchors exhibit significant differences. The "fungal" GPI-anchored insulins produced by *Pichia pastoris* and *Saccharomyces cerevisiae* induce very rapid and delayed onsets, respectively, of the plasma insulin increases compared to that elicited by the "mammalian" GPI-anchored insulin. The time courses of the plasma insulin increases are correlated well to the corresponded blood glucose decreases. Thus, apparently the composition of the glycan core determines the pharmacokinetic profile of the GPI-anchored insulin, at least in part, which presumably relies on its interaction with the recently identified phosphoinositolglycan (PIG) receptor (Müller et al. 2002a, b) and on its accessibility to cleavage by the serum phospholipases C/D (Bütikofer and Brodbeck 1993; Küng et al. 1997; Müller et al. 2005). Moreover, the glycan core harbors intrinsic immunogenic potential. Unfortunately, the complexity increases further since considerable variation in the time-action profiles for a given GPI-anchored insulin can be observed between different animals in the same experiment as well as between different experiments for the same animal (Müller et al. unpublished data). These deviations are, at least in part, caused by both intraindividual and interindividual variability in the concentrations of the serum hydrolases cleaving off the passenger protein from the GPI anchor within the spacer element. This spacer which separates the passenger, here insulin, from the anchor is thus critical for the pharmacodynamic profile and its variability elicited by a therapeutic GPI-anchored protein. Systematic variation of all structural elements of the GPI anchor by genetic manipulation and expression in different microorganisms, as already shown for *S. cerevisiae* and *P. pastoris*, will be crucial for their fine mapping. No doubt, these data will support the engineering of novel therapeutic proteins and antibodies with the crucial advantages of oral administration, selective targeting to and into the relevant diseased target cells, and curing their defects in the underlying susceptibility gene products with the desired pharmacokinetic and dynamic profiles.

Evaluation

The intention of this chapter is to introduce two novel strategies for oral delivery and tissue and intracellular targeting of therapeutic proteins and antibodies. They should facilitate the future development of personalized polypharmacy based on multiple and individual combinations of orally available protein drugs for correction of the multiple susceptibility genes defective in common complex diseases. Meanwhile the successful sequencing of the collective genome of all our resident microorganisms, the human microbiome (Nelson 2010; Zhao 2010), may support the identification of novel microbial biomaterials appropriate for nanoparticle formation and protein modifications enabling transcellular transport with potential for therapeutic application in humans. Ultimately, all these strategies should overcome the limitations inherent of injection of a single drug, only, to each patient affected by a given common complex disease. This is best exemplified by the current non-oral monopharmacy of type II diabetes with long-lasting insulin derivatives.

Oral Gene Therapy

In addition to the transport of therapeutic proteins and antibodies, the GPI modification could also

mediate the oral delivery, tissue-specific targeting, and cellular penetration of therapeutic nucleic acids (Xie et al. 2006; Kim and Rossi 2007; Martin and Caplen 2007; Gao and Huang 2009). Currently, the process of coupling siRNA or microRNA directed against diabetes susceptibility genes to the GPI anchor by standard chemical cross-linking procedures has been initiated. It will be interesting to see whether these constructs are taken up by the enterocytes and then transported via cytoplasmic lipid droplets to the cytoplasm and/or into the nucleus for specific, efficient, and stable downregulation of the expression of the corresponding gene products. Alternative systems for the efficient cellular delivery of therapeutic siRNA have been reported previously, among them polyelectrolyte complex micelles of six-arm polyethylene conjugates of the siRNA and a cell-penetrating peptide with a cross-linked fusogenic peptide (Choi et al. 2010). In general, GPI modification could enlarge our currently rather limited repertoire of subcellular targeting strategies for drug design and delivery (Rajendran et al. 2010).

Moreover, the GPI modification technology opens the possibility for oral nonviral gene therapy. The US BioTech company Genteric (Alameda, California) has previously demonstrated the efficient and reliable uptake of "naked" DNA, the so-called gene pill, by enterocytes in cell culture and in vivo (rats and mice) leading to marked expression and secretion into the intestinal lumen of the corresponding vector-encoded secretory gene products, e.g., growth hormone and insulin (press release). Possibly, the underlying molecular mechanisms involve caveolae-mediated endocytosis as has been elucidated for the promotion of uptake of plasmid DNA and efficient gene expression by poly (glycoamidoamine) vehicles (McLendon et al. 2010). In case of "oral transfection" with vectors encoding genes for therapeutic GPI-proteins and antibodies, their expression in enterocytes should result in incorporation into the outer leaflet of the apical plasma membrane lipid rafts (Fivaz et al. 2002). From there, i.e., from the cell surface, the described transcellular transport route across the enterocytes via caveolae and lipid droplets into the circulation presumably bound to secreted vesicles should be initiated. Upon release from the secreted vesicles and the GPI anchor, the therapeutic proteins and antibodies could exert their physiological action in the plasma or be further transported to relevant target tissues. So far this complex journey of ectopically expressed GPI-proteins starting at transiently nonvirally transfected enterocytes and proceeding via their apical plasma membrane, endocytic vesicles/caveolae, lipid droplets, and secreted vesicles into the circulation is pure speculation and has still to be demonstrated in vitro and in vivo. In case of operation of this path, a number of critical points remain to be clarified, such as stability and variability of the gene expression, choice of appropriate nonviral vectors, gastric degradation of the DNA, formulation, gene dosage precision, and the optimal time-action profile (presumably restricted to acute therapy). However, the putative benefits of nonviral gene therapy in general (Li and Huang 2007), and of its oral version, in particular, with its tightly regulated, specific, reversible, and short-acting control of gene expression on the basis of the rapid and efficient turnover of human enterocytes in vivo will justify corresponding future experimentation.

In addition to oral therapy, the use of GPI-modified siRNA or neutralizing antibodies may be useful for the rapid and reliable validation of putative drug targets in vitro and in vivo. So far target validation is frequently based on the use of small-molecule inhibitors which, however, typically suffer from their unknown to limited (at best) specificity toward the pharmacological target envisaged. In contrast, the introduction of siRNA or neutralizing antibodies into the nucleus and cytoplasm of relevant target cells in course of their GPI anchor-mediated transport across the plasma membrane will lead to highly specific inhibition of expression and activity, respectively, of the putative target. This technology could supplement our currently available tool box for state-of-the-art validation of novel pharmacological drug targets relying on in vitro incubation of primary or cultured target cells or on oral administration to relevant animal models of the disease.

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Personalized Diagnosis and Therapy

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Contents

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General Considerations

Personalized medicine, i.e., the use of information about a person's genes, proteins, metabolites, and environment to prevent, diagnose, and treat disease, has been much talked about in recent years. So some observers are wondering what the excitement is all about cumulating in the following statement: "Personalized health care is nothing new. Doctors have always tried to fit the therapy to the patient's need if possible." But what has happened more recently is that one has now begun to go a level deeper, i.e., to explore the biology of the disease and its treatment at the molecular level. However, molecular medicine does not per se define personalized medicine, but the molecular tools are important as they should enable greater relevance in the information provided by corresponding diagnostic tests (see below) (Edwards et al. 2008; Weedon et al. 2006; Romeo et al. 2007; Hegel et al. 1999; Wildin et al. 2001; Grant et al. 2006; Rothman and Greenland 2005; Raeder et al. 2006; Hegele et al. 2000; Capell and Collins 2006; Delepine et al. 2000; Janssens et al. 2006; Xiayan and Legido-Quigley 2008; Figeys and Pinto 2001; Müller 2002, 2010a, b; Pearson et al. 2007; Risch and Merikangas 1996; Janssens and van Duijn 2008; McCarthy 2003; McCarthy et al. 2003; Stumvoll et al. 2005; Lyssenko et al. 2005; Florez et al. 2003).

Traditionally, medical diagnostics and therapy follow standards of care based on epidemiological

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studies of large cohorts that do not take into account the variability of individuals within a population. Personalized medicine is the use of new methods of molecular diagnosis, i.e., biomarkers (see below), in order to better manage a patient's disease or predisposition toward a disease. Personalized medicine aims to achieve an optimal medical outcome by helping physicians and patients to choose the disease management likely to work best for a patient's unique genetic and environmental profile. Such approaches may include genetic screening programs that enable a more precise diagnosis of diseases and subtypes or help physicians to select the type and dose of medication best suited to certain patients. The latter approach is also named pharmacogenetics, which studies the influence of genetic variation on drug response in patients. The total diagnostic and therapeutic segment of the personalized medicine market is currently estimated to be $US 24 billion in the United States and forecast to grow by 10 % annually, reaching $US 42 billion by 2015 (The new science of personalized medicine, Price Waterhouse Coopers, 2009).

Personalized Diagnosis

Introduction

As personalized means different things to different people, additional complementary ways of characterizing diagnostics may further help to distinguish different shades of gray in the personalized medicine spectrum. In the strictest sense, personalized diagnostics may consist exclusively of companion diagnostics, which are by definition geared toward supporting a therapy decision for a particular drug, patient by patient. At a more permissive end of the spectrum, personalized diagnostic tests may include (i) early diagnostics, which encompasses diagnostic products permitting the detection of a disease at very early stages of its development and thus gives more treatment options; (ii) prognostics, which means diagnostics that provides a prediction or estimate of the risk of developing a particular condition based on phenotypic, e.g., transcriptomic, proteomic, lipidomic or metabolomic, parameters or genomic, e.g., hereditary or gene-based, characteristics; and (iii) all other types of diagnostics. One may indeed argue that if diagnostics were not designed to improve treatment decisions for individual patients, one way of defining personalized diagnostics is it would not have much sense.

During the past decade, knowledge about the involvement of a multitude of susceptibility genes and novel complete mechanisms in the pathogenesis of complex and common multifactorial diseases, such as obesity and T2D, is accumulating (see above). Nevertheless, currently these novel findings have not been translated into corresponding methods and technologies for their prognosis and diagnosis (Müller 2010). This may be due to the relatively moderate predictive power derived so far from the determination of the individual complete genetic profile (genomics) which, in part, is caused by inadequate numbers of (combinations of) SNPs identified in susceptibility genes so far. For principal reasons, this obstacle cannot be overcome by a mere increase in sample size (Rothman and Greenland 2005; Müller 2010). However, it may be weakened by the inclusion of additional "downstream parameters" encompassing the complete patterns of proteins (proteomics), lipids (lipidomics), metabolites (metabolomics), and their fluxes (fluxomics) of the patient versus control subject (Gibson 2009; McCarthy et al. 2003; Janssens and van Duijn 2008; Rothman and Greenland 2005). Unfortunately, the present technologies for genomics, proteomics, lipidomics, and metabolomics are relatively cost-intensive. Nevertheless, this burden would be accepted facing the enormous social and economical consequences for the community and the health problems for the affected individual patient which are associated in the long-term with common complex diseases, in general, and obesity and T2D, in particular. However, the acceptance by the society for the need of a timely and reliable diagnosis is based on the availability of adequate options for prevention and therapy, which may include but must not be limited to advices for a (more) healthy lifestyle, such as food restriction and intensified physical exercise, albeit there is no doubt about their

benefit for the prevention of the majority of common diseases in the western countries. Clearly, the expenditure for personalized diagnosis, which has to be supported by all (affected as well as non-affected) members of the health-care systems, cannot be justified by the currently implemented strategies of non-personalized prevention and therapy. They simply divide the population into normal ("control") and affected ("case") and recommend first-, second-, third-line, etc. treatments. Thus, the currently available diagnosis (as well as therapies, see below) for common complex metabolic diseases do not fit to the multitude and heterogeneity of the underlying disease mechanisms.

Biomarkers

During recent years drug approval agencies, including the FDA and EMEA, are encouraging greater use of biomarkers and diagnostics in drug development and prescribing decisions, thus promoting the concept of companion diagnostics for drugs. The FDA recently started reporting a list of genomic biomarkers that it considers valid to guide the appropriate clinical use of approved drugs. The list is being updated on a quarterly basis and counted 32 valid genomic biomarkers in mid-September 2009. Most drug labels in the list provide pharmacogenetic information with no immediate recommendation for genetic testing. However, testing is "recommended" or "required" only in a few cases. On March 20, 2009, four biomarkers were "required" to be tested, three for cancer and one for infectious disease indications, indicating that the "personalization" process has just been initiated (Müller 2002, 2010; McCarthy 2003; Stumvoll et al. 2005; Lyssenko et al. 2005; Florez et al. 2003).

However, the FDA was prompted to publish its list following a marked increase over the last decade of approved drug labels containing pharmacogenetic information which currently account for about 10 % of the approved drug labels. This portion is expected to increase continuously. The EMEA's communication on the requirement for biomarker testing is less transparent than the FDA's, but its initiatives should not be overlooked. For example, the EMEA played a key role in requiring biomarker testing for Amgen's Vectibix, following the FDA's accelerated approval without specific testing requirements. In mid-2009, the EMEA also had a larger number of drugs (at least 11) for which biomarker testing was required. Hopefully greater harmonization between different regulatory agencies will develop over time through greater consultation, but also following pressure from clinician communities as stakeholders in one country push to implement practices already included in drug labels in other countries.

It is thus essential to discover biomarker molecules that are objectively measured and evaluated as an indicator for normal biological or pathogenic processes as well as for pharmacological responses to a therapeutic intervention. Indeed, biomarkers can help to achieve many of the objectives of personalized medicine. They may improve diagnostics and help to understand how a drug works, allowing earlier decisions on whether to continue molecule development in clinical trials. This is why many pharmaceutical companies seek to acquire innovative biomarkers. Some companies already sell diagnostic kits and treatments based on biomarkers (e.g., Roche Herceptin® used in the treatment of HER2-positive breast cancer). Moreover, the strong growth of the biomarker market is a huge opportunity for start-up and value creation. In 2007, the global total biomarker market represented $US 5.6 billion and is expected to grow to 20 billion in 2014, among which the global core genetic testing market is projected to exceed $US 6.6 billion (The Global Biomarker Report, Markets and Markets, 2009). Certainly, the market is driven by both the growing incidence of genetic diseases and the rising awareness and interest of consumers for genetic testing (Müller 2010; Pearson et al. 2007).

Beyond their contribution to improving our basic knowledge, new generation GWAS (using arrays with low frequency SNPs found through the large resequencing efforts of the "1000 genomes" and other whole genome sequencing international projects) should also lead, when

associated with NGS of targeted diabetic populations, to breakthroughs in the pharmacogenetics of T2D. This is a new domain of major interest for both the European 7th framework program and for the drug industry through their common Innovative Medicine Initiative (the September 2010, 3rd call is focused on diabetes and personalized medicine), and there is hope that further research in the area will improve care for patients suffering from obesity and T2D.

Patient-Tailored Multiparameter Proteomics Using Protein Chips

Purpose and Rationale

Proteins are classically analyzed by a huge variety of electrophoretic, ELISA, and liquid chromatography procedures that are typically time-consuming and labor-intensive and, in general, are not compatible with the high-throughput scale. Furthermore, multiplex measurements for several protein analytes by the currently available methods necessitate multiple divisions of the original sample with multiple separate tests for each analyte which is accompanied by considerable costs. In contrast, protein chips enable the simultaneous determination of many different proteins in a single test without the need for splitting of the original sample and are therefore much faster (minutes vs. hours), more convenient (one test for the determination of multiple analytes), and less expensive (40–20 %) than the classical, in particular ELISA-based, technologies. The basic principle of microarray technology was first introduced more than 20 years ago (Ekins 1989). The underlying theory stated that a tiny spot of solid-phase purified antibody provides substantially better sensitivity than when used in conventional fluid-phase immunoassay formats. Driven by large-scale genome sequencing projects, DNA microarray technology became the first application of this theory and has been widely used for gene expression profiling (Pease et al. 1994; Schena et al. 1995; DeRisi et al. 1997; Schadt et al. 2003; Morley et al. 2004). However, biological functions are performed by proteins rather than nucleic acids. Moreover, RNA expression levels are not always correlated well to protein expression levels, and it turned out to be almost impossible to predict the functional characteristics of a polypeptide encoded by a given gene simply based on its expression profiles (Gygi et al. 1999). Therefore, the focus on studies on protein structures, functions, and protein-protein interactions should facilitate a more thorough characterization of the physiological function of a given gene (Lueking et al. 2005; Chen and Snyder 2010; Lynch et al. 2004).

During the last decade, a large body of evidence has accumulated that protein chips may revolutionize this area by their intrinsic capability of very rapid and simultaneous handling of many samples and after special adaption also of multiparameter analysis, in combination with validity, sensitivity, robustness, miniaturization, and relatively low costs. Moreover, protein chips cannot only be used for the evaluation of polypeptides of any size but also for the determination of small non-proteinaceous analytes, such as lipids, carbohydrates, and intermediary metabolites (Lin 2010; Malinowsky et al. 2010; Mueller et al. 2010; Yang et al. 2011; Merbl and Kirschner 2011). This considerably expands the application profile of protein chips from mere biotechnological process analytics to various research areas in diagnosis, drug discovery, and therapeutic monitoring (Nielsen and Geierstanger 2004; Dupuy et al. 2005; Henares et al. 2008): (i) personalized medicine with its scope of individualized diagnosis as well as therapy; (ii) systems biology with its aim of understanding the pathophysiology of common multifactorial diseases at the level of cells, tissues, and organisms and of interacting signaling molecules and metabolic enzymes that form complex networks rather than linear pathways; and (iii) tissue engineering with its potential to provide functional organs which develop in vitro from (e.g., adipose tissue-derived) mesenchymal stem cells isolated from the corresponding patient and have to be analyzed for maintenance of the differentiated and functional state by implantable protein chips and biosensors.

Conventional Protein Chips

A protein microarray also termed a protein chip is a solid surface, typically made of glass, on which

thousands of different proteins, such as antigens, antibodies, enzymes, and substrates, are immobilized in discrete spatial locations, forming a high-density protein dot matrix. Abundance-analyzing protein chips are typically composed of well-characterized biomolecules exerting specific binding activities, such as antibodies, for the qualitative elucidation of patterns of many to all (protein) analytes from a complex sample, such as serum and cell lysates, or for the quantitative determination of the presence of a single to a few proteins of interest. They have been applied for protein expression analysis, biomarker identification, cell surface marker profiling, and clinical diagnosis (Dupuy et al. 2005). Function-analyzing protein chips have been constructed by printing a large number of separately purified proteins and used primarily to comprehensively investigate the biochemical characteristics and activities of those immobilized proteins (Merbl and Kirschner 2011; Hu et al. 2011; Templin et al. 2002; Zhu et al. 2001).

On the basis of considerable differences in size, structure, charge, and hydrophobicity of individual proteins, the generation and handling of protein chips are much more complicated and less straightforward and standardizable than those of DNA chips. Unlike DNA molecules, full-length polypeptides cannot be directly synthesized in vitro at high efficacy per se and since most proteins have to fold and to be posttranslationally modified in correct fashion. These processes rely on complex molecular machineries consisting of a multitude of translation factors, chaperones, and accessory components, which are not easily amenable for the use in microarrays. In consequence, the proteins used so far for the construction of high-content protein chips have to be individually expressed and purified. Since proteins have to fold correctly in order to remain in the active state, they are susceptible to inactivation due to loss of their native conformation if immobilized directly on a solid surface. Polypeptides vary considerably in their accessibility for direct chemical cross-linking or non-covalent adsorption or indirect binding via antibodies to a matrix or carrier surface. To complicate the matter, chip surfaces can be modified by only one or two types of chemical or biological groups so far. Together these limitations pose high challenges for the optimization of the immobilization of (protein) analytes at the slide surface of the different protein chip configurations.

Procedure

Fabrication: Chip Printing

For the fabrication of two-dimensional microarrays, the principal and most critical step relies on the efficient dispensing process of biological fluids leading to the accessible arrangement of dense, yet spatially discrete, uniform and homogeneous nanoliter spots on a substrate surface which guarantees the structural and functional integrity of the probe (MacBeath and Schreiber 2000; Delehanty and Ligler 2003; Delehanty 2004; Moore 2001; Barbulovic-Nad et al. 2006). In addition, the dispensing process should be compatible with low costs and minimal sample volumes as well as with low risk for mutual sample contaminations and sample damage. Two basic categories of printing techniques, contact and noncontact ones, can be distinguished. During contact printing, the biological sample becomes deposited in the course of physical contact of the printing device with the substrate. At variance, during noncontact printing, there is no physical contact between the device and the substrate (e.g., laser writing, inkjet printing, photolithography). Both of these array fabrication categories can be further classified into serial or parallel. During serial deposition, consecutively repeated operations of the printing device considerably slow down the fabrication efficacy. In this regard, parallel deposition with simultaneous printing of all spots constituting the array in a single operation is preferred for large-scale fabrication. Nevertheless, for current application, the techniques for serial deposition are far more advanced than the newer and more complex parallel ones and therefore are considerably more common in practice (Morozov 2005).

The direct contact between the printing device and the substrate is provoked by either solid pins, spit pins, nano-tips, or microstamps. Historically, contact printing was initially performed using a

single pin. Subsequently, methods were developed involving a multitude of pins that, however, do not encompass the complete array. Printed microarrays guarantee accurate quantitative analysis only, if the spots were of uniform morphology, i.e., identical spot-to-spot size, shape, and surface characteristics and precision in the positioning (Morozov 2005; Wang et al. 2003; Rose 2000; Blawas and Reichert 1998). The morphology of the spots and its uniformity is predominantly affected by the sample viscosity, substrate planarity, substrate surface properties, pin surface properties, and pin contact area. Moreover, the control of the robotic forward and backward movement of the pin and the regulation of environmental factors for constant humidity, air pressure, and temperature as well as the minimization of contamination with dust represent the most critical factors for spot uniformity. A pin velocity of forward movement exceeding a critical threshold may cause inacceptable high inertial forces leading to the movement of considerable sample volumes out of the pin and thereby to enlargement of the spots and mutual spot-to-spot contamination due to a large size (Barbulovic-Nad et al. 2006; Morozov 2005; Wang et al. 2003; Rose 2000; Blawas and Reichert 1998). In addition, pin printing is determined by the surface tension of the sample solution as well as its wettability on the substrate. The inherent danger of sample evaporation and drying out from the wells and pin channels is efficiently prevented by maintenance of a high constant humidity. Sample viscosity and as a consequence also the dispensed volume are critically influenced by the temperature, which therefore has to be controlled very precisely. Finally, dust and contamination will seriously interfere with the fabrication of high-quality microarrays and have to be reduced with maximal care in order to minimize the risk of pin clogging.

In contrast to the serial deposition by pin printing that independently of the final practice always leads to direct contact between the substrate surface and a stamp or pin, noncontact printing technologies are of considerable heterogeneity ranging from photochemistry-based methods (that rely on the chemical treatment of the substrate and subsequent exposure with UV light using photomasks and can be categorized into photolithography and direct photochemical patterning) to laser writing to fluid droplet dispensing to microstamps (Hengsakul and Cass 1996; Mooney et al. 1996; Pritchard et al. 1995; Jones et al. 1998; Kusnezow et al. 2003; Kramer et al. 2004). Each of these technologies manages to deposit a large number of sample biomolecules at varying degree in parallel fashion. The most recent technology of nano-tip printing relies on scanning probe microscopy that enables nanoarrays of high density with spots of submicron size. Noncontact printing is characterized by two main advantages, considerably lowered risk for contamination and drastically increased throughput. The separation of the printing device and the substrate at each time during the analysis dramatically decreases the probability of cross-contamination and transfer of sample fluid from the primary spot to neighboring ones. This makes extensive washing steps and repeated cleaning of the printing device between the individual printing operations unnecessary. Moreover, noncontact printing technologies probably have the greatest potential for further upscaling of throughput in microarray fabrication. The majority of noncontact printers manage to deposit the sample fluids in parallel with production of the complete microarray in course of a single operation.

Taken together, the ideal profile for an array printing system, the reliable and durable generation of uniform and small-sized spots in a reproducibly dense and precise arrangement accompanied by the need for a minimal volume of solution, and the avoidance of contamination and biomolecular damage as well as high costs are hardly fulfilled by the currently existing technologies. In particular, contact printing with solid and split pins suffers from tedious time and preprinting requirements, problems of contamination, pin clogging, tip deformation, droplet uniformity, and high costs, but nevertheless represent the most broadly applied technology in research laboratories in academia and industry at present on the basis of its ability to provide reproducible results. At variance, noncontact printing and, in

particular, the variant of inkjet technology may contribute to significant cost reduction, but with the current document printers are hampered by printing inaccuracies and the resulting formation of smeared and cross-contaminated satellite droplets of irregular shape and size. These disadvantages are also shared by the alternative technology of electrospray deposition with the additional problem of putative damage of certain biomolecules upon exposure to electric fields. This possibility of biomolecular denaturation holds also true for photochemical noncontact printing which has the potential for microarray fabrication at very high throughput.

Fabrication: Chip Surface

Selecting a proper surface for protein immobilization is crucial to the success of protein chips. An ideal surface should be able to retain polypeptide functionality with relatively high signal-to-noise ratios and guarantee both high protein-binding capacity and long half-life. Different slide surfaces are in use, including aldehyde- and epoxy-derivatized glass surfaces, so-called Fullmoon slides, or Schott NHS-derivatized slides for random linkage of the proteins through amine- (Jones et al. 1998; Kusnezow et al. 2003), nitrocellulose- (Kramer et al. 2004; Stillman and Tonkinson 2000), or gel-coated slides for coupling through diffusion and absorption (Angenendt et al. 2002; Charles et al. 2004) and nickel-coated slides for non-covalent binding of His6-tagged proteins (Feilner et al. 2005). In any case, the immobilization step has to operate in efficient, reliable, and quantitative fashion. This means that each polypeptide contained in the sample has to be retained at the chip surface, irrespective of its nature and abundance. This is complicated by the nature of the interaction of the individual sample polypeptide with the chip surface, i.e., the type and number of the amino acids involved in cross-linking or secondary bond formation, which typically is heterogeneous and cannot be predicted. This may further contribute to variable and nonquantitative immobilization.

The typical substrate plate used is glass slides covered with polyvinylidene fluoride, nitrocellulose membrane, or polystyrene. These materials are relatively soft, not excluding lateral spread of printed proteins, and hence enable only a limited density of polypeptides to be printed. Moreover, nitrocellulose membranes tend to generate high background and low signal-to-noise ratios for most purposes (Bussow et al. 1998; Lueking et al. 1999; Holt et al. 2000a). To circumvent these limitations, three-dimensional matrix arrays have been developed, in which the glass slides are coated with polyacrylamide or agarose to build a porous hydrophilic matrix, in which the proteins or antibodies are captured within the pores. Thereby, lateral diffusion is restricted, and the size of the printed protein spots diminished, thus leading to elevated maximal complexity of the chip (Guschin et al. 1997; Afanassiev et al. 2000). Protein activity is typically well preserved in those matrix arrays, and their protein-binding capacity is relatively high. In these regard, as a further improvement, soft lithography has been introduced to fabricate nanowells on polydimethylsiloxane sheets fixed on top of microscope slides. These nanowell-based chips have been used for the immobilization of substrate proteins for profiling of the phosphorylation specificity of more than 100 protein kinases from budding yeast (Zhu et al. 2000) and mammalian tyrosine protein kinases (Nielsen et al. 2003). The open structure of the nanowells provides physical barriers and enables the consecutive addition of distinct buffers that is critical for multistep protein chips. The main disadvantage of this technique is the need for a specialized machinery for loading the nanowells at high density.

Alternatively, proteins, antigens, or antibodies may be printed directly onto the plain glass slides, which are usually coated with a bifunctional cross-linker harboring two distinct functional groups, one reacting with the glass surface and the other one with the desired protein. Those microarrays have been shown to have the advantages of high sensitivity, extended dynamic range, and considerable spot-to-spot reproducibility. Moreover, upon immobilization of about 1,000 of protein spots to aldehyde-activated plain glass surfaces, high-density protein chips have been created which enabled the detection of polypeptides belonging to completely distinct protein

classes and assessed by different types of assays (Bussow et al. 1998; Lueking et al. 1999; Holt et al. 2000b; Guschin et al. 1997; Afanassiev et al. 2000).

After the immobilization step, the protein chip becomes incubated with an appropriate well-characterized molecular probe which typically is a highly specific antibody (including single-chain or other variants) but may also consist of peptide-major histocompatibility complexes, carbohydrate-binding lectins, protein-interacting anti-/lipocalins, protein-nucleic acids, or RNA aptamers (Tao et al. 2007). For demonstration of binding of the molecular probe to the chip surface, the probes have to be coupled to a dye, a fluorophore, or an enzyme which catalyzes a luminescent reaction. The light, fluorescence, and luminescence signals are detected by a multichannel laser scanner or CCD camera with high-resolution power resulting in typical patterns of (directly or indirectly) colored spots in regular arrangement. The higher the spot intensity, the larger the amount of the probe bound to the chip, the larger the amount of analyte immobilized onto the chip surface and thus contained in the sample. Data generation encompassing incubation of the chip with the probe, binding of the probe to the analyte, washing of the chip for removal of unbound probe, reading out of the chip for the spot intensity and computer-based data transformation, and calculating the actual analyte content require short periods of time only (typically 2–5 min for a single cycle). The number of samples that can be processed in parallel depends on the power of the chip printer used (see above). With the currently available microarrays, up to 104 spots can be applied onto a typical light microscopic glass slide. Future nanoarrays will enable the spotting of 106 samples per slide with a diameter as small as 250 nm and at distances as low as 100 nm, limited only by the resolution of the currently available scanners.

Categories and Configurations

After successful printing and immobilization onto the slide surfaces, the protein analytes are evaluated for diverse parameters, which classify the protein chips into two categories, analytical configurations for the elucidation of molecular identity, structure, or amount and functional configurations (Chen et al. 2008; Alhamdani et al. 2010; Jones et al. 2006; Hsu and Mahal 2006; Tao et al. 2008; Kumble 2003; Knezevic et al. 2001). In the case of functional protein chips, a large number of proteins contained in complex biological samples, such as body fluids, or the total proteome of a cell or tissue for a systems biology approach are typically spotted. There is no need for extensive biochemical characterization of the proteins prior to immobilization and chip analysis. The systematic screening for specific and divergent activities and functions encompasses protein-protein, protein-DNA, protein-carbohydrate, protein-lipid, protein-metabolite, and protein-drug interactions as well as the identification of enzyme substrates or the detection of (undesired) immune and toxicological responses.

During the past decade, different configurations of protein chips have been introduced into academic and industrial research applications which are basically discriminated by the mode of immobilization and detection of the sample analyte. Each of these configurations is characterized by specific patterns of advantages and issues. The so-called forward protein chip critically depends on the quantitative immobilization of the protein analytes by secondary bonds or covalent cross-links at the chip surface as well as their quantitative detection by the labeled molecular probes, such as antibodies. However, even in case of quantitative recovery of the analytes, it cannot be excluded that unspecific interactions and modifications involved herein will lead to the masking of certain protein epitopes which are recognized by the detecting probe, e.g., the antibody. By nature, the amino acids involved in the interaction of the protein analyte with the chip surface cannot be predicted and may vary with each procedure/cycle of the currently used immobilization techniques. As a consequence, the detection of the analyte will not be quantitative leading to underestimation of the analyte content with considerable variation between distinct measurements.

To circumvent these problems, the so-called reverse protein chip has been introduced which relies on the direct immobilization of the protein

analytes by binding to well-characterized immobilizing probes. This type of analytical or functional protein chip represents the most convenient and powerful multiplexed detection platform and is commonly used for determining protein expression, cell surface markers, and biomarkers as well as for clinical diagnosis. To correct for putative differences in the efficacy of the immobilization of the analytes and of the readout of the signal (e.g., "edge-effects" within the spots) in comparative studies (e.g., diseased vs. normal tissues) and in analogy to the typical two-color mRNA/gene expression profiling approaches, sample and control analytes are labeled with the two distinct dyes, e.g., Cy3 and Cy5, by chemical means, then combined at equivalent ratio, and finally transferred to the array. This procedure provides ratiometric quantitative data for the relative changes in protein abundance (Knezevic et al. 2001; Miller et al. 2003; Haab et al. 2001). The immobilizing probes, most often antibodies, are themselves coupled onto the chip surface by secondary interactions with coat materials (e.g., nitrocellulose) or covalent cross-linking to functional (e.g., amino) groups of the glass slide (Hamelinck et al. 2005; Wingren et al. 2007; Chen and Zhu 2006). In contrast to "forward" chips, the "reverse" chips can be controlled and normalized for the coupling of the immobilizing antibodies under standard conditions in order to compensate for eventual nonquantitative recovery of the protein analytes. This solely depends on the number of functional immobilizing antibodies coupled to the chip surface. In any case, the immobilizing antibodies have to be characterized for their ability to capture proteins out of the biological sample, since the arrayed antibodies may be prevented from interacting with their cognate protein analytes under the prevalent experimental conditions.

Within the "reverse" chip configuration, the analyte can be detected by its direct labeling which, however, enables only the determination of the relative abundance between distinct samples rather than their absolute quantitative measurement. For the evaluation of the absolute amount of the protein analyte, the so-called sandwich configuration has to be used that relies on the specific binding of a detecting 2nd antibody that recognizes an epitope distinct from the immobilizing 1st antibody and is labeled with a dye, fluorophore, or luminescent enzyme. This chip configuration, which in principle represents a multiplexed version of standard ELISA immunoassays, combines the high sensitivity, accuracy, and specificity of the "sandwich" approach with the throughput capability of microarray procedures (Nielsen and Geierstanger 2004; Dupuy et al. 2005; Henares et al. 2008). Determination of hundreds of proteins in complex biological samples, such as body fluids, can be performed by a single experiment using sample amounts which often enable only a single assay in the well of a microtiter plate. The specific and quantitative determination of the amount of protein analytes by abundance-based microarrays relies on two distinct analyte-specific probes, most often antibodies. The use of two probes/antibodies recognizing different epitopes of the same analyte, such as in typical ELISA sandwich immunoassays, circumvents the requirement for reliable labeling of the analyte and results in a highly specific and sensitive signal. However, as a consequence, the "reverse" configuration is less convenient for simultaneous measurement of many analytes and critically depends on the availability of two analyte-specific antibodies with nonoverlapping epitopes for each analyte. Importantly, "sandwich" protein chips have the advantage of exquisite selectivity for the protein analyte due to the simultaneous operation of two different antibodies.

At the end of the "sandwich" protein chip procedure, the signal is generated by chemically modified, e.g., fluorescently labeled, secondary antibodies, resulting in a convenient two-step procedure without the need for a separate staining step (Nielsen and Geierstanger 2004). Alternative signal generation strategies are based on commercially available biotinylated antibodies. The detection requires the incubation of the sandwich complex with Cy3-/Cy5-labeled streptavidin or other streptavidin variants, such as Texas Red conjugates (Wang et al. 2003) or streptavidin-R-phycoerythrin (SAPE)(Huang et al. 2001; Tam et al. 2002). The reported limits of detection are

in the 10-pg/ml range. A further increase in sensitivity was achieved by amplification of the fluorescent signal with the help of a second layer of SAPE linked to the first layer through an anti-SAPE antibody resulting in a fourfold elevation of the signal (Wodicka et al. 1997). Moreover, signal amplification in course of horseradish peroxidase-triggered tyramide radical formation has been demonstrated to considerably increase the number of biotin labels at the antibody spot (Woodbury et al. 2002). The tyramide radicals provoke the cross-linking of biotin (or a fluorophore) to all exposed tyrosine residues of any protein analyte. In contrast, classical ELISA-based technologies rely on streptavidin-horseradish peroxidase (HRP) or species-specific antibodies linked to HRP or alkaline phosphatase in combination with chemiluminescent substrates. Their enzymic cleavage results in the generation of light around the antibody spot that is recorded by a CCD camera. Both the sensitivity and the accuracy of "sandwich" configurations with chemiluminescent signal generation are typically comparable to those of plate ELISA and higher than those of standard fluorescence readouts (Moody et al. 2001; Wiese et al. 2001). Dynamic ranges for concentration measurements of typically two to three orders of magnitude (with any of these signal generation strategies) have been reported with intra- and inter-assay coefficients of variation usually below 3 % and 10 %, respectively. Their multiplexing capacity is limited by the maximal spot density only, which by no doubt will be increased in the course of future instrumental progress.

Nevertheless so far, "reverse" protein chips of either the direct labeling or "sandwich" configuration have not reached the robustness and accuracy of classical ELISA-based assays. These problems are caused in part by technical hurdles in the generation of antibody spots during microarray production which are uniform in amount, morphology, and surface characteristics (Nielsen and Geierstanger 2004). In this regard, gel-type antibody spots have considerable advantages compared to flat ones since they allow larger spaces between the immobilized capturing antibodies as well as exposure of their epitope-binding domains in the gel interior within a well-hydrated environment. This seems to ensure a higher portion of antibodies remaining in the correct three-dimensional functional conformation compared to antibody spots immobilized and dried up at the spot surface. In addition to isolated gel pads, glass slides uniformly coated with "HydroGel" have also been used for the printing of "reverse" antibody microarrays (Wang et al. 2002). This "HydroGel" is characterized by low intrinsic fluorescence background which leads to a further increase in their sensitivity. However, longer washing times are required (>30 min) for the gel-based arrays which for the sake of elimination of piercing are typically printed using a noncontact piezoelectric microarrayer. In addition, the immobilization capability of the capturing antibody may rely on its orientation toward the chip surface with the epitope-binding domains facing the bulk solution (Peluso et al. 2003). A major portion of the Fab' fragments which have been coupled via biotinylation of the reduced thiols at their hinge regions to a streptavidin-coated chip surface was demonstrated to remain in the fully active state, in contrast to the drastically reduced binding activity of randomly immobilized Fab' fragments. However, the analogous optimization of the orientation of full-length antibodies turned out to be accompanied by less pronounced advantages compared to Fab' fragments.

In any case, the overall performance of antibody microarrays critically depends on the quality of the capturing antibodies used for the "reverse" configuration. Even the highest sensitivity of the signal detection system will not compensate for low antibody performance. In principal, polyclonal, monoclonal, and recombinant antibodies are appropriate for "reverse" sandwich chips with high binding affinity being most important for efficient capturing antibodies. For multiplex detection, polyclonal antibodies can be used which, however, have to be purified by affinity chromatography due to their potential cross-reactivity with the capturing antibodies of the different analytes.

The identification of a pair of antibodies directed against the protein analyte, which are specific for distinct nonoverlapping epitopes, i.

e., with no or very little cross-reactivity of the detection or capturing antibody of one analyte with the corresponding antibodies for the other analytes in the multiplexed assay, and do not mutually impair binding to the analyte due to steric hindrance, is often tedious, long-lasting, and expensive and sometimes may even fail. Putative (partial) incompatibility of 1st and 2nd antibodies will be recognized in time during the chip development in course of assaying at varying titers in a single multiplexed experiment with minimal sample consumption. Putative cross-reactivity between the antibodies could result in false-positive signals or in reductions of the dynamic range of the array and thereby is critical for the development of the "sandwich" configuration. As a consequence, every "sandwich" assay has to be optimized, e.g., by decreasing the concentration of the detection antibody as much as possible to reduce cross-reactivity. The accompanying loss of signal may be compensated for by elevating the concentration of the capturing antibody. Thus, the proper adjustment of the concentrations of both detection and capturing antibodies is required for optimal performance of microarrays in the "sandwich" configuration.

For many secretory proteins, such as cytokines and hormones, appropriate reagents are commercially available, mostly from ELISA kits comprised of pairs of monoclonal or affinity-purified polyclonal antibodies, with demonstrated adequate performance in the microarray format. Those cytokine-/hormone-specific antibody pairs developed for ELISA applications during the last two decades can be easily transferred to microarray assays. At variance, very few ELISA antibody pairs have been developed so far for intracellular proteins. The majority of the commercially available antibodies against cellular proteins were introduced for different applications, such as immunoblotting, do not operate as pairs, and fail to capture the proteins out of total cell or tissue lysates, often due to the presence of detergent at high concentration. However, it is believed that antibody reagent companies will increase their efforts in the identification of antibody pairs for ELISA and bead assays, which should improve the availability of validated antibody pairs for the use in arrays. Nevertheless, in some cases, compensation for partial interference and cross-reactivity by normalization may be feasible and useful for a given antibody pair in case of lack of alternatives ones. Thereby, underestimation and high variance in the determination of the analyte content would be minimized. Faced with the problems of antibody cross-reactivity, insufficient sensitivity, and inadequate assay linearity during the development of multiplexed microarray assays, the generation of recombinant antibodies or antibody-like fragments will facilitate the availability of antibody reagents with adjusted affinity and specificity in the course of optimization of the epitope-binding domains (Schier et al. 1996; Chames et al. 1998). In addition, alternative capturing reagents, such as DNA or RNA aptamers (Brody et al. 1999), or alternative protein-binding scaffolds, such as anticalins and lipocalins (Beste et al. 1999; Hanes et al. 2000; Holt et al. 2000a, b; Lohse and Wright 2001), have been used in pilot experimental setups. However, their utility for assaying low-abundance proteins in complex biological samples remains to be demonstrated.

Evaluation

In summary, protein chips in "sandwich" configuration resemble standard ELISA immunoassays, also with regard to the lower limits of detection and accuracy, but enable measurement at high throughput and parallel scale, which is based on the considerable differences in the signal generation process. In theory, the sensitivity of miniaturized assays should be higher compared to macroscopic ones since the concentration of the signal-generating molecules on the surface of a microspot should result in a higher signal-to-background ratio at the identical analyte concentration (Ekins 1998). In practice, however, the "sandwich" configuration does not dramatically improve the sensitivity compared to standard ELISA plate measurements, even in case of operation of very sophisticated signal generation technologies. Nevertheless, "sandwich" microarrays have the potential to become a valuable tool for the measurement of clinical and diagnostic markers as well as infectious agents, which are

often detected with immunoassays in ELISA plate format, on the basis of very low costs per data point, requirement of low sample volume (µl-range), and handling of complex samples (body fluids and tissues). Thus, antibody microarrays in "sandwich" configuration have huge advantages for diagnostic purposes, when the amount of sample is limited (e.g., single drop of blood) and multiple analytes (e.g., 50–100 disease markers) have to be evaluated. In addition, the random screening of general patient populations without detailed indication for these biomarkers is facilitated by "sandwich" microarrays, also on the basis of low reagent consumption.

At variance to the "sandwich" configuration, the "direct labeling" configuration of antibody microarrays circumvents the need for a 2nd detection antibody (and, in consequence, may be of lower selectivity than the "sandwich" chip). For this the dye, fluorophore or (luminescent) enzyme is coupled directly to the protein analyte through secondary interactions or covalent cross-linking prior to incubation of the samples with the protein chip.

A principal problem with antibody-based microarrays of each category, i.e., forward or reverse, and configuration, i.e., "direct labeling" or "sandwich", represents the possibility of masking of the relevant epitopes that are exposed by the protein analytes and recognized by the capturing and/or detection antibodies by polypeptides also contained in the biological sample. The interaction of the protein analyte with a masking protein via secondary bonds may occur by chance or fulfill a physiological role. One example is represented by the matrix metalloproteinase-9 (MMP-9), that is involved in the disassembly of the basement membrane and promotion of angiogenesis. MMP-9 levels are elevated in tissues, blood, and urine of tumor patients, as revealed by quantitative zymography and immunoassays. This has been associated with the malignancy of various tumor types, e.g., gastric cancer, and with worse survival of the patients (Kubben et al. 2006; Fernandez et al. 2005; Roeb et al. 2001). Interestingly, extra protease activity bands were detected in the zymograms of urine samples from cancer patients and most often result from complex formation of MMP-9 with lipocalin-2 (Yan et al. 2001; Hofmaier et al. 2011). In vitro and in vivo studies demonstrated a physiological function of lipocalin-2 in the protection of MMP-9 toward autodegradation. Most importantly, the enzymic activity of the MMP-9/lipocalin-2 complex, but not of the levels of single MMP-9 and lipocalin-2, has recently been found to be significantly correlated with the depth of tumor invasion in esophageal squamous cell carcinomas, the survival of gastric cancer patients, and the prognosis for breast cancer patients (Deng et al. 2012; Sier et al. 1996; Kubben et al. 2007). Together these findings suggest that urinary MMP-9/lipocalin-2 complex together with the separate constituent components may be used as novel biomarkers for various types of cancer and for the noninvasive cancer diagnosis and prognosis.

For the rapid and reliable analysis of the free MMP-9/lipocalin-2 constituents and/or complexes thereof in patient samples, it is of crucial importance that (i) the protein chip manages to unequivocally discriminate between the complex and its constituents and (ii) the data obtained are not affected by the presence of free constituents and the formation of the complex, respectively, in the biological samples. For MMP-9 and lipocalin-2, these prerequisites have been fulfilled by two microarrays reported so far for clinical use. A reverse phase protein lysate array for the measurement of sole MMP-9 in patient tumor tissue samples was constructed by robotic printing of the serially diluted protein lysates onto PVDF-coated glass slides, subsequent probing with validated (by immunoblotting) and commercially available anti-MMP-9 primary antibodies, biotinylated secondary antibodies, streptavidin-biotin-peroxidase complexes (for signal amplification), and biotinyl-tyramide/hydrogen peroxide and streptavidin-peroxidase (for signal amplification) and final development using hydrogen peroxide (Jiang et al. 2006). A microplate-based ELISA has been introduced for the independent determination of sole MMP-9, sole lipocalin-2, and MMP-9/lipocalin-2 complexes in tumor tissues from gastric cancer patients as well as the urine from breast cancer patients by immobilization of the complexes via anti-MMP-9 antibodies

followed by detection using anti-lipocalin-2 antibodies. Importantly, the MMP-9- and lipocalin-2-specific microarrays did not detect the complex and, vice versa, the complex-specific microarray did not recognize MMP-9 and lipocalin-2 in their free forms (Kubben et al. 2007). As a general conclusion, the possibility of interference of antibody-based microarrays in the course of complex formation of the protein analytes, which may lead to negative or false-positive results, has to be investigated rigorously prior to their application.

GPI-(Anchored) Protein Chip

Unfortunately, the fabrication and operation of conventional protein chips of both "forward" and "reverse" configuration as described above may lead to nonquantitative detection and immobilization, respectively, of the (antibody- or directly labeled) analytes in course of (partial) masking of the epitopes recognized by the immobilizing antibody and detection antibody, respectively. The problems may be (partially) overcome by a novel type of protein chip that is based on glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-proteins). These GPI-protein chips are currently being evaluated and validated for numerous applications in "point-of-care-testing" for individualized diagnosis and health care as well as monitoring of the individual therapeutic outcome.

GPI-Anchored Molecular Probes

Typical transmembrane proteins span the phospholipid bilayer of the cellular plasma membranes through a single or several stretches of hydrophobic amino acids, the transmembrane domain(s), with large amino-terminal (carbohydrate- and disulfide bridge-harboring), and carboxy-terminal polypeptide domains facing the cell surface and cytoplasm, respectively. In contrast, GPI-proteins lack (a) transmembrane domain(s) but are embedded exclusively in the outer extracellular leaflet of the phospholipid bilayer by a covalently linked GPI structure (Müller et al. 1994; Nosjean et al. 1997; Ikezawa 2002). This glycolipid anchor consists of phosphatidylinositol and several distinct and specifically linked carbohydrate moieties, the glycan portion, and is coupled via its terminal ethanolamine residue through an amide linkage to the carboxy-terminus of the extracellular protein domain. Thus, none of the amino acids of the GPI-protein moiety is in intimate contact to the plasma membrane. Rather, the GPI-protein is associated with the cell surface solely through its GPI anchor, which can be specifically cleaved by a GPI-specific phospholipase C leading to release and solubilization of the protein moiety. GPI-proteins are expressed in all eukaryotic cells studied so far, from yeast to man, and fulfill diverse functions as cell surface receptors, enzymes, antigens, transporters and signaling, and cell adhesion molecules (Nosjean et al. 1997).

For application in the protein chip technology, it is important that in principle each soluble passenger protein, such as a binding protein, receptor, enzyme, or antibody, can be expressed ectopically as GPI-protein at the surface of eukaryotic host cells, such as the yeast *Saccharomyces cerevisiae*, Chinese hamster ovary cells, or human embryonic kidney cells, using recombinant DNA technology. For this, the relevant host cells have to be transfected with a plasmid harboring the cDNA derived from the corresponding passenger protein gene and appropriate 5′- and 3′-regulatory elements for its inducible/repressible transcription and translation. In addition, for biogenesis of the passenger protein as GPI-protein two targeting signals, signal sequences I and II have to be placed at the 5′-/amino- and 3′-/carboxy-termini of the corresponding gene/protein constructs (Caras and Weddell 1989). Signal sequence I directs the nascent GPI-protein through the endoplasmic reticulum, Golgi apparatus, and secretory vesicles to the plasma membrane along the typical secretory pathway. Simultaneously, signal sequence II drives the covalent coupling of the GPI anchor, prefabricated at the endoplasmic reticulum by stepwise glycosylation of phosphatidylinositol, to the GPI-protein precursor in the course of removal of signal sequence II by a transamidase reaction occurring in the endoplasmic reticulum (Orlean and Menon 2007). The molecular machinery including the genes involved as well as the structural features of the signal sequences I and II has been elucidated during the past two decades (Tiede et al. 1999; Kinoshita

et al. 2008). This knowledge will be helpful for the ectopic expression of GPI-modified/anchored versions of any soluble protein at the surface of host cells by preparing a chimeric cDNA in which its endogenous 5′- and 3′-terminal sequences are substituted for by those encoding signal sequences I and II, e.g., derived from the native GPI-protein. Moreover, the efficacy of the recombinant expression of any given (soluble) protein as GPI-protein variant will increase considerably upon the use of optimally engineered signal sequences I and II instead of its native counterparts derived from the authentic GPI-protein as well as of genetically engineered host yeast or mammalian cells which maximally express the rate-limiting molecular components (e.g., transamidase) of the GPI-protein biosynthetic pathway (Kinoshita et al. 2008; Englund 1993).

In the case of GPI modification/anchorage of antibodies as capturing/immobilization reagents for the construction of microarrays, intact mouse (monoclonal) antibodies suffer from the main disadvantage of their complex multichain structure and large size (∼150 kDa). Therefore, it has been tried to bypass these limitations of intact antibodies by the use of genetically engineered small (∼30 kDa) single-chain variable fragment antibodies (scFv) as demonstrated first for anti-CD20 scFvs as a strategy to target CD20-positive tumor cells (Hamdy et al. 2005). They are constructed by coupling of the variable heavy chain (VH) and the variable light chain (VK) domains of the intact antibody by a short flexible peptidic linker (Huston et al. 1991). Importantly, the exquisite specificity for the epitope-binding site and the pronounced affinity for the authentic epitope have been demonstrated to be preserved in scFvs (Hudson 1998). Unfortunately, due to the limited understanding of the impact of amino acid exchanges on protein folding, no a priori method is currently available for the prediction of the capability of a selected antibody to operate with the desired selectivity and affinity when produced as an scFv. As a consequence, prior to use for immobilization in protein chips, the scFvs in their GPI-anchored and non-anchored versions (since the former often will react nonspecifically with all cellular membranes) have to be analyzed and confirmed for the specific recognition of the relevant protein analyte epitope in comparison to the original antibody from the selected hybridoma. In conclusion, on the basis of their size and single-chain structure with the expression from a single transcript, scFvs are more accessible for the recombinant coupling to GPI anchors than intact immunoglobulin molecules. In fact, the strategy of modification of a scFv with a carboxy-terminal GPI structure and its efficacy was first demonstrated for the expression of an immune modulatory signal (5H7) at the surface of T- and B-lymphoid cells as GPI-anchored 5H7 variant (Kulkarni et al. 2000).

In general, for the conversion of an intact antibody molecule selected for capturing/immobilization of a given protein analyte during a microarray procedure into a GPI-tagged scFv by genetic engineering using standard recombinant technologies, DNA fragments encoding the VH and the VK domains were amplified from the total cDNA using degenerate primers which had been prepared from the corresponding hybridoma cells secreting the anti-analyte monoclonal antibody. For the design of the primers, the VH and the VK framework domains conserved between the immunoglobulin subfamily members were selected. The predicted VH and VK domains cloned from the hybridoma were coupled by a linker sequence resulting in the basic scFv construct (VH-Linker-VK) that was then modified to facilitate (i) its targeting to the typical secretory pathway (amino-terminal signal sequence I), (ii) its purification by immobilized metal affinity chromatography (carboxy-terminal His6-tag), (iii) its immunological detection (hemagglutinin [HA] 11-amino-acid peptide tag located carboxy-terminal to the His6-tag), and (iv) its anchorage at the cell surface by the posttranslationally added GPI structure (signal sequence II derived from the GPI-protein alkaline phosphatase and located carboxy-terminal to the HA-tag).

The GPI-modified scFv proteins can be expressed in High FiveTM insect cells with high yield and at relatively low costs and then easily affinity-purified in the native active state (Hamdy et al. 2005). In contrast to bacterially expressed scFvs which often require the solubilization and

subsequent refolding from inclusion bodies formed during the fermentation, insect-expressed scFvs do not require renaturation (Hamdy et al. 2005; Schodin and Kranz 1993; Nagarajan and Selvaraj 1999). In contrast to the expression of GPI-proteins in mammalian cells, insect-expressed scFvs are not inserted into detergent-insoluble lipid rafts of the endoplasmic reticulum, Golgi, and plasma membranes along the secretory pathway, but can be solubilized from the membranes by nonionic detergents at low concentration.

Alternatively, baker's yeast (e.g., *Saccharomyces cerevisiae*) and cultured cell lines (e.g., HEK-293) represent appropriate host cells for the expression of GPI-modified scFv proteins. After the upstream processing, which includes fermentation of the host cells in appropriate bioreactors and subsequent removal of the culture medium, total GPI-proteins will be extracted from the cell surface, preferably in selective fashion without total solubilization of the cells. For this, nonionic detergent, such as Triton X-100 or Triton X-114, is added at low concentration at low temperature. This results in disintegration of the "nonlipid" raft domains of the host cell plasma membranes under concomitant aggregation of the GPI-proteins together with cholesterol and (glyco)sphingolipids into the lipid rafts (Varma and Mayor 1998), which can be collected and enriched by sucrose gradient centrifugation on the basis of their low buoyant density. Finally, the GPI-proteins are solubilized by high concentration of detergent at room temperature and, if required, further purified by (several rounds of) conventional column chromatography. An alternative to this selective detergent extraction has recently been developed, the so-called magnetic extraction. It is based on antibodies which are directed against the glycan portion of the GPI anchor and covalently coupled to metal beads (Müller 2010).

In addition to the recombinant engineering of GPI-anchored scFv proteins acting as high-affinity capturing probes for protein analytes of any desired type, in principle, naturally occurring GPI-proteins with physiological receptor or binding function may be used for the immobilization of the corresponding ligands at the protein chip surface. The number of GPI-anchored receptor and binding proteins is steadily increasing. In fact, some of them are possible candidates for the immobilization at protein chips on the basis of their known ligands, which may serve as biomarkers of diagnostic or therapeutic relevance, such as the glypicans for the heparin-binding growth factors (Matsuda et al. 2003), the Ly-6/urokinase-type plasminogen activator receptors for the urokinase-type plasminogen activator (Shetty et al. 2003), the FcγRIIIB low-affinity IgG receptor for the Fc portion of immunoglobulins of the IgG class (Meknache et al. 2009), and the glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein-1, GPIHBP1, for lipoprotein lipase (Olafsen et al. 2010).

Immobilization of GPI-(Anchored) Proteins

The development of a stable and universal immobilization method for the capturing probe, e.g., antibody, which does not grossly affect its structure, affinity, and selectivity, is one of the most critical aspects and challenges of microarray fabrication. So far, a number of different methods have been introduced for the immobilization of GPI-proteins on solid chip surfaces, such as non-covalent adsorption, covalent binding, and affinity capture. With regard to the adsorption technique, the GPI anchor biosynthetically attached to antibodies or scFvs, which have been raised against the desired protein analytes, manages the non-covalent immobilization of the capturing probes at the chip surface. The underlying principle is based on the structure of biological membranes which represent natural nanostructures separating the intracellular components from the extracellular environment. They consist mainly of phospholipids, spontaneously assembling as a continuous spherical lipid bilayer structure where the hydrophilic polar heads shield the hydrophobic fatty acid tails from the surrounding polar cytosolic and extracellular environments. One of the major phospholipids of biological membranes is phosphatidylcholine (PC), which has a zwitterionic head group. The monomers comprising the PC heads have been synthesized with attention to the chemical

structure of the phospholipid molecules, and polymers derived thereof have been applied in the preparation of cell membrane-like structures and biomaterials. A polymer family containing 2-methacryloyloxyethyl PC (MCP polymers) is of particular interest for the modification of both SiO2-based and polymer-based chip surfaces because of its high performance in the suppression of nonspecific adsorption of proteins; subcellular structures, such as organelles; and cells, such as platelets. Consequently, MPC polymers have been used to form cell membrane-like interfaces for chip applications via adsorption (Sibarani et al. 2007).

The relevant phospholipid monomers can be synthesized with high yield and excellent purity. One of the representative MPC monomers is a methacrylate with a PC unit as a phospholipid polar group. Many phospholipid polymers based on MPC chemistry have been developed and studied for functionalized surface modification. The MPC monomer can copolymerize with various vinyl monomers to form phospholipid polymers having a wide variety of molecular architectures. They can be transformed to cell membrane-like surfaces by coating the polymer, blending with the polymer, or grafting to the polymer chains. Thereby they provide biointerfaces capable of suppressing many biological responses, such as nonspecific interaction with proteins, organelles, and cells (Müller 2010; Sibarani et al. 2007). In addition, with the incorporation of functional moieties enabling bioconjugation, these MPC polymers also form PC-covered surfaces capable of selectively interacting with specific biomolecules, such as GPI-proteins, among them antibodies and scFvs.

For the immobilization of GPI-antibodies, the microscopic glass slide is coated with a monolayer of phospholipids, which is facilitated by hydrophobic interactions between the saturated long-chain fatty acids and the glass surface and mimics the structure of the extracellular leaflet of the plasma membrane phospholipid bilayer. Upon addition of the anti-analyte GPI-antibodies/scFvs embedded in detergent micelles to the phospholipid- or MPC-coated glass slide, which can meanwhile be managed with sufficient reliability by piezoelectric standard printers commercially available for conventional protein chips, the antibodies/scFvs become spontaneously inserted into the phospholipid or MPC monolayers, solely in response to adequate dilution. This will result in the defined orientation of the GPI-antibodies/scFvs with the epitope-binding domain facing the chip surface at high density, which resembles that of GPI-proteins at the outer face of biological membranes upon their incubation with intact cells, liposomes, or reconstituted model membranes (Nosjean and Roux 2003; Sesana et al. 2008; Ilangumaran et al. 1996). In comparison to covalent cross-linking, the GPI anchorage of the anti-analyte antibodies/scFvs to this special type of "reverse" protein chip has the distinct advantage of higher efficacy and selectivity in combination with lower background due to unspecific adsorption of the analyte. This often represents a problem with bifunctional chemical cross-linkers due to their hydrophobic nature and broad specificity. Moreover, the covalent coupling of the GPI anchor to the carboxy-terminus of the capturing probe, such as scFvs, does not interfere with epitope recognition, in general, and by the amino-terminal VH and VK domains, in particular. This feature will guarantee high efficacy, robustness, reliability, and reproducibility of analyte immobilization with low variance between different measurements using the same chip or different chips, which have been prepared by independent spotting procedures with different batches of capturing probes.

With regard to spotting of the GPI-proteins, the predominant procedures evaluated so far are the contact and the noncontact printing. Metal pins with solid or quill tips are applied in contact printers to transfer volumes in the sub-nanoliter range of the GPI-protein samples to the slide surface. Quill pins have a higher sample capacity and enable the printing of hundreds of spots continuously after each sample loading. The printed spots are typically circular with the size depending to a major degree on the dimension of the tip, the material, and the chemistry of its surface and the buffer used. A considerable advantage of this type of printer is their speed and high throughput with loading of up to

48 pins and printing of up to 250 slides simultaneously. However, those pins are very fragile and expensive. Furthermore, the tip of the pins may damage the slide surface, in particular, in the case of using complex three-dimensional substrates, such as MPC polymer-coated slides and biointerfaces. In addition, GPI-proteins may expose hydrophobic surface domains, including the GPI anchor, causing unspecific adherence to metal. The typical routine washing steps may not be sufficient to get rid off them completely from the pins. Altogether, these features may result in considerable cross-contamination of GPI-protein samples and in carryover problems.

To overcome these issues, noncontact dispensing techniques have been introduced for printing GPI-protein microarrays, by which a small droplet of GPI-protein sample becomes delivered to the slide surface without touching it. The droplets are generated by conventional inkjet, piezoelectric pulsing, or electrospray deposition (Roda et al. 2000; Sloane et al. 2002; Avssenko et al. 2002). Unlike contact printing, the amount of liquid deposited by noncontact printers does not rely on the surface characteristics of the slide and the morphology of the spots on hydrophobic surfaces, such as the phospholipid or MPC monolayers (Dufva 2005). The major advantage of noncontact printers is their ability to print on artificial membranes, such as phospholipid and MPC monolayers, in addition to standard glass slides. However, those instrumentations typically suffer from longer printing times and from equipment with a lower number of pins, which represent major drawbacks in case of printing a large number of GPI-protein samples. In addition, noncontact printers are sometimes faced with the problem of misplacing the spots and/or the generation of satellite spots, that usually result in increased rate of failure (Hartmann et al. 2009). Furthermore, in comparison to contact printers, their noncontact counterparts typically require larger sample volumes, which is challenging and often leads to considerable expenditure and costs for the high-throughput recombinant production of GPI-proteins.

Upon implementation of the required tools and equipment, the expenditure for the generic production of the anti-analyte GPI-antibodies/scFvs and their immobilization onto the chip using versatile cassette GPI-protein expression vectors, magnetic extraction, and an automated standard printer for embedding into the phospholipid or MPC monolayer is usually lower compared to covalent cross-linking with regard to both time and costs. Cross-linking requires intensive testing of a multitude of chemicals and reaction conditions and a careful quality control for successful coupling of functional capturing probes to the chip surface.

Procedure

Operation of GPI-(Anchored) Protein Chips

Upon addition of the sample to the GPI-protein chip, the immobilized (protein) analyte is routinely detected by binding of an analyte detection probe, e.g., antibody, labeled with a dye, fluorophore, or luminescent enzyme, as is the case for conventional reverse protein chips of the "sandwich" configuration. This directly leads to corresponding light, fluorescence, or luminescence signals at "positive" spots of the array. To circumvent the need for the anti-analyte detection probe that has to be identified, produced, and labeled individually for each analyte and must not interfere with the immobilization of the analyte by the analyte capturing probe, i.e., GPI-anchored scFvs, the immobilized analyte can be detected by an indirect "competitive" mode (Müller 2010). For this, a GPI-modified version of the protein analyte is prepared by cell surface expression in recombinant immobilized yeast or adherent mammalian host cells using one of the versatile cassette GPI-protein expression vector systems. Thereafter, diacylglycerol is removed and a soluble version with phosphoinositolglycan (PIG) structure instead of GPI attached is generated by cleavage of the GPI structure with a GPI-specific phospholipase C. Importantly, the PIG moiety covalently coupled to the carboy-terminus of the protein analyte via a phosphodiester ethanolamine bridge harbors a terminal inositol cyclic phosphate moiety generated during the bacterial phospholipase reaction. The resulting PIG-analyte is recognized

by anti-PIG antibodies which specifically react with the inositol cyclic phosphate moiety and are labeled with a dye, fluorophore, or luminescent enzyme, similar to the 2nd anti-analyte antibodies used for conventional "reverse" protein chips of the "sandwich" configuration.

These labeled anti-PIG antibodies once raised can be generically used for the binding to any recombinant GPI-analyte upon its lipolytic conversion into the corresponding PIG-analyte. The labeled anti-PIG antibodies are incubated in excess with the PIG-analyte. The resulting complexes of anti-PIG antibody and PIG-analyte are then added in slight excess to the GPI-protein chip coated with a phospholipid or MPC monolayer and anti-analyte GPI-antibodies/scFvs. After removal of unbound complexes by washing, the immobilization sites, i.e., the anti-analyte GPI-antibodies/scFvs, of the chip are all saturated which will result in uniform light, fluorescence, or luminescence signals at each spot of the array. Subtle differences in signal strength between individual spots of the same chip or between the overall signals of different chips are caused by variations in spotting, embedding, and binding efficacies of the anti-analyte GPI-antibodies/scFvs and will be compensated for by normalization of the data provided by the laser scanner. Thereafter, the reference or sample probes containing the authentic, i.e., unmodified, protein analyte is added to the chip. This causes the displacement of the labeled anti-PIG antibody-PIG-analyte complexes from binding to the anti-analyte GPI-antibodies/scFvs. Thereby the signal which is emitted by these complexes at the corresponding array spot positions becomes reduced. The competition curve with the signal strength decreasing with increasing concentrations of reference analyte is calibrated similar to typical RIA/ELISA procedures and then used for calculation of the analyte concentration in the sample.

Single-Parameter GPI-(Anchored) Protein Chips

The data available so far for GPI-protein chips up to the microarray format (2–5 $\times$ 104 spots) demonstrate that the "competitive" and "sandwich" modes are comparable with regard to sensitivity and reliability. However, the GPI-protein chip of the "competitive" mode has the huge advantage of operating independent of a 2nd anti-analyte antibody for detection. It becomes substituted for by the labeled anti-PIG antibody-PIG-analyte complexes. The distance between the labeled anti-PIG antibody and the protein moiety of the PIG-analyte within the complex is quite large and certainly exceeds that between the analyte and the detecting anti-analyte antibody in a conventional "reverse" protein chip of the "sandwich" configuration. This topology will guarantee the absence of sterical hindrance between the immobilizing anti-analyte GPI-antibody/scFvs and the anti-PIG antibody and, in consequence, ensure the immobilization of the PIG-analyte and the authentic unmodified analyte with identical efficacy.

The defined modification of the protein analyte with the PIG remnant of the GPI anchor usually does not interfere with the structure and function of the analyte. The carboxy-terminus is often located at the surface of a polypeptide or, if buried in its interior, the overall protein conformation will not be disturbed grossly by the relatively small and hydrophilic glycan moiety. In consequence, masking or inactivation of epitopes of the protein analyte in the course of the PIG modification with relevance for its subsequent immobilization by anti-analyte GPI-antibodies/scFvs and detection by anti-PIG antibodies is rather unlikely. Nevertheless, moderate impairment in the immobilization of the PIG-analytes would be acceptable since it can be determined and compensated for by normalization prior to their use. This advantage relies on the uniform labeling of each analyte molecule at the same site, i.e., at the carboxy-terminus, independent of the batch and production cycle. In contrast, chemical cross-linking as used for the conventional "reverse" protein chips of the "labeling" configuration will always result in heterogeneous modification of the various reactive amino acids and accompanying structural deteriorations, which can barely be predicted. A preliminary comparative evaluation of advantages and disadvantages of GPI-protein chips in the competitive configuration as so far available versus conventional RIA and ELISA

procedures on the basis of the determination of human insulin in a matrix of rat serum demonstrated clear-cut strengths of the GPI-protein chip with regard to selectivity, capacity, throughput, and the options for multiplex and online measurements, but also revealed weaknesses in the sensitivity, dynamic range, and precision. These will presumably be solved in the near future in course of further development of the current pilot version of the GPI-protein chip fabrication and instrumentation technologies.

Multiparameter GPI-(Anchored) Protein Chips

The (GPI-)protein chips presented so far have been designed for the determination of a single parameter of the corresponding sample analyte, i.e., molecular identity and/or amount. This is based on the immobilization and/or detection of the analyte by binding proteins, such as antibodies, scFvs, or anticalins, which specifically recognize one or several epitopes of the analyte. Epitopes are typically constituted by continuous stretches of a few to up to several hundred amino acids. The successful immobilization/detection of the analyte, i.e., its cross-reactivity with the binding protein with high affinity and selectivity, demonstrates the presence of the epitopes in the analyte polypeptide and, in consequence, strongly argues for its molecular identity with regard to the complete amino acid sequence. However, identity in amino acid sequence as indicated by this type of (GPI-)protein chip does not necessarily demonstrate the structural integrity of the protein analyte. The epitopes recognized by the capturing probes, e.g., GPI-anchored scFvs or other binding proteins, which are routinely used for protein chips, usually have been directed toward synthetic short peptides rather than large polypeptide domains with defined three-dimensional structure and therefore do not cover the complete amino acid sequence.

For the parallel or successive (within a short period) analysis of multiple parameters, such as identity, amount, structure, function, immunogenicity, and toxicity, using (GPI-)protein chips, the microscopic glass slides have to be replaced for special gold-coated glass prisms that are exposed to light of defined wavelength in a special instrumental setup, called "BiaCore." The beam is reflected under a certain angle and with larger wavelength thereby generating a baseline signal which is composed of the reflection angle and the wavelength, the so-called surface plasmon resonance (SPR). SPR is probably the best known method for the characterization kinetics of protein interactions (Boozer et al. 2006). SPR requires a gold surface which is derivatized with a protein layer. Binding of macromolecules to the gold surface can be detected as a change in incident angle or wavelength that is dependent on the refractive index of the interface. Mass transfer to the gold surface is detected in real time, allowing on-rates and off-rates to be measured. SPR has been successfully integrated into microfluidic devices several times (Jin-Lee et al. 2001; Wegner et al. 2002; Wang et al. 2007; Thorsen et al. 2002; Quellet et al. 2010), for instance, for the realization of 264 independent and simultaneous SPR measurements (Thorsen et al. 2002; Quellet et al. 2010). Importantly, any change in the mass that becomes intimately associated with the chip gold surface opposite to the prism will result in alterations of this baseline SPR signal. This is already caused in course of coating of the "BiaCore" chip with a phospholipid monolayer or MPC polymeric (or other bioconjugated) phospholipid analogues (Nakai et al. 1977; Nishizawa et al. 2008) and subsequent insertion of the anti-analyte, such as anti-insulin, GPI-antibodies/scFvs. The addition of the sample containing the protein analyte, such as human insulin, will lead to a further specific increase in mass at the chip gold surface that is reflected in a corresponding increment of the SPR signal. Thus, the immobilization of human insulin by anti-insulin GPI-antibodies/scFvs and thereby the demonstration of its molecular identity are monitored as upregulation of the SPR signal in real time. Thereafter, a new equilibrium will be achieved for the immobilization reaction resulting in a higher "baseline" value. This represents the "starting point" for the following specific associations of molecules with, i.e., mass increases at, the "BiaCore" chip gold surface.

Next, for analysis of the structure of the analyte, such as human insulin, which has already been immobilized at the "BiaCore"-based

GPI-protein chip, one takes advantage of its specific high-affinity binding to a "biosensor." For insulin, the "biosensor" is the insulin receptor, which is a heterotetrameric transmembrane protein complex of high-molecular mass (Sommerfeld et al. 2011). Following recombinant expression, solubilization, (partial) purification, and reconstitution into detergent micelles, the structurally and functionally intact human insulin receptor is incubated in excess with the chip. Only in the case of human insulin exhibiting the authentic three-dimensional conformation, the insulin receptor will bind with high affinity and thereby cause a further increase in the "BiaCore" chip-associated mass. This is accompanied by a pronounced upshift of the SPR signal with time up to achievement of the next "baseline" value in course of equilibrium binding of insulin to its receptor. Finally, the function of the structurally intact protein analyte already immobilized onto the "BiaCore" chip gold surface is assessed. The non-covalent cross-linking of the insulin receptor αβ-subunit halves by insulin is known to stimulate its intrinsic protein kinase activity which ultimately leads to autophosphorylation of the receptor β-subunits at certain tyrosine residues in the "trans" configuration Thus, upon addition of ATP, phosphate residues will be incorporated into the human insulin receptor, but only in the case of complete functionality of the insulin analyte. The resulting increase in mass will be moderate, but yet sufficient to elicit a small but significant upregulation of the SPR signal.

Evaluation

Taken together, the sequential and rapid (10–15 min for three parameters) multiparameter analysis of molecular identity (by successful immobilization), three-dimensional structure (by successful receptor binding), and function (by successful receptor activation) of human insulin, e.g., derived from biotechnological production, becomes feasible with the "BiaCore"-based GPI-protein chip. A number of additional important advantages have meanwhile been recognized during the initial calibration and validation experiments, among them (i) low time requirement, (ii) high sensitivity, (iii) high precision and accuracy, (iv) high sample throughput, (v) extended dynamic range, (vi) low variance, and (vii) sufficient robustness toward matrix components. Moreover, in contrast to alternative (GPI-)protein chips, e.g., those based on glass microscopic slides, the "BiaCore"-based (GPI-) protein gold chips do not necessitate any washing step for the removal of unbound ligands, such as the insulin receptor or ATP. Only ligand molecules which tightly interact with the gold surface of the chip rather than those freely floating in the incubation medium even in immediate neighborhood to the gold surface will succeed in inducing a significant SPR signal. Altogether these features enable the reliable determination of multiple parameters for protein analytes in a large number and in short time by "BiaCore"-based (GPI-) protein chips. No doubt, in the future this technology will considerably facilitate process analytics for the biotechnological production of protein therapeuticals.

However, for multiparameter analysis, the (GPI-)protein chips have to be processed along a defined sequence of different steps. In the case of human insulin, it encompasses the addition of insulin receptor and ATP and the change of buffers, several incubations, etc. and does not only require the simple exposure of the chip to the sample and shortly thereafter the measurement of the light, fluorescence, or luminescence signals. Of course, this multistep handling, if performed with many "hands," counteracts the efforts for acceleration, simplification, and automation of process analytics, personalized diagnosis, systems biology, and therapeutic monitoring. The integration of the "BiaCore"-based GPI-protein chips into a "chip-on-the-chip" represents an example for the possible automation and miniaturization of multiparameter analysis based on the recently developed "lab-on-a-chip" technology.

Modification of the Method

Automation of GPI-(Anchored) Protein Chips

For automated handling of all the processing steps required for multiparameter analysis, the complete reactor device, encompassing the "BiaCore"-based (GPI-)protein chip, the SPR

detector unit, and the temperature control unit have to be installed together with small reservoir chambers, which harbor all the reaction components and ingredients fuelling the reactor, within a compact glass/silicon monolith device in a so-called (GPI-)protein chip-on-the-chip (Gardeniers and van den Berg 2004; Hawtin et al. 2005; Maerkl 2011). In the case of analysis of human insulin for identity, structure, and function, these reservoir chambers have to be filled with the recombinant human insulin receptor, ATP, and two different buffers supporting the binding and kinase reactions. The sample(s) are contained in one to several chambers, the number of which critically depends on the size of the monolith and the complexity of the multiparameter analysis. Prior to transfer of the reaction components and samples to the reactor device, they are combined in a mixing device installed at the influx channel of the reactor device.

The transfer of the various reaction components to the reactor device is mediated by the continuous and constant flux of an inert carrier fluid from a corresponding reservoir chamber via the reactor device to a vacuum device. The latter operates as a combination of a membrane pump and a waste container and is placed at the efflux channel of the reactor device (Müller 2010; Gardeniers and van den Berg 2004; Hawtin et al. 2005; Maerkl 2011; Manz et al. 1994; Ros et al. 2006). The distinct reservoir chambers and devices are interconnected by microfluidic channels forming a circuit network under field-effect flow control (Schasfoort 1999; Sun et al. 2005; Unger et al. 2000). The channels are often fabricated in poly(dimethylsiloxane) (Sia and Whitesides 2003) and coated with phospholipid polymer biointerfaces (Xu et al. 2010) for minimizing unspecific interactions between the microfluidic device and the bioanalyte. The carrier fluid-driven flux of samples, reaction components, and buffers into the reactor device is controlled by electrically driven microvalves. These are installed at the efflux channel of each reservoir chamber and regulated in time-dependent fashion by a software-based process leading unit. For this, the microvalves as well as the devices are connected via electrical circuits to electronic chips integrated into the same monolith. In the (GPI-)protein "chip-on-the-chip" of the first generation, which was based on the pioneering work of the company Agilent Inc., the monolith harbored all reservoir chambers and devices, including the vacuum pump and waste container, and served as a one-way cartridge (Luedke and Preckel 2001). Only the data registration and analysis were performed by a separate central readout unit which becomes connected with the (SPR) detector of the reactor device via an electrical interface between the one-way cartridge and the central unit.

For reasons of sustainability, considerable efforts have been undertaken during the past decade to shift the interface between the one-way cartridge and the central readout unit "to the left," i.e., to leave only the reservoir chambers for the samples and reaction components in the cartridge and to install the reactor, detector, vacuum, and waste devices in the central unit. With regard to the cost-intensive "BiaCore-based" GPI-protein chips, it was of critical importance to implement them in the central unit for multiple cycles of regeneration and reuse. Regeneration is easily accomplished by removal of the immobilizing anti-analyte GPI-antibodies/scFvs (with the analytes and detecting antibodies bound) from the phospholipid- or MPC-coated gold surface of the "BiaCore" chip in course of treatment with low concentrations of nonionic detergent. After washing with aqueous buffer, a fresh phospholipid or MPC monolayer with embedded anti-analyte GPI-antibodies/scFvs is rapidly formed. Following control of the immobilization, binding affinity, and capacity by measurement of the SPR baseline, the GPI-protein chip is restored for the next round of multiparameter analysis. Taken together, the configuration of reservoir chambers and devices installed within a (GPI-)protein "chip-on-the-chip" enables automated multiparameter analysis of protein analytes with the considerable advantages of low time requirement (typically less than 15 min per sample), reduced sample volume, reduced reagent volume, and low personal costs. Certainly, for validation (GPI-)protein "chip-on-

the-chips" have to meet all the criteria typically requested for conventional bioanalytical methods, such as signal-to-noise ratio, sensitivity, resolution, precision, accuracy, selectivity, dynamic range, and variance. This has to be demonstrated for each parameter and analyte, as well as for each batch of the "chip-on-the-chip" produced since the fabrication process may underlie considerable variability (Andersson and van den Berg 2004).

Nanoparticle-Based (GPI-Anchored) Protein Chips

The chip technologies described so far rely on the immobilization of the protein analyte at the chip surface as well as on the use of anti-analyte antibodies/scFvs or other specific capturing probes for their immobilization and/or detection. Therefore, intensive efforts have been made during the past decade to establish alternative protein chips which operate with all components, including the analyte, in solution and do not depend on antibodies or specific capturing probes (Miranda et al. 2010a). Instead, these innovative methods are based on nanoparticles (NPs) and soluble reporter enzymes (Mallick and Kuster 2010; De et al. 2009a, b; Miranda et al. 2010a, b, 2011; Wright et al. 2005; Baldini et al. 2004). In 2007, a sensor array consisting of six cationic functionalized gold NPs and an anionic PPE polymer was introduced by the Rotello group (You et al. 2007), which manages to identify seven distinct polypeptides with high sensitivity and reliability. The gold NPs provoke quenching of the polymer fluorescence. This NP-polymer interaction and thus the quenching are abrogated by the presence of the protein analytes which thereby results in unique fluorescence response patterns relying on the differential NP-analyte affinity. The efficacy of these "chemical noses" has been attributed to both the quenching characteristics of the gold NPs and the "molecular wire" effect of the PPE polymer (Bunz and Rotello 2010). On the basis of the dependence of the analyte-NP interactions on their respective structural properties, such as surface charge, hydrophobicity/hydrophilicity, and hydrogen-bonding sites, the differential affinities will result in differential fluorescence response fingerprint patterns for distinguished proteins (De et al. 2009) which are quantitatively evaluated by linear discriminant analysis (LDA) of the raw data obtained (Jurs et al. 2000). The limit of detection reported for the NP-PPE system, that succeeded in the correct elucidation of 52 out of 55 unknown protein analytes, was 4–215 nM depending on their size (You et al. 2007).

As an alternative to PPE-NPs as scaffolds for the sensor construction, a separate class of polymeric NPs has been introduced with a polymeric micellar nanosystem which manages to respond toward electronic complementarity and thereby guarantees selectivity for metalloproteins (Sandanaraj et al. 2006). For this eight different fluorescence dyes were non-covalently coupled to the micellar interior of an homopolymer eliciting a specific fluorescence pattern that enables the discrimination of four different metalloproteins with high sensitivity.

Another approach is based on a micellar disassembly process for signal transduction (Savariar et al. 2008). For this five different non-covalently assembled receptors were used for analysis of the disassembly by following the liberation of the encapsulated dye in response to five different non-metalloproteins. The resulting shift in fluorescence due to the disassembly of the receptor-analyte complexes resulted in analyte-specific patterns with a limit of detection of 8 μM. In extension of this principle of differential response by a single polymer-surfactant complex, two novel variants relied on the disassembly and analyte-induced release pathways and the photo-induced quenching of charge/energy transfer, i.e., on excited state quenching (Gonzalez et al. 2009). By variation of non-metalloprotein or metalloprotein transducers, the limits of detection for the non-metalloproteins and metalloproteins were lowered to 8 μM and 80 nM, respectively. In addition, the application of a fluorescent anthracene-core dendrimer system with carboxylic acid residues on the particle periphery enabled a differential protein pattern in response to differential binding energy transfer steps at analyte concentrations ranging from 1 to 5 μM (Jiwpanich and Sandanaraj 2009). In the course of binding, the energy transfer step induced led to quenching of the fluorescent core. This

interchange between quenching and binding triggered differential responses enabling the unequivocal identification of three different metalloproteins.

The most recent variation of the "chemical nose" approach takes advantage of the amphiphilic nature of GPI-proteins. For this a reporter enzyme has to be chosen that catalyzes an easily detectable reaction and exhibits a large surface area with exposed polar as well as hydrophobic amino acid side chains and/or posttranslational modifications of heterogeneous nature. The bacterial hydrolase, β-galactosidase, converts the dye precursor, 4-methylumbelliferyl-β-D-galactopyranoside, into the dye, methylumbelliferone, which can be followed by simple photometric measurement. It is characterized by a negative net surface charge at physiological pH due to a multitude of negatively charged amino acids protruding into the aqueous environment. To increase the apolar surface area of β-galactosidase without drastically decreasing its (in the wild-type form high) solubility or causing its aggregation, the GPI-anchored ß-galactosidase was generated by recombinant expression in mammalian host cells (e.g., HEK-293, CHO) and purification using magnetic extraction (see above). In contrast to the wild-type form, GPI-β-galactosidase displays amphiphilic character (according to its partitioning between a Triton X-114 detergent and an aqueous phase). This is caused by the carboxy-terminal GPI structure harboring two saturated long-chain fatty acids and the glycan moiety, which protrude from the core of the polypeptide chain onto its surface, in combination with the negatively charged surface-exposed amino acids. Importantly, the GPI modification does not significantly impair the hydrolytic activity of β-galactosidase and is compatible with its solubility in the absence of detergent at the concentrations required for the assay.

The NPs which are used for the antibody-free protein chips and in a certain sense replace the antibodies will serve as "chemical noses." They have to manage the smelling of the surfaces of both the reporter enzyme and the protein analyte in differential fashion (Anslyn and Rotello 2010). For this, the NPs of 2 nm core size and about 10 nm hydrodynamic diameter consist of a gold core and an outer shell of covalently coupled organic molecules. These shell structures are built by a terminal positively charged ammonium residue with different functional groups of varying hydrophobicity as substituent and bound to the gold core via a constant hydrophobic spacer. In consequence, the NPs bind with high selectivity and avidity, rather than affinity, to protein surfaces through electrostatic and hydrophobic/apolar interactions to the major part and hydrogen and van der Waal bonds to the minor part. In fact, upon incubation those cationic/hydrophobic NPs bind to the surface of the GPI-β-galactosidase through salt bridges between the terminal ammonium groups and the negatively charged amino acids as well as hydrophobic interactions between the spacer/functional groups and the fatty acids of the GPI moiety. These multiple interactions between the NPs and the GPI-β-galactosidase lead to its complete inhibition. Importantly, binding of the NPs to GPI-β-galactosidase does not cause its denaturation and is reversible. Upon release of the NPs, e.g., in course of high dilution of the binding reaction, the hydrolase activity of GPI-β-galactosidase was shown to be completely restored.

Protein analytes added to an incubation mixture which consists of the reporter enzyme and the NPs under appropriate binding conditions have two options: (i) they do not interact with the NPs and thus the NPs remain bound to the reporter enzyme and continue in blocking its activity, i.e., the generation of the corresponding light signal. (ii) They interact with the NPs with certain selectivity and avidity and thereby cause displacement of the NPs from the reporter enzyme, thereby leading to its activation, i.e., the generation of the corresponding light signal. The signal critically depends on the relative strength of the interactions of the NPs and the reporter enzyme versus the protein analyte, which is determined by avidity and selectivity rather than mere affinity. Thus low and high signals, i.e., β-galactosidase activities measured, indicate relatively high and low interaction, respectively, between the NPs and the GPI-β-galactosidase and vice versa low and high interaction, respectively, between the NPs

and the protein analyte. In consequence, the signal strength is positively and negatively correlated to the efficacy of the "smelling" of the surface characteristics of the protein analyte and GPI-β-galactosidase, respectively, by the NPs. These "chemical noses," here constituted by the ammonium and functional groups of the shell structures and by the spacer of the NPs, "smell" the negatively charged amino acids and the GPI moiety of the reporter enzyme, here the GPI-β-galactosidase.

It is easily conceivable that a single type of NPs enables only limited differentiation between the different protein analytes contained in a complex biological sample, such as human plasma. This is exemplified by the analysis of human reference insulin versus insulin variants versus the serum protein albumin. NPs equipped with the distinct functional group, NP5, allowed the discrimination of human insulin from albumin as well as unfolded and mutant insulin, but not from degraded insulin, with NP4 from degraded, unfolded, and mutant insulin, but not from albumin, NP2 from albumin, as well as degraded and unfolded insulin, but not from mutant insulin, etc. It was the combination of the six distinct types of NPs equipped with the functional groups, NP1-6, that enabled the unambiguous discrimination of the four distinct insulins and albumin from one another on the basis of the unique signatures provided by NP1-6.

The minimal number of different types of NPs required for the unambiguous discrimination of a given, but defined, number of protein analytes very critically depends on the nature of the analytes and the NPs used and can therefore hardly be predicted. Certainly, gross structural differences between the analytes on one side and between the NPs on the other side will facilitate the analysis and limit the expenditure to the use of a rather low number of NPs. In contrast, subtle deviations in the biophysical/biochemical characteristics of the analytes will necessitate the use of a higher number of NPs of more intricate structural variation. The currently available data already indicate that four to eight NPs may enable the differentiation of protein analytes at similar number (Anslyn and Rotello 2010; Rana et al. 2010). It remains to be clarified whether the determination of a distinguished analyte out of a complex sample originating from a body fluid, such as plasma, and harboring several hundred proteins, among them several high-abundant ones, will be feasible at all and, if so, will necessitate high expenditure, i.e., very many NP types for the unequivocal identification of a single low-abundance protein. An interesting possibility to increase the number and types of interaction between the NPs and the reporter enzyme and, in consequence, to support the competition between the protein analytes and the reporter enzyme for binding to the NPs relies on the carboxy-terminal modification of the reporter enzyme with a GPI structure (see below). Its amphiphilic nature determined by the saturated long-chain fatty acids and the core glycan component may facilitate or impair the interaction of the GPI-modified reporter enzyme with the NPs and thereby enhance its accessibility for differential competition by the different protein analytes.

Nevertheless, taking into consideration, the current experimental experience with non-GPI-coated NPs, the convenient, straightforward, rapid, and inexpensive determination of protein analytes in complex biological samples on the basis of NP-based protein chips using a limited set of NP types seems to be feasible. It may be driven in the near future by considerable progress in the development of parallel synthesis of NP materials starting from a common core or scaffold structure and varying the functional building groups at the NP surface by chance. Combinatorial chemistry may turn out as efficient tool for the generation of a huge variety of NP types around a common scaffold structure as has already been demonstrated in medicinal chemistry during the last two decades. In addition, it seems feasible to identify NP types for a given protein analyte that manage to discriminate between the analyte in the free and complexed state, e.g., MMP-9 unbound and bound to lipocalin-2 (see above), as has been reported previously as a potential drawback for typical (non-NP-based) protein chips (see above). From an experimental point of view, the hurdles for the development of protein chips with the capability to discriminate between free and

complexed protein analytes are presumably significantly lower for NP-based compared to conventional protein chips.

Taken together, protein chips based on NPs rather than on antibodies or other capturing/detection probes and operating with soluble rather than immobilized analytes have the potential for sensitive, quantitative, selective, and reliable determination of protein analytes from complex sample mixtures. The feasibility, in particular their resolution, critically depends on the availability of appropriate "chemical noses," i.e., NPs, capable of "smelling" subtle differences at the surface structure of protein analyte(s) versus the reporter enzyme. The functional groups required can hardly be predicted. In fact, there is no need for elucidation of the specific structural determinants that may be appropriate for highly selective and avid interactions between the analyte and the NPs. In most cases, the set of appropriate NPs will be identified just by trial and error. For this, functional groups are synthesized by high-throughput combinatorial chemistry, subsequently automatically coupled to the gold core, and finally assayed for interaction with the reporter enzyme by simply assessing its activity under throughput mode. In any case, the probability for finding appropriate NPs should considerably increase, i.e., the number of NPs to be synthesized and tested decrease, upon the introduction of the GPI moiety at the carboxy-terminus of the reporter enzyme and the consequent increase in its surface hydrophobicity. No doubt, so far NP-based (GPI-)protein chips are less far advanced with regard to the (automated) rapid analysis of many samples under routine conditions, e.g., for the personalized measurement of biomarkers. Certainly, for validation and calibration, NP-based (GPI-)protein chips have to fulfill the same requirements as conventional protein chips.

In addition to assaying the surface integrity of the protein analyte, which typically reflects the combination of molecular and structural identity, NP-based (GPI-)protein chips are capable of detecting any posttranslational modifications with great sensitivity, in particular those which alter the surface hydrophobicity of the protein analyte, such as myristoylation, acylation, prenylation, phosphorylation, glycosylation, denaturation, and aggregation.

Moreover, during the past decade, NP-based protein chips have already been successfully used for the "smelling" of the surfaces of various bacterial and mammalian cell types for different purposes (Miranda et al. 2011; Rana et al. 2010). For instance, pathogenic bacteria were detected with extremely high sensitivity at 102 particles per ml drinking water. Human tumor and healthy cells were discriminated from one another by NPs on the basis of subtle yet adequate differences in their surface characteristics. These differential surface patterns are most likely caused by the combination of many distinct small structural features rather than the predominance of one or a few striking markers protruding from the bacterial cell wall and plasma membrane, respectively (Wright et al. 2005). In fact, in case of tumor cells, the critical surface antigens or markers often remain ill-defined. Yet the tumor cells may be identified without knowledge about primary or secondary consequences for the plasma membrane structure on the basis of NPs which succeed in "smelling" the overall cell surface landscape. Nevertheless, in any case the cell types to be discriminated have to be clearly defined (however, often a tremendous problem in tumor biology) prior to efforts for their differential recognition by (a set of) NPs.

At variance with the non-NP-based chip-on-the-chip described above, the reactor for the NP-based (GPI-reporter) protein chip is just a temperature-controlled incubation chamber that becomes filled with binding buffer, reporter enzyme, NPs, sample, and dye in a defined sequence. The chamber walls have to be coated with materials compatible with the sample analyte and the matrix (Chapman 1993; Matsuda et al. 2003). The dye formed during the incubation is then measured in a distinct detection unit equipped with a photometer and installed at the efflux channel. This configuration enables the sequential filling and emptying of the reactor with the various types of NPs for each cycle in rapid ($\sim$2 min per cycle) and precisely controlled fashion. Eventually, the reactor may be washed between two successive cycles with carrier fluid

to get rid of the GPI-reporter protein or the NPs adhering to the chamber walls. Again, for maximal sustainability, only the reservoir chambers for the samples, NPs, and consumables are installed together into the one-way cartridge, whereas the instrumentation encompassing the mixer, reactor, detector, vacuum pump, and electronic chips are combined in the central unit. Again, one-way cartridge and central unit are interconnected via fluidic and electrical interfaces (Whitesides 2004).

Typically, the determination of the unambiguous signature for a protein analyte will be completed within 15–45 min, corresponding to 5–15 cycles with distinct types of NPs each. The time requirement depends on the complexity of the sample mixture and the number and quality, i.e., selectivity and avidity, of the NPs used. The full automation, including the filling of the samples into the one-way cartridge, its connection with the central unit, and the reading out of the central unit, enables (i) the parallel measurements of the same sample with distinct types of NPs or of distinct samples with the same type of NPs and then (ii) the successive measurements of the next samples or with the next types of NPs. Central units equipped with up to 96 reading channels are currently being under validation for pilot process analytics of biotechnological production and have already demonstrated the reliable and sensitive determination of human insulin in plasma with low time requirements (~200 samples per hour) and acceptable running costs.

Overall Evaluation

The standard approach in biology has mainly been described as reductionist procedure, where a biological system is broken down into the components, and these components in turn are analyzed separately to interpret their effect on the system's behavior (Sorger 2005). Various genetic and pharmacological tests are then carried out to confirm that this analysis is correct. While this reductionism has turned out to be powerful, it is rarely quantitative, and the explanations are only descriptive or qualitative. Recent advances in network analysis enabled the investigation of biological systems from a new perspective, relying on the integration of multiple parameters or factors into larger-scale models. These become very powerful when they are combined with standard mechanistic studies. While systems approaches per se are limited by measurement and computational tools, they have the potential to generate a physiological dimension that with mechanistic analyses per se can hardly be obtained.

The next generation of drugs for common multifactorial diseases, such as cancer, type II diabetes, obesity, and neurodegenerative diseases, will approach a novel level of efficacy if the therapy is tailored to the molecular characteristics and composition of each patient's aberrant cells and affected tissues. Over the past 6 years, significant progress has been achieved in the application of genetic and genomic profiling to individualize and stratify drug administration, such as for chemotherapy during the treatment of cancer based on kinase inhibitors that target multiple signaling pathways for the regulation of growth and cell division (McCarthy et al. 2010; Chow 2010; Gangadhar and Schilsky 2010). This should lead to the incorporation of protein microarray procedures into future clinical trials. Importantly, molecular therapy will require a novel class of technologies for proteomic profiling since genomics typically fails to decipher the activation state of specific signaling pathways that harbor the relevant target of the newly identified inhibitor or activator. Protein chips and, in particular, those relying on GPI-proteins represent a broadly adopted technology which meets the requirement for profiling of the functional state of cellular signaling pathways and networks. The (GPI-) protein chip has graduated from the realm of basic science to the level of clinical trial design and application.

The systems approaches critically depend on proteomic analysis at large-scale and multiparameter level (Liotta et al. 2001; Müller 2010b). Whereas the chemical tools for this analysis are still primitive, different combinations of (GPI-)protein chips and printed proteins enable the assessment of binding interactions, such as antibody-epitope, receptor-ligand, and enzyme-substrate, on a genome-wide scale (Di Carlo and Lee 2006). While major progress in the area of conventional protein chips has been achieved

during the past decade, the future development of microarrays based on GPI-proteins and NPs offers hope for better quantitative and multiparameter analyses at acceptable expenditure in time, costs, skills, and equipment. In a broader sense, (GPI-) protein chip technology may fill a critical missing component of molecular profiling, i.e., the quantitative evaluation of the function of signaling proteins and their posttranslationally modified versions. Measurement of this class of protein analytes, which includes important drug targets, provides information about the disease state of cells that cannot be obtained by genetics or genomics. The need to measure those analytes from small biopsy samples will continue and expand in the future. New signaling molecules and drug targets are continuously identified and become validated. Consequently, the complexity of the analyte repertoire will increase as is true for the demands on sensitivity, precision, reproducibility, and versatility of (GPI-)protein microarrays. They will utilize nanotechnology to an increasing degree, as already indicated here for the NP-based GPI-protein chip, the third-generation amplification technologies, and the novel detection procedures. Ideally the ultimate clinical embodiment will be a one-step technology from the user's perspective, with completely electronic readout. It will not rely on an array imaging step if the construction of a multiparameter protein chip with high sensitivity and the precision in a homogenous (solution phase) NP- and/or GPI-based format were successful.

Thus the utility of (GPI-)protein chip technology resides in signaling pathway analysis and profiling. The outcome of this knowledge will be a new form of diagnostic report for individualized therapy and its monitoring. So far, the signaling network map of a given cell could not be delineated to a fixed circuit board which unequivocally hints to abnormally abundant or functional network proteins like a marked route on a street map. Instead, signaling network proteins coalesce into pathways following a stimulus. Once the stimulus is removed, the interconnected proteins disperse and the network organization dissolves. As more will become known about how signaling networks are continuously remodeled under the influence of the tissue microenvironment, it will turn out that the diagnostic report of the future will be an individualized network map or the equivalent of a distinct street map for each patient. The most meaningful measurements may rely on the strength of connections and functional interactions between nodes in the network, not just the amount of each node or protein in the network. In course of the integration of genomics, transcriptomics, proteomics, metabolomics, and fluxomics into a systems biology approach, dynamic individualized pathway maps will provide improved visualization of the cell signaling networks for designing a rationale personalized therapy.

The ambitious goal of in vivo multiparameter analysis should enable or at least facilitate the reliable tracking of spatial and temporal determinants of cell function encompassing protein expression, structure, activity, and interactions at the level of tissues, organs, and whole organisms (Picollet-D'hahan 2011). Ultimately, the medical application of protein chips may not be limited to in vitro routine diagnosis and to pharmacological and preclinical research but rather be extended to in vivo diagnosis on the basis of implantation of the microarrays into patients as physiological probes (Tang 2011; Kohl et al. 2011). The data about the patients' physiology measured continuously and collected in real time by the implanted protein chip will considerably support the diagnosis with improved reliability and compliance compared to routine hospital visits and accompanying time-consuming invasive tests.

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Determination of (Phospho)Proteins Using a Multiplex Bead-Based Capture Immunoassay

Purpose and Rationale

Multiplex phosphoprotein assays and multiplex total target assays are bead-based multiplex assays (e.g., Bio-Rad Luminex xMAP technology) that detect the phosphorylation of proteins in lysates derived from cell culture or tissue samples. These 96-well plate-format assays allow profiling of the specific phosphorylation state of up to 100 different proteins using as few as two wells and as little as 25 µl of lysate per well. The multiplex total target assay reports the abundance of the target protein in one well, while the multiplex phosphoprotein assay reports the level of phosphorylation of that protein in a separate well. These instructions apply to both assays.

Singleplex or Premixed Multiplex Assays

Multiplex phosphoprotein and total target assays are available as singleplex or premixed multiplex assays. Singleplex assays are designed to be flexible. They can be used individually to test for a single phosphoprotein at a time, or they can be combined to create a multiplex assay to test for a specific set of phosphoproteins in a single sample. Premixed multiplex assays are the more convenient format for repeat testing of a specific set of phosphoproteins. These assays are only available through Bio-Rad's online x-Plex assay service

The principle of these 96-well plate-format bead-based assays is similar to a capture sandwich immunoassay. An antibody directed against the desired target protein is covalently coupled to internally dyed beads. The coupled beads are allowed to react with a lysate sample containing target protein. After a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope is added to the reaction. The result is the formation of a sandwich of antibodies around the target protein. Streptavidin-phycoerythrin (streptavidin-PE) is then added to bind to the biotinylated detection antibodies on the bead surface.

Data from the reaction are then acquired using the multiplex suspension array system (or Luminex 100 system), a dual-laser, flow-based microplate reader system. The contents of the well are drawn up into the reader. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent signal on the bead surface. This identifies each assay and reports the level of target protein in the well. Intensity of fluorescence detected on the beads indicates the relative quantity of targeted proteins. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity on Bio-Plex ManagerTM Software, the accompanying software package. If specific wells are identified for comparison, the ratio of fluorescence intensity between those wells is automatically calculated.

Procedure

Samples are rinsed with cell wash buffer as follows. For adherent cells, the treatment reaction is terminated by aspirating the culture medium and quickly rinsing the cells with ice-cold cell wash buffer. The volume of cell wash buffer required is the same as the volume of aspirated cell culture medium. Keep the cells on ice. For suspension cells, the treatment reaction is terminated by adding ice-cold wash buffer to the cells. The volume of cell wash buffer required is twice that of the culture medium. The cells are centrifuged at 1,000 rpm for 5 min at 4 °C. Then the supernatant is aspirated. For tissue samples, the sample is rinsed with cell wash buffer once. Then the tissue is cut into 3 × 3-mm pieces, which are transferred

to a 2 ml tissue grinder. An equal volume of assay buffer is added to the lysate. If the lysate is not tested immediately, it is stored at −20 °C. The lysate is stable for up to five freeze-thaw cycles.

For preparation of the lysate, lysing solution is added immediately to the cells. The amount of lysing solution needed depends on the cell concentration in the culture vessel. The samples are briefly sonicated (e.g., with a Sonifer 450 as follows: Duty cycle = 40, Output = 1, Pulse sonicating = two 10 min pulses with a 1 min break in between). Thereafter, the cells are agitated. The cell lysate is transferred to a centrifuge tube and rotated at 4,500 × g for 20 min at 4 °C. The supernatant is collected without disturbing the pellet. Subsequently, the lysate protein concentration is determined. The protein concentration should be 200–900 μg/ml. For determining total protein concentrations, Bio-Rad's DC protein assay kit may be considered (Bio-Rad catalog #500-0112). It may be necessary to test-lyse the samples with different volumes of lysing solution to obtain the specified protein concentration range. An equal volume of assay buffer is added to the lysate, which is stored (overnight) frozen at −20 °C and thawed before testing. The lysates are thawed at room temperature and then placed on ice. If necessary, it is possible to further dilute the lysates by using a 1:1 mixture of lysing solution and assay buffer (both freshly prepared).

The following instructions apply to multiplex phosphoprotein and total target singleplex, custom-premixed, and multiplex assays. Phosphoprotein assays should not be mixed with its corresponding total target assays (e.g., phospho-Akt and total Akt). For the design of an experiment, the wells of a 96-well plate that will be used for each lysate should be assigned correspondingly. It has to be considered that the instrument reads wells down the plate and not across. Thus, the wells have to be assigned vertically. The total number of wells that will be used in the assay is 82. A 25 % excess (or two wells for every eight wells used) should be included to ensure that enough diluted coupled beads, detection antibodies, and streptavidin-PE are prepared. These numbers are recorded on the worksheet since they will be referenced throughout the assay.

For the preparation of coupled beads, the beads are protected from light by covering the tubes with aluminum foil. All tubes have to be kept on ice until ready to use. Coupled beads must be mixed manually prior to use when combining singleplex or premixed assays. The coupled beads are mixed (50x) at medium speed for 5 s. A sufficient volume of coupled beads (1x) using wash buffer is prepared. When preparing a multiplex assay, equal volumes of each bead should be used. Each well requires 1 μl of coupled beads (50x) for each target adjusted to a final volume of 50 μl. These calculations can be done on the worksheet.

For calibration of the vacuum apparatus, the vacuum apparatus must be calibrated at the beginning of the assay to ensure an optimal bead yield. More detailed instructions can be derived from the Bio-Plex suspension array system hardware instruction manual. All the wells of a 96-well filter plate are pre-wetted with 100 μl of wash buffer. The filter plate is placed on the vacuum apparatus, and the vacuum is turned on to the maximum level. The filter plate is pressed on. The time required to remove the buffer from the wells by vacuum filtration is noted. The evacuation time should be 2–5 s. If the evacuation time is below 2 s, the pressure is too high. In this case the vacuum control valve is opened slightly, and previous steps are repeated. If the evacuation time is above 5 s, the pressure is too low. The vacuum control valve is closed slightly, and previous steps are repeated.

For start of the assay procedure, all buffers should have room temperature and be devoid of air bubbles. The desired number of wells in a 96-well filter plate is washed. If fewer than 96 wells are required, the unused wells are covered with sealing tape for later use. The coupled beads are vortexed (1x) for 5 s at medium speed. Fifty microliters are added to each well and immediately vacuum-filtered. After two washing cycles, the thawed lysates are gently vortexed for 3 s. Fifty microliters of lysate are added to each well with changing of the pipet tip after every volume transfer. After incubation for 15–18 h (or overnight), a sufficient volume of detection antibodies (1x) is prepared using detection antibody diluent. Each well requires 1 μl of detection

antibodies (25x) for each target adjusted to a final volume of 25 µl. After the incubation, the sealing tape and then the vacuum-filter are slowly removed and discarded. After three washing cycles, the detection antibodies are gently vortexed and then added in 25 25 µl portions to each well with changing of the pipet tip after every volume transfer. After incubation for 30 min, the plates are washed three times. The plate has to be kept in the dark. A sufficient volume of streptavidin-PE (1x) is prepared using wash buffer. Each well requires 0.5 µl of streptavidin-PE (100x) adjusted to a final volume of 50 µl. The diluted streptavidin-PE is mixed vigorously, and 50 µl are added to each well. After incubation for 10 min, the sealing tape and then the vacuum-filter are slowly removed and discarded. The plates are rinsed three times. Subsequently, 125 µl of resuspension buffer are added to each well. The plates are incubated for 30 s. If the data are not acquired immediately, the assay may be stored in the dark at 4 °C for up to 24 h.

For data acquisition, the assay plate is shaken at 1,100 rpm for 30 s immediately before acquiring data. Failure to do so will result in increased data acquisition time due to bead settling. It has to be checked that the filter plate is flat. While pressing on one end of the plate, the distance that the opposite end of the plate is raised off a flat surface is observed. If the distance is >1 mm, all contents have to be transferred to a flat-bottom 96-well plate or another filter plate. The plate has to be visually inspected to ensure that the assay wells are filled with buffer prior to placing the plate in the Bio-Plex microplate platform. The sealing tape has to be removed slowly and any plate cover before placing the plate in the reader. An appropriate running protocol has to be selected with specified data acquisition for 25 beads per region. In Advanced Settings, the default DD gate values have to be adjusted to 4,335 (low) and 10,000 (high). When using a Luminex 100 instrument, the gates have to be set according to the Luminex procedure (see Manual). "Start" has to be selected and the ".rbx file" saved. Then the instructions for data acquisition have to be followed. If acquiring data from more than one plate, the waste bottle has to be emptied and the sheath bottle refilled after each plate. "Wash Between Plates" has to be selected and the instructions for fluidics maintenance followed. Then the "Prepare Protocol" and "Acquire Data" steps have to be repeated. When data acquisition is complete, "Shut Down" has to be selected and the instructions have to be followed. It is possible to acquire data from a well or plate a second time using the Rerun/Recovery mode located below Start (Run Protocol). The wells should be analyzed where data will be acquired a second time. Any previous data will be overwritten. The buffer is removed by vacuum filtration, and 125 µl of resuspension buffer are added to each well. The filter plate is covered with a new sheet of sealing tape. Then "Acquire Data" steps to acquire data a second time are repeated. The data acquired should be similar to the data acquired initially. However, the data acquisition time will be extended since fewer beads are present in each well.

References and Further Reading

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410:37–40

For a complete list of publications using Bio-Plex Phosphoprotein Detection Assays, refer to bulletin 5394

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Detection of Exosomes and Microvesicles (EMVs)

Purpose and Rationale

Many cell types release exosomes and microvesicles (EMVs) with a size range from 0.05 to 5 µm, harboring receptors, bioactive and signaling proteins, molecular mediators, and nucleic acids for cell-to-cell communication. Microvesicles bud directly from the plasma membrane, in contrast to exosomes which are derived from multivesicular bodies within the cell. Increased levels of EMVs have been observed in plasma, urine, and other body fluids in patients suffering from a wide range of common complex diseases, including vascular, metabolic, lung,

autoimmune and neurodegenerative diseases, chronic inflammation, and cancer. EMVs may affect target cells directly by surface-bound ligands, transferred surface receptors, and membrane-associated enzymes, such as glycosylphosphatidylinositol-anchored proteins, or delivered cytoplasmic or membrane-associated constituents, such as cytosolic proteins, micro/mRNAs, bioactive lipids, and even mitochondria. The use of EMVs as diagnostic markers for the prediction, diagnosis, therapy monitoring, and prognosis of complex diseases is becoming increasingly attractive. Novel technologies for analysis of the size, density, and molecular composition of EMVs are currently emerging together with methods for their improved isolation and purification out of heterogenous vesicle populations. In addition, the recent revolution in mass-spectroscopy, (micro-)flow cytometry, atomic force microscopy, nanoparticle tracking, and biosensing will considerably facilitate the quantitative and qualitative analysis of all constituents assembled in EMVs. Technologies will be preferred that provide signatures specific for EMV subsets rather than a single or a few parameter(s) averaged for the total EMV population. Those EMV signatures have to be correlated to specific disease states along cross-sectional and longitudinal clinical studies. Moreover, it has to be tested which signatures and molecular components, i.e., EMV subspecies, are most informative to obtain actionable disease information. Ultimately, the reliable, rapid, and low-cost analysis of EMVs will support systems biology-based approaches for the diagnosis and therapy of complex diseases and supplement the analytical power of conventional biomarkers.

The increased release of exosomes and microvesicles (EMVs) and their accumulation in body fluids including plasma, liquor, urine, saliva, mucus, and interstitial fluids (e.g., ascites, pleural fluids) is often directly related to cell activation (Stoorvogel et al. 2002; Cocucci et al. 2008; Simons and Raposo 2009) and the pathogenesis of a variety of diseases (Freyssinet 2003). While the exact mechanisms inducing EMV release still remain obscure (Stoorvogel et al. 2002; Record et al. 2011; Thery 2011; Morel et al. 2004a; Piccin et al. 2007; Muralidharan-Chari et al. 2010; Lee et al. 2011a), the releasing efficacy is often modulated by (receptor-dependent) extracellular signals, such as ligands or viral infection (Booth et al. 2006; Fevrier et al. 2004; Welsch et al. 2007; Chairoungdua et al. 2010; Aupeix et al. 1997). Apparently, the underlying vesicular trafficking, exocytotic, and plasma membrane shedding processes (Thery et al. 2002) operate in constitutive (Ca2+-independent) (Brasoveanu et al. 1997) or regulated (Ca2+-dependent) fashion (Zitvogel et al. 1998; Blanchard et al. 2002; Laulagnier et al. 2004; Martinez et al. 2004; Hogan et al. 2009) that may be induced by specific signals, such as certain physiological (e.g., fatty acids, reactive oxygen species) or pharmacological (e.g., antidiabetic sulfonylurea drug glimepiride) stimuli (Müller 2010; Müller et al. 2008a, b, 2011a, b, c). The use of EMVs as predictive, diagnostic, therapeutic, and prognostic biomarkers under fulfillment of all criteria commonly accepted for their use (Lee et al. 2011b; Müller 2012a; Cheruvanky et al. 2007; Conde-Vancells et al. 2010; Miranda et al. 2010a; Simak and Gelderman 2006; Pisitkun et al. 2004; Hunter et al. 2008; Taylor and Gercel-Taylor 2008; Michael et al. 2010; Keller et al. 2011; Dowling and Clynes 2011; Lathia 2002; Colburn 2003; Naylor 2003; Zolg and Langen 2004; Ciesla et al. 2011; Lee et al. 2011b; Zineh and Huang 2011, Müller 2012b) is of increasing interest for many, in particular common complex diseases, such as metabolic and cardiovascular disorders (Kolberg et al. 2009; Schulze et al. 2009, Herder et al. 2011; Younus and Rodgers 2011; Lyons and Basu 2012; Bonauer et al. 2010; Dignat-George and Boulanger 2011; Martinez and Andriantsitohaina 2011; Siljander 2011; Boulanger et al. 2001; Nomura et al. 2001; Bernal-Mizrachi et al. 2003; Ferreira et al. 2004; Jimenez et al. 2003; Preston et al. 2003; Horstman et al. 2004a, b; Brodsky et al. 2004; Nomura et al. 1998; Tan et al. 2005; Esposito et al. 2006a; Setzer et al. 2006; Agouni et al. 2008; Freyssinet and Toti 2010; Nomura et al. 1995; Diamant et al. 2002; Omoto et al. 2002; Sabatier et al. 2002; Esposito et al. 2006b, 2011; Goichot et al. 2006;

Koga et al. 2006; Leroyer et al. 2008; Helal et al. 2010; Tramontano et al. 2010), inflammation and cancer (Horstman 2007; Mitchell 2009; Rabinowits et al. 2009; Simpson et al. 2009; Qazi et al. 2010; Baj-Krzyworzeka et al. 2007; Deregibus et al. 2007; Skog et al. 2008; Al-Nedawi et al. 2009; Yuan et al. 2009; Castellana et al. 2010; Liu et al. 2010; Kogure et al. 2011; Yang et al. 2011) and under the systems biological point of view (Morel et al. 2004b; Naylor 2005).

Analytical Prerequisites

The predominant scientific and technological criteria for future research and development of omics- and nanoparticle (NP)-based EMV analytics to achieve these objectives are as follows:

(i) High-throughput/high-content analytical platforms have to be implemented and validated (standard operation procedures) that allow the quantitation and discrimination of EMV composition and heterogeneity as related to their physicochemical properties (size, density, refractive index) on the basis of both single-parameter and signature analysis of their proteomic (by MS [mass spectrometry], single/multiplex enzyme-linked immunosorbent assays [ELISAs]), lipidomic (by MS), metabolomic (by MS), and nucleic acid (DNA, mRNA, miRNA [microRNA] by reverse transcription-polymerase chain reaction [RT-PCR]) composition. (ii) The pre-analytical procedures, including the calibration and standardization of flow cytometry (FCM) platforms, appropriate for EMV analytics have to be improved and simplified. (iii) Multiparameter combinations of fluorescently labeled monoclonal antibodies and nucleic acid staining and fixation/permeation pre-analytical protocols for simultaneous multiparameter FCM analysis of cell type-specific EMVs have to be implemented and validated. (iv) Quantitative MS-based proteomics as reference method and as bridging technology for the determination of protein composition and content in EMVs released from different cell types under normal and disease conditions has to be used to facilitate the final selection of antibody targets for single and multiplex ELISA platforms or multiparameter FCM. (v) Quantitative MS-based lipid species analytics for EMVs from different cell types has to be introduced and validated on the basis of the high-throughput/content lipid species profiler (>800 lipid species) already developed (see www.lipidomicnet.org) with special focus on regulatory and high-affinity lipid ligands (e.g., fatty acids, lysophospholipids, sphingolipids) for G protein-coupled receptors, annexins, nuclear hormone receptors (e.g., PPARγ, LXR, RXR), and other transmembrane receptors and transporters (e.g., CD36, integrins, selectins, glycosylphosphatidylinositol-anchored proteins). (vi) Novel highly membrane-permeable dequencher dyes to increase the specificity, sensitivity, and accuracy of fluorescence staining of nucleic acids (DNA, mRNA, miRNA) in EMVs with excitation/emission profiles suitable for multiparameter FCM have to be implemented and validated. (vii) The pre-analytics (extraction, inhibitor elimination, etc.) and analytics (primer design, assay format) of array-based and quantitative RT-PCR Taqman high-throughput measurement of the miRNA contents in EMVs have to be improved and validated. (viii) Multi-omics-, nanoparticle tracking analysis (NTA)-, and nanoparticle (NP) biosensing-based software tools and graphic data presentation for single-parameter and signature-fuelled analytics of EMVs have to be implemented for routine (clinical) use. (ix) The levels and composition of EMVs released by specific cell types in vitro have to be determined for the use of specific EMV preparations appropriate as standard materials to be added for the normalization and validation of the assay for EMV analytics (sensitivity and linearity determination). (x) For validation as novel biomarkers or surrogate markers reflecting the systems biology-based risk assessment, diagnosis, therapy monitoring, and prognosis, the disease specificity, sensitivity, and predictive value of cell type-specific EMVs defined by single markers or complex signatures have to be established in patient cohorts with complex common diseases. (xi) A public formate for multiparameter omics-, FCM-, signature-driven, as well as cell type- and disease-specific data bases has to be developed as "EMV platform" to provide connectivity and algorithms to synergize published and newly generated knowledge about proteome-, lipidome-, metabolome-, and

nucleic acid-based EMV targets. (xii) Finally, the knowledge gathered by the "EMV platform" has to be transferred to preclinical and clinical research, practical medicine, and national and international health-care authorities as well as to companies engaged in diagnostics and drug discovery and development.

Procedures

For the majority of biomarker projects, correlations between the modulation of candidate plasma EMV biomarkers and disease-specific stages by measuring target cell-specific EMVs including their biological functionality and activation status have to be elucidated in order to identify and validate novel biomarker candidates for appropriate clinical cohorts. In a pilot project dedicated to cancer diagnostics, this could already be achieved by finding correlations between the individual variability in the stress response (in particular, in the level of preexisting total and cell surface expression of the heat shock protein, Hsp70) (Chung et al. 2008; Gehrmann et al. 2008; Vigh et al. 2007; Stangl et al. 2011; Xie et al. 2010) in a heterogeneous melanoma cell population (Peter et al. 2012) and their metastatic as well as invasive capabilities by using state-of-the-art ultrasensitive high-content imaging techniques.

The specific objectives are (i) to link the phenotypic population heterogeneity of the stress response (during development of the disease and/or its treatment) and the membrane structural variability (structure and dynamics of lipid rafts and EMVs) (Schmitz and Grandl 2008) and the resistance against apoptosis and cell lysis (Babiychuk et al. 2011a, b; Potez et al. 2011; Draeger 2011; Draeger et al. 2011) in cell cultures derived from the tumor patients and (ii) to characterize cell populations responding to stress at the level of individual cells by proteomic and lipidomic methods. The identification of specific alterations in membrane domain, in particular lipid raft, structures (Babiychuk and Draeger 2006) leading to selectively induced expression of heat shock proteins in a heterogeneous cell population may facilitate the understanding why a small subpopulation of cells apparently determines the outcome of cancer development and/or treatment. Standardized technologies will be required to profile heterogeneous cell populations and to characterize EMVs derived from individual cells by combining microscopic, flow cytometric, light scattering, particle tracking, and biosensing technologies (Horstman et al. 2004b).

Enrichment and Purification

A number of workflows already have been and still have to be developed for the validation of the enrichment of EMVs from a cell population. They encompass several readouts, such as size and density, for the discrimination of exosomes, microvesicles, and apoptotic vesicles themselves as well as of contaminating entities, lipid (droplets), lipoproteins, protein aggregates, protein-phospholipid micelles, and cell and membrane fragments but also broken and intact cells. In general, EMV enrichment and purification relies on three principle workflows. (i) Adsorption to nanomagnetic beads through ionic interactions between the negatively charged phospholipid surface of EMVs and positively charged beads (Soo et al. 2012; Coren et al. 2008) or EDTA-coupled beads complexed to Ca2+ (Momen-Heravi et al. 2012; Tauro et al. 2012; Jayachandran et al. 2012); (ii) differential centrifugation using sequentially increasing centrifugal forces for the removal of cells, cellular debris, larger particles, and organelles from conditioned cell culture supernatants or body fluids and final collection of the microvesicles (10,000–20,000 × g, 10–30 min) or exosomes (>100,000 × g, >120 min, subsequent to deprivement of the microvesicle fraction by the 10,000–20,000 × g spin) during the last centrifugation step (Raposo et al. 1996; Thery et al. 2006); (iii) size-exclusion chromatography involving a low-speed centrifugation step for the removal of cells, cellular debris, larger particles, and organelles, followed by pre-concentration with two filtration steps (0.2 μm pore size, 100 kDa MWCO) and subsequent size-exclusion chromatography of the concentrates and final collection of the separated exosome and microvesicle populations from the individual fractions by high-speed centrifugation (>100,000 × g, 120 min) (Taylor et al. 2002, 2011).

Importantly, systematic investigation of the impact of the storage conditions of preparations of EMVs on their size and integrity demonstrated (Sokolova et al. 2011) that the vesicle diameter significantly declines with the duration of the storage period (e.g., within 2 days) and with increasing temperature during the storage (from 4 °C to 37 °C). It is of practical interest that storage at −20 °C, repeated freezing-thawing cycles (up to 10), and ultracentrifugation per se do not appear to affect the size and composition of EMVs to any significant degree (Sokolova et al. 2011).

Detection, Differentiation, and Quantification

In vitro models to generate EMVs from the main cell lineages implicated in the pathogenesis of the disease of interest should encompass the following steps: (i) Cell culture protocols and standard operation procedures have to be implemented and optimized for the routine generation of EMVs from relevant and appropriate human cell culture models. (ii) Cell type-specific EMV markers have to be validated using FCM, immune blotting (using commercially available and/or newly generated mono-/polyclonal antibodies), lipid/protein/DNA arrays and nucleic acid probes, as well as NTA and biosensing on EMVs generated in vitro from the corresponding cell type. (iii) Candidate markers for relevant cell-derived EMVs have to be selected for the demonstration of their presence in the circulation and validated as candidate biomarkers in future clinical studies.

Filtration

A convenient, cheap, and rapid procedure for the differentiation of EMVs, which does not depend on sophisticated and labor-intensive technology, represents filtration. The samples are passed through filters of appropriate pore size (0.1–0.2 μm) and made of materials which do not support unspecific adhesion. In general, the filtration methods for the isolation of EMVs have the distinct advantage compared to procedures based on differential centrifugation of being capable of processing larger sample volumes, in particular, of cell culture supernatants. The subpopulations of the fractionated EMVs can be further analyzed and separated according to density or function which considerably increases the resolving power compared to filtration alone (Jy et al. 2010; Lawrie et al. 2009). For instance, a filtration method has been developed that enables the quantification of phosphatidylserine-positive EMVs from human plasma with diameter >200 nm and relies on staining with annexin-V (Grant et al. 2011). The levels of EMVs in the venous plasma of healthy donors were calculated as one vesicle released from 32 blood cells on average. This releasing efficacy is considerably lower than that determined for cultured cells with one vesicle released per eight cells. Importantly, this study identified some parameters critical for the releasing efficacy of blood cells, in particular, of peripheral blood monocytes. The majority of the variables investigated, including low temperature (−20 °C), proband age, and gender, does not have detectable impact on the absolute number of EMVs. In contrast, fasting and smoking lead to significant (about 2.7-fold and 1.9-fold, respectively) increments in the levels of released EMVs which were amenable to staining with annexin-V compared to the fed and nonsmoking state, respectively (Grant et al. 2011).

Size-Exclusion Chromatography

Five hundred microliter samples containing microvesicles are subjected to the size-exclusion chromatography using a Sephacryl S-300 column (0.9 × 60 cm), equilibrated with 20 mM Tris/HCl (pH 7.4), 1.5 M NaCl, 0.01 % sodium azide, 10 μg/ml leupeptin, and 20 units/ml RNAsin. Fractions of 0.2 ml are collected, centrifuged (Beckman Airfuge, A-110 fixed-angle rotor, 110,000 rpm, 30 min, 4 °C), and then suspended in 100 μl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM MgCl2, 2 mM MnCl2, 1 mM DTT, and protease inhibitor mix.

Centrifugation

EMVs contained in biological sample fluids can be collected by differential centrifugation, often including initially a single or two sequential

"cleaning" step(s) at low speed and for short time (e.g., 300 × g, 5 min) for the removal of intact and broken cells, cell debris, huge membrane fragments, and subcellular organelles (e.g., mitochondria) followed by the "collection" step for microvesicles at medium speed and for intermediary time (e.g., 12,000 × g, 20 min) and terminated by the "collection" step for exosomes at high speed (in an ultracentrifuge, e.g., 150,000 × g, 120 min) for prolonged time (Momen-Heravi 2012; Tauro et al. 2012; Jayachandran et al. 2012). The fractionated microvesicle and exosome pellets can be further purified by several washing cycles using appropriate centrifugation conditions adapted from the "collection" steps and modified for optimal differentiation between the separated EMV subspecies. The identity of the pelleted EMV subspecies can then be determined by immune blotting for known EMV protein components. However, this procedure does not provide information about the number and size of the separated EMV subspecies.

Typical protocol for EMV released from adipocytes: After induction (14 h, 37 °C) of the adherent adipocytes with stimuli known to trigger release (e.g., high concentrations of fatty acids or of the antidiabetic sulfonylurea drug, glimepiride, or of reactive oxygen species) in 12-well plates filled with 1 ml per well of DMEM containing 5.5 mM glucose, 10 % FCS, 0.2 % BSA, 1 mM sodium pyruvate, 25 μg/ml gentamicin, 50 units/ml penicillin, and 20 μg/ml streptomycin sulfate by the addition of palmitate, glimepiride, or GO (for the production of H2O2 in the glucose-containing incubation medium) to final concentrations as desired, the cells are chilled and collected by spinning (200 × g, 3 min). Total EMVs are prepared from the incubation medium (0.9-ml portions) by two sequential passages through 5 5 μm mu;m meshes to remove detached cells. The flow-through is supplemented with DTT (final conc. 0.5 mM) and protease inhibitor mix "complete" (final conc. 1 tablet per 100 ml), then centrifuged (1,000 × g, 10 min, 4 °C), and finally filtered through 1 1 μm mu;m PVDF meshes to remove residual cell debris. The flow-through is centrifuged (2,000 × g, 20 min, 4 °C). The resulting supernatant is recentrifuged (5,000 × g, 30 min, 4 °C). From the resulting supernatant, total EMVs are collected by centrifugation (Beckman Airfuge, A-110 fixed-angle rotor, 110,000 rpm, 1 h, 4 °C). After careful aspiration of the supernatants, the pellets are suspended in 100 μl of EMV buffer (10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM MgCl2, 2 mM MnCl2, 1 mM DTT, protease inhibitor mix "complete").

Typical protocol for EMV released into serum: Rat serum is centrifuged (15,000 × g, 15 min, 4 °C) to remove cell debris and aggregates. The resulting supernatant is centrifuged (100,000 × g, 2 h, 4 °C). After washing with PBS, the resulting pelleted vesicles are suspended in PBS and purified by sucrose density gradient centrifugation (200,000 × g, 18 h, Beckman SW41, Beckman-Coulter, Brea, CA) on 10–70 % sucrose in PBS prepared with Gradient Mate device (BioComp, Fredericton, New Brunswick, Canada). Fractions with densities of 1.12–1.22 mg/ml are combined, diluted tenfold with PBS, and centrifuged (100,000 × g, 2 h, 4 °C). The pelleted vesicles are suspended in the initial volume of MV buffer and recentrifuged as above.

Enzyme-Linked Immunosorbent Assay

ELISAs typically fail to quantitatively capture all the EMVs present in biological sample fluids; detect soluble antigens, such as cytoplasmic enzymes and nuclear proteins, which may represent the soluble contents of EMVs and are prone to loss during the EMV preparation procedure; and cannot differentiate between microvesicles and exosomes (Tauro et al. 2012). The latter two disadvantages could be overcome by the use of detecting antibodies raised against surface, in particular, transmembrane protein components that are specific for EMVs.

Affinity Purification of a Subset of Adipocyte-Derived EMVs

For affinity purification of Gce1-/CD73-harboring EMVs, total EMVs are adsorbed to cAMP- or AMP-Sepharose beads. For this, 100 μl of EMV suspension is supplemented with 200 μl of (c) AMP-Sepharose beads (50 mg in 1 ml of

100 mM Hepes/KOH, pH 7.4, 140 mM NaCl, 1 mM MgCl2, 0.5 mM DTT, and protease inhibitor mix "complete") and then incubated under continuous head-over rotation (60 cycles per min) of the tubes. Thereafter, the incubation mixtures are centrifuged (2,500 × g, 5 min, 4 °C). The collected Sepharose bead-EMV complexes are washed twice and then suspended in 100 μl of EMV buffer containing either 100 μM AMP or cAMP for dissociation of the EMVs from the beads. After incubation (30 min, 4 °C) and centrifugation (5,000 × g, 5 min, 4 °C), the supernatant is recovered and centrifuged (Beckman Airfuge, A-110 fixed-angle rotor, 110,000 rpm, 30 min, 4 °C) for collection of the Gce1- or CD73-harboring EMVs.

Electron Microscopy

In general, electron microscopy manages to reveal the presence of EMVs in the pellets obtained by ultracentrifugation of the biological sample fluids, such as plasma, urine, or cell culture medium. However, in general, this method is not quantitative and necessitates extensive efforts for appropriate sample preparation. Nevertheless, transmission as well as scanning electron microscopy has been used in many studies published during the last two decades and is therefore commonly regarded as the gold standard for the size determination and classification of EMVs (Sharma et al. 2010). However, closer analysis of the multitude of electron microscopic micrographs and pictures available in the public domain reveals the inherent difficulties and pitfalls in the interpretation of the results obtained by different research groups. In particular, the considerable variability in the extent of heterogeneity as delineated from the size and morphology of the EMVs reported to be released from the same cell type in response to the same stimulus (e.g., calcium ionophores) is surprising and disappointing. It may be explained by (subtle) differences in the methods used for preparing the EMV samples and/or taking the micrographs as well as in the biased selection of the photographic fields to be analyzed and published. In any case, these study-to-study variations significantly impair the quantitative evaluation and classification of the portions of EMV subspecies released from specific cell types upon challenge with specific stimuli. An additional disadvantage represents the pronounced time requirements for a complete cycle of the electron microscopic procedure, which do not match the demands for routine and clinical EMV analytics.

Atomic Force Microscopy

Atom force microscopy has recently been introduced for the analysis of the size (distribution), number, and morphology of EMVs (Sharma et al. 2010), in general, and of CD41+ vesicles in plasma, in particular (Yuana et al. 2010). The data presented so far are of exquisite quality with regard to both the precision of the size measurement and the clarity and illumination of structural details that have never been detected up to now. Certainly, these exciting results have to be corroborated using distinct cell types and stimuli with emphasis on analysis of the degree of variation. Unfortunately, at present the equipment requires high expenditure regarding the technological complexity, knowledge, and experience in instrument handling, labor intensity, and costs, which will limit the broad availability of atomic force microscopy.

Conventional Flow Cytometry (FCM)

The degree of light scattering recognized by commercially available (analogue or first-generation digital instruments of) flow cytometers (e.g., Becton Dickinson Facs CaliburTM Flow cytometer) is adequate for certain routine clinical applications, such as cell sorting and counting of particles with diameters above 500 nm, but not sufficient to support the detection of smaller particles below 300 nm (van der Pol et al. 2010; Perez-Pujol et al. 2007). For future routine analysis of microvesicles (rather than exosomes) by FCM, it will be essential to compare plasma samples with identical numbers of EMVs on two or more of instruments of the same type for standardization and calibration of EMV analytics per se as well as evaluation of the reliability of the chosen instrument series. Further improvements in resolution may result from adsorption of the EMVs to latex beads as well as the use of CD surface markers, such as CD61, CD8, or FasL, to identify,

characterize, and categorize EMVs released from different cell types (Thery et al. 2006; Abusamra et al. 2005; Andreola et al. 2002).

A solution for the apparent failure of FCM to differentiate between vesicle sizes in the range of 100–500 nm, i.e., for the differentiation between microvesicles and exosomes, may come from the use of fluorescent signals instead of visible light, which are generated by excitation with a laser beam and become highly amplified by sensitive photomultipliers. The monitoring of scattered fluorescence results in a significantly elevated signal-to-noise ratio compared to scattered visible light. In consequence, the counting efficacy of vesicles in the range of 100–500 nm becomes significantly increased upon fluorescence measurement which leads to a considerable improvement of the reliability of detection and assignment of microvesicles. In contrast, at present typical exosomes fail to be detected by FCM with satisfying precision and reproducibility, even if operating with the fluorescence principle.

Multiparameter FCM

Numerous FCM studies using body fluids have shown a correlation between the number of EMVs and certain human diseases. However, due to the optical performance of the currently used standard FCM platforms (scattering, frequency), microvesicles (≈1 μM diameter) are accessible for measurement by conventional FCM, while the smaller exosomes (50–100 nm) remain below the detection limit. Moreover, other protein complexes, especially circulating immune complexes, protein aggregates, membrane fragments, and subcellular organelles, overlap in biophysical properties (size, scattering, density, and sedimentation) with EMVs. However, with the most recent FCM technology (NAVIOS Beckman-Coulter, FACSVerse Becton Dickinson, Apogee A50), the instrumental requirements to reliably quantify EMVs have greatly been improved. Moreover, important progress has been made toward standardization of sample preparation, immunostaining, and vesicle size protocols. Currently, there is little agreement on the actual concentration of EMVs in blood in healthy subjects ranging from 200/μl to over 1.000.000/μl. These discrepancies are mostly due to variation in sample processing and in the calibrator beads used in order to distinguish EMVs from cell debris, organelles, or protein aggregates. A recently published protocol using 0.5–0.9 μm-polystyrene microspheres (megamix) allowed to define a forward scatter EMV-size gate for standardization of EMV-measurements in body fluids. In combination with latex beads, the Apogee A50-Micro Flow Cytometer now manages to resolve EMVs down to about 100 nm by light scattering and to discriminate exosome and microvesicle subpopulations. With the most recent instrumentation introduced into the market (Beckman-Coulter NAVIOS, Becton Dickinson FACSVerse), the sensitivity, speed (20-bit processors), and optical resolution (scattering performance) now enable a more standardized and quantitative EMV analysis in pre-centrifuged patient blood samples.

Dynamic Light Scattering

For more than five decades, light scattering has been commonly applied for the determination of the molecular weights of large protein complexes and the sizes of small particles. This range of resolution which has been further broadened on the basis of recent technological progress seemed to be well suited for the analysis and differentiation of the individual EMV subspecies. In fact, an interesting comparison of the performance in size determination is performed between two commercially available instruments, Zetasizer Nano S (Malvern Instruments, Ltd., Malvern UK) and N5 Submicron Particle Size Analyzer (Beckman-Coulter, Brea, CA), that are both based on dynamic light scattering but different technical realization, with EMVs from fresh-frozen human plasma samples (Lawrie et al. 2009). In fact, the detection principle seems to be compatible with the resolution requirements for the discrimination between exosomes and microvesicles and to offer the additional advantages of simple practical application and routine use. Meanwhile, the Delsa Nano series of analyzers has been introduced into the market, which will replace the Beckman instrument NP5 and relies on Brownian motion of the EMVs, in the presence or absence of an applied electrical field, that causes

Doppler effects with the light scattered from the vesicles. This instrument provides a valuable combination of information about zeta potentials, electrophoretic mobility, and size distribution. In theory, the huge detection range from 0.6 nm to 7 μm should guarantee the reliable classification and characterization of EMVs, which remains to be demonstrated in future studies.

Electrochemical Impedance Spectroscopy (EIS)

A novel label-free method for EMV detection and quantification represents EIS which reaches detection limits as low as several EMVs per μl. Impedance-based detection and quantification of EMVs is based on fundamental interfacial electrochemistry at the sensing electrode, which is driven by electrochemical activities of EMVs that can be separated by applied electrochemical potential. Compared to light scattering in FCM, the integration of the EIS method into an automated, more reproducible, and accurate EMV counting platform is conceivable. However, the detection limit of $\geq$300 nm may limit its use and introduce a bias toward the detection of larger vesicles, i.e., of microvesicles versus exosomes (Lawrie et al. 2009).

Coulter Principle

The so-called Coulter technology monitors the considerable changes in the electrical impedance through a narrow channel with a defined aperture that is caused by the passage of single cells or particles and enables the determination of their number per volume unit as well as of their absolute size and size distribution with the help of appropriate signal and data processing software. The diameter of the aperture is critical for the size range of particles and cells that are amenable to Coulter analysis. For instance, the Coulter counter, "Multisizer 4" (Beckman-Coulter, Brea, CA), was designed for the general determination of particle size with a lower cutoff of 400–600 nm presumably for technical reasons which limits the lower diameter to be analyzed at present. Consequently, this instrument will not enable the reliable counting of exosomes.

Nucleic Acid Analytics

A multitude of methods have been reported for the identification of nucleic acids associated with EMVs, which are based on routine next-generation sequencing, RT-PCR, Taqman analysis, and chip/microarray technologies (Palma et al. 2012; Bala et al. 2012; Gallo et al. 2012; Lässer et al. 2012; Zhou et al. 2012; Rani et al. 2011; Taylor et al. 2011; Michael et al. 2010; Rosell et al. 2009; Valadi et al. 2007) and will not be covered in this review. Instead, the potential use of fluorescent probes and chromophores directed against EMV-associated nucleic acids is proposed. For this, standard fluorescent nucleic acid probes could be used that detect the complementary sequence information by enhanced emission intensity or quenching (Ehrenschwender and Wagenknecht 2011). However, such changes potentially result from side effects leading to artifacts in the fluorescence readout. Undesired quenching of fluorescence emission represents a considerable problem in cell biology. Hence, dual (or even multiple) labels, that change their emission maximum, operate as superior probes for the detection and imaging of nucleic acids, as it has been shown with wavelength-shifting molecular beacons, for instance (Ehrenschwender et al. 2010; Holzhauser et al. 2010; Holzhauser and Wagenknecht 2011). However, the disadvantage of commercially available systems is that often the signal-to-noise ratio is rather low. It has been proposed based on published results (see below) that the RNA base surrogate pairs of cyanine dyes promote a resonance dipole-dipole interaction mechanism within a ground-state complex (Berndl et al. 2009, 2010). This assembly follows a static quenching mechanism yielding a better signal-to-noise ratio. It is important to point out that the ground state of dye-dye interactions is determined by the RNA duplex framework holding the dyes close together and is not due to an inherent affinity of the dyes for each other since this provides the basis to discriminate optically between miRNA strands (Berndl et al. 2009).

From most recent results with combinations of cyanine dyes, it becomes clear that the type of chromophore attachment to the oligonucleotides

is critical for efficient changes in emission color. In commercially available probes, for instance, molecular beacons, fluorescent dyes are attached typically via flexible and long alkyl chain linkers (Holzhauser and Wagenknecht 2011). Energy transfer can occur only inefficiently by collisional quenching. In contrast, in ongoing approaches, the DNA architecture around the fluorescent chromophores incorporated as DNA base substitutions forces the two dyes in close proximity and thereby enhances the energy transfer efficiency by static quenching (Ehrenschwender and Wagenknecht 2011). During the detection of the corresponding miRNAs, the architectural force becomes released by opening the hairpin conformation step by step, and the resulting separation of the dyes gives the characteristic fluorescence color change, for instance, from red to green (Holzhauser et al. 2010). In comparison with conventional probes, this approach is characterized by two major advantages: (i) the fluorescence readout allows a clear and distinct discrimination simply by the emission color (about 140 nm shift). (ii) On the basis of the well-separated emission bands, the high contrast between the open and closed forms increases the signal-to-noise ratio. These two properties hopefully characterize the corresponding newly developed probes as powerful tools for the nucleic acid analytics of EMVs. Moreover, the application of synthetic chemistry will allow the tuning of the applied chromophores. In consequence, the design, synthesis, biophysical characterization, and application of chromophore-labeled oligonucleotides as specific and bright fluorescent probes for the detection and imaging of EMV-derived RNAs promise the distinct advantage of the avoidance of sophisticated and cost-intense technologies and thus justify future research efforts for the confirmation of this hypothesis.

However, each of the above procedures necessitates the disruption of the isolated EMVs in order to gain access to their luminal contents. While the extraction and purification of mRNAs and miRNAs out of EMVs for their subsequent analysis have turned out to be feasible even for routine purposes, the use of NP-based biosensing (see below) in theory may offer the opportunity to identify the nucleic acid signatures of EMVs without the need for their cracking and nucleic acid preparation due to the intrinsic capability of NPs to cross biological membranes. This hypothesis and the associated advantage of a considerably simplified mRNA/miRNA analytics merit rigorous testing by future experimentation.

RNA Isolation and qRT-PCR

For the removal of exogenously adherent nucleic acids, the EMV pellets are suspended in 1 μg RNase A (DNase- and protease-free; Roche Biochemicals, Mannheim, Germany) and DNase I (RNase-free; Qiagen, Hilden, Germany) per ml of EMV buffer and then incubated (1 h, 25 °C). The samples are centrifuged (Beckman Airfuge, A-110 fixed-angle rotor, 110,000 rpm, 1 h, 4 °C), then suspended in EMV buffer containing RNase inhibitor (1 U/ml), and finally washed by three resuspension/washing cycles with 1 ml of EMV buffer each. The washed EMV pellets are disintegrated by the use of TRIzol (Invitrogen, Carlsbad, USA). Total RNA is isolated with the Qiagen (Hilden, Germany) RNeasy kit including proteinase K digestion, DNase digestion, and an additional RNeasy cleanup step, as recommended by the manufacturer. Total RNA (1 μg) is reverse-transcribed using appropriate primer sets. The specific transcripts are quantitatively evaluated as described previously (Müller 2010).

For quantitative RT-PCR, total RNA (1 μg equivalent to 50 ng mRNA) is reverse-transcribed with the AMV-RT first-strand cDNA synthesis kit (Roche Biochemicals, Mannheim, Germany) in 20 μl reaction volume. Reverse-transcribed single-strand cDNA (2 μl) is used as a template for amplification in a LightCycler using FastStart DNA master SYBR Green according to instructions of the manufacturer (Roche Biochemicals, Mannheim, Germany). 18S RNA is used to normalize for the starting amount of cDNA. For amplification of microRNAs, DNase-I-digested total RNA is reverse-transcribed using a Mir-X^{TM} miRNA First-Strand Synthesis Kit (Takara Bio Inc. Otsu, Japan) and subjected to quantitative RT-PCR using a published protocol (Miranville et al. 2010) with the following modifications: one cycle (60 s at 94 °C), 40 cycles each (30 s at 94 °C

and 30 s at 55 °C and 90 s at 72 °C), and one cycle (10 min at 72 °C) after an initial activation step for 10 min at 95 °C. Primer sets for individual mRNAs and miRNAs used are given in the relevant literature. The correct size of the resulting fragments and generation of single products are monitored by agarose gel electrophoresis. Total RNA contents are calculated using a concentration standard curve of the respective amplified fragments and normalized to expression levels of the housekeeping gene ß-actin. The comparison of the amounts of mRNAs and microRNAs between different EMVs is based on equivalent amounts of total RNA in each experiment due to lack of appropriate "housekeeping" RNAs in the microvesicles. The Ct-values are used to calculate the relative abundance for each mRNA and microRNA. For normalization of mRNA and microRNA, 36B4 and snoRNA202 are used, respectively. Quantitative comparison of different mRNAs and microRNAs is determined with the 2-DDCt method (Aoki et al. 2007; Livak and Schmittgen 2001).

Nanoparticle Tracking Analysis (NTA)

Many of the problems and disadvantages associated with the technologies mentioned above could be overcome by the recently introduced method of NTA. It enables the direct and real-time visualization as well as quantitative evaluation of nanoparticles (NPs) in fluidic samples (Carr et al. 2009) and has already been established in other areas, such as the size determination of engineered NPs, such as inks, pigments, carbon nanotubes, protein aggregates, and viral particles. NTA relies on the light-scattering characteristics of laser light on vesicles that undergo Brownian motion when in solution. Thus, the underlying principle is a correlation between the Brownian motion that is monitored by light scattering using a light microscope connected to a video system and the vesicle size. The NTA software uses the video data for tracking the Brownian motion of individual EMVs and thereof calculates their size and total concentration. For this, a video recording of the tracked EMVs is analyzed, and a mean-squared displacement is calculated for each vesicle (Filipe et al. 2010; Dragovic et al. 2011).

Conventional NTA

Conventional NTA allows the determination of the hydrodynamic radius and thereby of the size distribution of the EMVs. For this, the diffusion coefficient and sphere-equivalent hydrodynamic radius have to be calculated on the basis of the Stokes-Einstein equation. First experimental experience is now available for the NanoSight LM10 and NS500 instruments (NanoSight Ltd., Amesbury, UK) that introduce an exactly focused laser beam via a glass prism into the sample fluid harboring the EMVs in suspension at diluted state. Following refraction of the beam at a low angle when it hits the sample, a thin beam of laser light is generated at the interface of the glass prism and the sample fluid which illuminates the EMVs through the thickness of the sample chamber (about 500 μm). EMVs which reside within a beam as it crosses the sample layer are visualized with a conventional optical microscope connected to a video camera. These instruments are typically installed in parallel to the beam axis and collect all the light which becomes scattered by all EMVs present in the field of view at the time point of the photography. The beam depth is about 20 μm at the point of analysis and thereby matches with the distance of the imaging optics guaranteeing optimal focus. Typically, the recording data for the video are 60 s total duration and 30 frames per sec. The NTA software (NanoSight Ltd.) for monitoring the movement of the individual EMVs has been designed to first identify and classify each vesicle and then to follow each selected vesicle by frame-to-frame comparison of its tracked Brownian motion. The relative velocity of the individual EMVs calculated from the frame-to-frame comparisons and measurements is correlated to the vesicle size on the basis of the two-dimensional Stokes-Einstein equation.

In a series of studies, the potential of NTA for the determination of size distribution and concentration of EMVs and their classification into exosome and microvesicle subspecies was demonstrated with biological fluidic samples (Carr et al. 2009; Filipe et al. 2010; Dragovic et al. 2011). The identification and tracking of a particular EMV contained in the sample can be optimized by adjustment of a multitude of

parameters affecting the video capture, such as shutter speed and camera gain, as well as the data analysis, such as background subtraction, filter setting, minimal track length, and frame-to-frame search area. Importantly, the refractive index of the individual EMV critically determines the range of its size which is compatible for its detection by NTA. In general, high refractive indices, such as for colloidal gold, allow the tracking of very small particles, whereas low indices, such as for cellular vesicles including EMVs, require larger diameters of the particles for their reliable visualization. The reason for this is the strong dependence of the lower detection limit on the signal-to-noise ratio of the image. The latter is critically affected by the amount of the scattered light that for EMVs at the size limit of detection will follow the Rayleigh scattering equation. In consequence, the lower limit of detection of EMVs with their low refractive indexes by the NTA system is in the range of 50 nm. The upper limit of detection is at about 1 μm since for larger particles their Brownian motion becomes too low and thereby would limit the accurate tracking. Taken together, this theoretical range of detection makes NTA perfectly suited for the discrimination of EMV subspecies according to their size distribution.

In fact, the experimental evidence so far available (Soo et al. 2012; Sokolova et al. 2011; Dragovic et al. 2011) has clearly demonstrated that the size and concentration of EMVs can be measured using NTA accompanied by a number of decisive advantages compared to the methods discussed above: (i) In comparison to electron microscopy and atomic force microscopy, a complete operation cycle of NTA requires less time compatible with a higher throughput of sample processing and analysis and, in addition, does not contribute to shrinkage artifacts as caused by the fixation procedure, since it is performed in suspension. (ii) In comparison to conventional FCM, the demonstrated lower size limit for detection of EMVs by NTA is considerably lower (50 vs. 300 nm), and thereby NTA manages to detect vesicles as small as about 50 nm with significantly higher sensitivity. It remains to be shown whether the improved resolution power of the latest generation of digital flow cytometers with the reported discrimination of 100 nm and 300 nm latex/polystyrene beads from one another in a mixture (Lacroix et al. 2010a, b) holds true for the minimal sizes of EMVs for their detection and separation by FCM. This seems to be questionable given the lower refractive indexes of EMVs and associated underestimation of their size compared to latex/polystyrene beads (Lacroix et al. 2010a, b; Foladori et al. 2008).

It is thus of considerable advantage that the detection principle of NTA relies on the Brownian motion of the EMVs rather than on their refractive index (Carr et al. 2009). Interestingly, according to FCM, the major portion (>90 %) of placental vesicles, which are commonly assumed to represent a mixture of exosomes, microvesicles, and apoptotic bodies over a broad size range (Orozco and Lewis 2010), have a diameter of less than 1 μm with a peak distribution shifted leftward to the 300 nm cutoff. According to NTA, the major portion of the EMVs actually has diameters below 300 nm, albeit larger vesicles of 500–600 nm are identified in that preparation, too, however to a minor degree only (Dragovic et al. 2011). This apparent underestimation of the number of larger compared to smaller vesicles, i.e., microvesicles versus exosomes, in complex EMV samples may be due to the requirement for their dilution in case of a high ratio of exosomes versus microvesicles in those mixtures. NTA is operating most accurately with EMV concentrations of 2–20 × 108/ml. Samples harboring higher numbers of EMVs have to be diluted before measurement and the relative EMV concentration calculated according to the dilution factor. Thus, albeit vesicles with a size up to 1 μm can be tracked by NTA, a low concentration of them caused by dilution will inevitably lead to a more than proportional decrease in signal strength over background due to the limited Brownian motion of large vesicles. To overcome the resulting possibility of underestimation of vesicles from 0.5 to 1 μm, such as microvesicles in particular, alone or in complex mixtures, the determination of their numbers should be performed by FCM rather than NTA.

(iii) In comparison to dynamic light scattering, NTA is capable to differentiate the EMV

subspecies in complex sample mixtures taking into consideration the limitations as discussed above. Very small vesicles are accessible for detection by dynamic light scattering if present in the sample alone. However, in combination with other vesicles of different sizes, the very small ones resist resolution by this method since the light emitted from all the vesicles together becomes collected and measured simultaneously by the single detection element of the instrument. The calculated average value for the scattering of a given sample tends to overestimate the absolute size and size distribution of the vesicles.

At variance, NTA measures simultaneously the scattering intensity and velocity of the movement of individual vesicles and thereof calculates their size individually. This operation mode is compatible with maximal resolution in size even out of heterogeneous EMV sample mixtures. In addition, the direct monitoring of individual EMVs supports the determination of the vesicle concentration through extrapolation of the number of vesicles detected at any given time point in any microscopic field to the concentration of the vesicles per volume unit with regard to the defined and known scattering volume. Certainly, the possibility of calculating the concentration of individual EMVs, which remains unique for NTA so far, represents a huge advantage for the analysis of the EMV subspecies and their composition of biological fluids, since the physiological relevance of EMVs of different size (and composition) in health and disease apparently critically depends on alterations in their concentration and shifts in their size distribution.

In addition, the possibility to quantitatively evaluate the number of EMVs in a given sample by NTA using the NanoSight instrument allows for the first time in combination with protein determination to know how many EMVs are being added to an experimental setup for studying their biological effects. So far, the input of EMVs into a test system has typically been reported as the amount of EMV protein, which often ranges from a few to several 100 μg (Abusamra et al. 2005; Webber et al. 2010), rather than as a precise number. NTA is now likely to support a more reliable standardization for biological test systems. Furthermore, the physical state of a given EMV preparation prior to its addition to a test system has often remained uncharacterized so far. Now NTA analysis of EMVs prior to the assay may reveal whether (even minor) portions of the vesicles contained in the sample to be assayed are in the monodispersed or aggregated state, which both could considerably affect the biological activity of the EMVs in the test system. Finally, the knowledge of the average size and size distribution of EMVs as determined by NTA will help to judge about the (biophysical) quality of a given EMV population as well as the reproducibility of its preparation.

A major disadvantage of NTA represents the considerable height and variability of the background signal. To keep that within acceptable limits in many experiments, serum-free medium seems to be required since serum supplements (e.g., fetal calf serum) often harbor vesicles and particulate materials which have to be removed by ultracentrifugation prior to their addition to the culture medium. Moreover, serum proteins may tend to aggregate and thereby elicit false-positive signals during NTA analysis. Unfortunately, many cell systems do not tolerate extended periods of time under serum-free conditions without changes in physiological behavior or loss of vitality, which both could affect the NTA results. As a consequence, the analytics of EMVs released from those cultured cells cannot be assessed in serum-positive medium since it will be very difficult or even impossible to discriminate between cell-derived and serum-derived EMVs on the basis of conventional NTA. However, it is conceivable that the measurement of EMV signatures rather than single parameters in course of NP-based biosensing may enable the "subtraction" of serum-derived EMVs from the total EMV population prepared from the culture medium of the releasing cells and thereby result in the identification of the cell-derived EMVs (see below).

Fluorescent NTA

The unmodified version of NTA does not include the possibility to characterize the EMVs at the molecular level, in particular with regard to the

presence of marker proteins and the cellular origin. Such phenotyping of EMVs is of crucial importance for our future understanding of their biological roles, since the easily accessible sources for EMVs, such as plasma, urine, saliva, and mucus, consist of complex mixtures of EMVs released from many different cell types. To tackle this challenge, Dragovich and coworkers recently modified and further developed the conventional NTA technology with the combination of fluorescent labeling (Dragovic et al. 2011). Thus fluorescent NTA has been designed for the simultaneous detection of both microvesicles and exosomes that had been labeled with stable fluorophores (quantum dots) conjugated to antibodies or other biological probes for excitement with a 405 nm violet laser and measurement of fluorescence emission using a matched 430 nm long-pass fluorescence filter and a sensitive fluorescence camera. Under the fluorescence mode, only the fluorescently labeled EMVs are tracked, individually and in real-time for the subsequent calculation of the size and concentration of the labeled EMVs. The light-scatter mode can operate in parallel for the evaluation of the total number of EMVs and/or other light-scattering particles. For instance, three videos of either 30 or 60 s may be recorded for each EMV sample with shutter speeds of 30, 6, and 1 ms for the 100, 200 , and 400 nm control beads and with shutter speeds of 30 and 15 ms for the EMVs samples. NTA 1.1 and 2.1 software (NanoSight) may be used for data analysis.

Dragovic and coworkers showed for the first time that the combination of NTA with the use of fluorescent antibodies directed toward EMV protein components or fluorescent quantum dot-labeled cell tracker peptides and fluorescence measurement allows the phenotyping and classification of EMVs with regard to their cellular origin, even from total pelleted vesicles out of complex biological fluids, such as human plasma (Dragovic et al. 2011). For this, EMVs released from human placenta in course of perifusion in vitro or EMVs recovered from human plasma are incubated with a fluorescent mouse monoclonal antibody specific for the placental EMV marker protein, NDOG2 (Reddy et al. 2008), and linked to quantum dots. Alternatively, a fluorescent cell tracker peptide conjugated to quantum dots is used for the incubation. The concentrations of NDOG2-positive placental or cell tracker-positive plasma EMVs are found to be slightly and drastically, respectively, lower compared to those of the corresponding total EMVs as revealed by light scattering. These differences are presumably due to contaminations of the perifusate with blood cells and/or EMVs derived thereof, which are recognized by light scattering rather than the anti-NDOG2 antibody, and of the plasma with lipidic structures, such as very-low-density lipoproteins, chylomicrons, and lipid droplets, which are detected by light scattering rather than the cell tracker dye, respectively. Upon separation of those lipidic structures with a size similar to, but a buoyant density significantly below, that of EMVs (Ruf and Gould 1998; Cantero et al. 1998) from the EMVs in course of ultracentrifugation, the numbers of (cell-derived) fluorescence and (total) light scattering counts are determined for the pelleted EMVs and found to be very similar (Dragovic et al. 2011). These data suggest for the first time the principal feasibility of NTA-based single or multiple component analysis of EMVs.

In an analogous set of experiments, Soo and coworkers (Soo et al. 2012) compared the numbers of EMVs released from various human T-cell lines that can be detected by NTA before and after immunodepletion of the EMVs using antihuman CD45 antibodies coupled to magnetic beads (Grant et al. 2011). Only about 50–60 % of the total EMVs detected by NTA are accessible for immunodepletion (Soo et al. 2012), raising the possibility that the EMV population studied is heterogeneous with vesicles of comparable size either expressing or lacking CD45. An alternative explanation is that within an EMV subspecies, the variation in size of the exosomes or microvesicles affects the expression of a given canonical marker protein. Thus a (too) small size of exosomes or microvesicles may prevent the inclusion of a certain canonical marker commonly accepted for either exosomes or microvesicles, respectively, in each individual member of that particular EMV subspecies. In consequence, a series of

proven markers should be tested in order to define a complete EMV-specific pattern for the protein equipment of EMV subspecies. Altogether, these results are very encouraging for the potential routine analysis of the cellular origin and for further phenotyping of EMVs from complex biological fluids using fluorescent NTA. These objectives may be realized in course of more detailed labeling studies with plasma EMVs and (combinations of) fluorescently labeled antibodies and peptides directed against specific markers for erythrocytes, leukocytes, platelets, macrophages, and endothelial cells as well as for a selected panel of tissue cells.

Conventional NP-Based Biosensing

The generation of signatures reflecting in completion or in part, at least, the combination of different components, such as proteins, phospholipids, and nucleic acids, that constitutes the specific types of EMVs and EMV subspecies, rather than the identification of single or a few components as is true for the methods described so far represents the objective of biosensing. In principle, biosensors could perfectly fulfill this job of pattern recognition based on the following assumptions and theoretical considerations, but clearly the successful operation of biosensing for the analytics of EMV signatures remains to be demonstrated in the future and will encompass the following steps. The initial step for achieving this goal represents the design of NPs consisting of a gold core with covalently attached tentacle-like aliphatic, positively charged, and aromatic substituents at their surface. They have to be produced with multiple structural variations for differential interaction with EMVs through their proteinaceous and phospholipidic surface components and possibly luminal protein as well as miRNA/mRNA constituents after the passage of uncharged or amphiphilic NP variants across the EMV membranes. With regard to the biosensing of luminal EMV components, NPs of certain size and structure have been amply documented to efficiently cross biological membranes (and reach the cytoplasmic compartment of many mammalian target cells) through yet incompletely understood molecular mechanisms (Aschner 2009; Garcia-Garcia et al. 2005; Elder et al. 2009; Bouwmeester et al. 2011). A multitude of hydrogen, electrostatic, hydrophobic, and van der Waals bonds will contribute to the high-avidity interactions of the EMV protein and nucleic acid constituents with the substituted NPs. Analogous NPs which interact with selected serum proteins, viruses, bacteria, or cancer cells in selective fashion have already been successfully developed and used as so-called chemical noses in very sensitive and specific detection assays (De et al. 2009; Anslyn and Rotello 2010; Miranda et al. 2010b). For the subsequent step, a readily assayable reporter enzyme, such as glucose oxidase, has to be selected and engineered via recombinant means which interacts with those EMV-specific NPs on the basis of high avidity rather than high affinity and selectivity.

It is thought that upon incubation, the reporter enzyme, such as glucose oxidase, and the sample EMVs will compete with one another for binding to the NPs depending on the relative avidity and cooperativity (Müller 2012a). Two possibilities can be envisaged: (i) NPs interact with the glucose oxidase rather than with the EMVs and thereby cause its inhibition, presumably through steric hindrance of substrate (i.e., glucose) binding, or (ii) NPs interact with certain subspecies of the sample EMVs rather than with the glucose oxidase thereby leaving it in the active state. Theoretically, this can be determined through the measurement of the produced protons, preferably by an ion-sensitive field-effect transistor (ISFET) assembled onto a biosensor chip and providing small currents which are transformed by appropriate data software into signatures (Müller 2011, 2012b). This technology allows very precise quantitative evaluation of the activity state of glucose oxidase or of alternative proton-generating reporter enzymes. ISFET is optimal for the precise and reliable recognition of subtle differences in avidity of the NP-EMV interaction for different NP and EMV species and thereby contributes to the differentiation of distinct EMV types in a body fluid sample. An additional advantage of the biosensor technology is the possibility for measurements in the high-throughput format upon installing of a microarray of ISFETs onto

microfluidic cards and lab-on-a-chips. These configurations should greatly facilitate the analysis of huge numbers of NP-EMV interactions with NP structural variants that is required for the delineation of signatures specific for each EMV subspecies.

In general, the unequivocal identification of EMV subspecies out of total sample EMVs cannot be achieved with the use of a single or a few NP structural variants. This will lead to almost identical or similar signatures provoked by overlapping recognition patterns of the various NPs as well as by similar molecular composition and structural features of the various EMV subspecies in the sample. Rather, EMV analytics by NP-based biosensing necessitates the reliable assignment of a multitude of structurally distinct NPs to the EMV subspecies. In fact, the intelligent and systematic design of NPs with structurally distinct surface substituents considerably differing in hydrophobicity, hydrogen donors/acceptors, charges, and size by combinatorial chemistry and their inclusion in the biosensing array will drastically improve its resolving power. In the future, it will be of great interest to compare EMV signatures between healthy and diseased humans, such as those suffering from T2D and obesity, along distinct and sequential pathogenetic stages.

NTA-Based Biosensing

Currently attempts are ongoing to integrate the NTA- and NP-based biosensing technologies into a single assay format in order to combine the unique advantages of the former, i.e., rapid and reliable determination of the size and number of individual EMVs in a body fluid sample, and the latter, i.e., unequivocal annotation of identity and cellular origin of the EMVs released from a multitude of different tissues/cells into a body fluid. For this, the NPs, which have been designed for differential interaction with the sample EMVs, have to be coupled to distinct fluorophores. Only those NPs will be selected for the fluorescent labeling that are known to recognize surface markers specific for a subset of EMVs, such as those released from a specific cell type or in response to a specific stimulus. Those cell type-specific EMV candidate markers have already been elucidated for rodent and human adipocytes, such as Gce1, CD73 (Müller et al. 2009a, b, 2010a, b, 2011, 2012c), MFG-E8 (Aoki et al. 2007; Ogawa et al. 2010), murine, and human macrophages and T cells, such as IL-1ß, caspase-1 (Qu et al. 2007), TCR/CD3 (Blanchard et al. 2002), CD45, Alix, and Tsg101 (Soo et al. 2012), as well as human dendritic tumor cells, such as ERBB2 (Napoletano et al. 2009), and hopefully their number will increase in course of the ongoing characterization of EMVs released from other cell types using NTA and biosensing. However, it is the current paradigm that cell type-specific EMVs are characterized by specific signatures constituted for by quantitative rather than qualitative differences in the expression level of many (but not all) EMV components and do not differ in the presence or absence of a single typical "marker" component. The ongoing efforts for the introduction of NTA- and NP-based biosensing for EMV analytics will ultimately reveal the validity of this paradigm and thereby the usefulness of EMVs as "systems biology-based" biomarkers.

The number of EMV-associated components, which can be analyzed simultaneously, depends on the number of different fluorophores available for conjugation to the differently substituted NPs. For consecutive analysis, it will be sufficient to label the different NPs with the same fluorophore. The interaction of NPs with EMVs is monitored by fluorescence scattering, which replaces the measurement of reporter enzyme activity by ISFET as performed for NP-based biosensing. In addition to receiving the EMV-specific signatures after multiple cycles of measurement with differently substituted NPs, as is the case for conventional NP-based biosensing, NTA-based biosensing could allow concomitant monitoring of the movement (velocity) of individual EMVs and thereby determination of their size and size distribution as well as physical state. Again, future intense experimentation will be required for the clear-cut demonstration of the compatibility of NP-based biosensing and NTA with the resulting advantages as predicted above.

Moreover, in an attempt to overcome the need for multiple fluorescence labeling of the EMVs,

we are currently evaluating the possibility to follow the EMV-NP interaction by the putative reduction in Brownian motion in course of binding of the NPs to the EMVs. To increase the impact of NPs on EMV movement/velocity, the hydrodynamic radius of the NPs has to be adapted to the size of exosomes or microvesicles, respectively. From a simplifying point of view, NTA- (in its fluorescent version) and NP-based biosensing differ only in the use of fluorophore-conjugated antibodies versus NP, respectively. But this makes the critical difference. The creation of EMV-specific signatures follows an "evolutionary" process according to the needs at the time point of assay implementation. Their informative value, i.e., resolving power, can be gradually increased with the number and quality (with regard to avidity and selectivity) of the NPs included in the assay. The synthesis of NP surface substituents starting from previously adequate structures follows the rules of combinatorial chemistry and can be automated thereby resulting in a huge variety of structural variants in short time. This optimization process is conveniently followed, for instance, by NP-based biosensing, and terminated upon fulfillment of the present criteria for the signature. Those NPs may form the substrate for further optimization in the case of future challenges. Thus there is no need for reinventing the wheel in order to meet higher demands for EMV interaction/recognition. In contrast, this case would require the generation and identification of novel antibodies with improved affinity and specificity by chance in time-consuming and cost-intensive processes.

In the ultimate and ideal experimental setting of NTA-based biosensing, the following operation modes are conceivable: (i) determination of the size distribution, total number, and physical state of EMVs in the absence of NPs, (ii) determination of the signature/identity and number of specific EMVs by consecutive analysis using unlabeled NPs, (iii) determination of the identity and amount of EMV-specific markers by consecutive analysis using NPs labeled with the same fluorophore, (iv) determination of the identity and amount of EMV-specific markers by parallel analysis using NPs labeled with different fluorophores, and (v) determination of the signature exerted by a multitude of distinct EMV-specific markers by parallel analysis using NPs labeled with different fluorophores and quenching/shifting of the emitted and scattered fluorescence. For gaining a complete picture about the nature of the EMVs in a body fluid sample, it may be useful to integrate two or more of these operation modes into automated sequential (or if possible parallel) cycles. Future experimentation will reveal which of these hypothetical operation modes can actually be realized on the basis of the currently available instrumentation and whether the NTA-based biosensing will considerably improve our tool box for a more thorough understanding of the systems biology of EMVs (Taylor and Gercel-Taylor 2011; Minagar et al. 2001; Kim et al. 2003; Eilertsen and Osterud 2005; Marzesco et al. 2005).

Evaluation

EMVs released from many cell types into body fluids in response to specific (environmental) stimuli during the pathogenesis of complex diseases form the intermediary scale between (macro)molecules and cells with regard to size, volume, and complexity in their molecular composition and thus are difficult to analyze. Importantly, the size and composition of EMVs seem to rely on their biogenesis and to determine their biological roles. The development of novel technologies, in particular those that are based on the recognition of signatures encompassing the protein, lipid, and nucleic acid components, as well as physical characteristics, such as size, curvature, and surface charge, rather than on the measurement of single or a few parameters only, will significantly facilitate the unambiguous assignment of EMV subspecies, their cellular origin, and their relevance for the prediction, diagnosis, and therapeutic monitoring of metabolic diseases and contribute to a better understanding of their pathogenesis from a systems biological point of view. A prerequisite for this challenging task is the clarification of a number of pre-analytical issues and problems with preparation of the samples harboring the EMVS which critically depend on the relevant source (e.g., blood urine, saliva, tissues). A number of studies have been published

with the aim of the standardization and validation of source-dependent preanalytics of EMVs (Yuana et al. 2011; Jayachandran et al. 2012; Trummer et al. 2008, 2009; Zhang et al. 2010; Wang et al. 2012; Moon et al. 2011; Dey-Hazra et al. 2010; Mrvar-Brecko et al. 2010; Leong et al. 2011; Holme et al. 1994; Turchinovich et al. 2011; Merchant et al. 2010; Pattanapanyasat et al. 2004; van der Pol et al. 2012; Robert et al. 2009; Matic et al. 2011; Shah et al. 2008; Chandler et al. 2011; Mullier et al. 2011; Lvovich et al. 2010; Chen et al. 2010; Hind et al. 2010).

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Biosensing of Biomarker Complexes

General Considerations

Currently used biomarkers for the prediction of T2D: At present, the risk assessment for T2D in nonobese and obese subjects lacking any symptoms and in those probands displaying first signs of the disease as well as its definite diagnosis often after 5–15 years of pathogenetic progression and prognosis of the future disease outcome are all based on the measurement of the levels of typical and easily accessible serum parameters, such as glucose, intermediary metabolites, such as 2-hydroxybutyrate, as well as surrogate entities, such as glycated hemoglobin HbA1c. These so-called traditional biomarkers in combination enable prediction of T2D with a probability of about 0.65–0.75 (Böhm et al. 2014; Cheng et al. 2012; Lyons and Basu 2012; Colagiuri 2012; Lipska and Kosiborod 2011; Rhee and

Gerszten 2012; Herder et al. 2011; Rychetnik et al. 2012). The supplementation with information about physical body parameters (BMI, waist-to-hip ratio, sex) as well as the general health state and lifestyle of the probands (e.g., blood pressure, smoking, fitness, food consumption as evaluated in the Deutsche Risikoabschätzung) leads to a further increase in the predictive power to about 0.85–0.90. In the future, functional assays, such as glucose and insulin tolerance tests, as well as noninvasive imaging procedures, currently in the stage of clinical testing and approval, such as magnetic resonance imaging of the ß-cell mass and positron emission tomography of the brown adipose tissue mass, will further improve predictive power. These phenotypic biomarkers in combination reflect the complex interactions between diabetes susceptibility genes and environmental influences including the individual life stage and style and therefore have to be determined at a rather late stage during the pathogenesis (Basu and Lyons 2012; Hieronimus et al. 2014). By nature, the earlier the time points of measurement of phenotypic biomarkers, the less predictive they are. Moreover, the established phenotypic biomarkers do not allow classification of T2D into multiple subtypes according to the different underlying pathogenetic mechanisms and resulting late complications. In consequence, they fail to support individualized preventive and therapeutic efforts.

Currently Used Biomarkers

In current research clinical studies, the determination of so-called novel biomarkers, predominantly proteins, such as cytokines (e.g., TNFα), adipokines (e.g., leptin), incretins (e.g., GLP-1), and others (e.g., cross-reactive protein), in combination but not as single entities, led to prediction values approaching but not exceeding those achieved with combinations of traditional biomarkers (Pradhan et al. 2001; Inzucchi et al. 2012; Dastani et al. 2012; Hara et al. 2014; Jacobs et al. 2014). Not surprisingly, the highest prediction probabilities have been reported for combinations of traditional and novel biomarkers, as, for instance, those evaluated by the EASD Risk Score (Lango et al. 2001). However, because of the partial overlap of the pathogenetic pathways covered by these biomarkers, the predictive values upon their combination did not reach the sum of their individual contributions. It may be argued that the future increase in the number of susceptibility genes identified for T2D in course of genome-wide association studies will lead to considerable improvement of the predictive power of combinations of polymorphic genotypic biomarkers affecting multiple target tissues and pathogenic pathways. However, the path from genotype to phenotype with the underlying gene-gene and gene-environment interactions, genomic plasticity, and epigenetic modifications is complex and not subject to simple cause-effect relationship. Therefore, it remains questionable whether complete genetic profiling per se will enable prediction of T2D with the desired probabilities (higher than 0.90) (Müller 2010; 2012; Kolberg et al. 2009).

Demands for Future T2D Biomarkers

Certainly, the value of a biomarker for the prediction of T2D in obese subjects increases with (i) the time point of its initial functional expression and technical detectability;, (ii) detection in easily accessible body fluids, such as plasma, prior to disease onset; and (iii) detectable differences between obese individuals with regard to life stage, lifestyle, and disease onset as well as diabetic late complications (Cho et al. 2011; Wagner et al. 2014; Stefan et al. 2014). Furthermore, with regard to drug discovery and development, the efficacy of new antidiabetic drugs has been evaluated traditionally in clinical trials using late diabetic complications and mortality as the clinical endpoints. Such trials usually require 10,000–20,000 subjects and at least 5 years of follow-up to demonstrate significant therapeutic benefits. Smaller and shorter studies based on biomarkers which enable the stratification of T2D into multiple subtypes and their individualized prediction for obese subjects with high power for monitoring the therapeutic efficacy have the potential to revolutionize the complete drug discovery process (Rathmann et al. 2013).

Rationale and Purposes

"Reductionistic" approaches for the identification of novel biomarkers are based on the measurement of the levels of a single or a few dominant and defined protein, lipid, or metabolite species (e.g., by PCR or ELISA methods) and provide a complete but biased understanding of the underlying seemingly linear metabolic pathway. In contrast, "holistic" approaches rely on the determination of changes in the levels and fluxes of all relevant components amenable to (un) targeted "omics" technologies and lead to a complete but unbiased understanding of the underlying network of interacting pathways. Unfortunately, so far both approaches have demonstrated limited predictive power for T2D only. This may be, in part, due to the limited information depth intrinsic to both "reductionism" and "holism" (loss of "interactome" and "biophysical" properties), which does not support stratification and individualized therapy of lifestyle diseases, in general. A novel "phenomenological" approach may lead to biomarkers of higher predictive power reflecting the intimate interplay between susceptibility genes and environmental cues in a more direct and precise fashion than previous reductionistic and holistic approaches. It relies on the demonstration and biophysical characterization of extracellular complexes in plasma, which harbor glycosylphosphatidylinositol-anchored proteins (GPI-APs) and phospholipids and possibly additional proteinaceous and lipidic components (ECGAPP) and may be released from almost each cell type through nonclassical secretory mechanisms.

The rationale of this "phenomenological" approach is as follows: (i) ECGAPP are released from metabolically relevant tissues (e.g., adipose, muscle, liver, ß-cells, endothelial cells) into the circulation in response to metabolic stress (e.g., high levels of glucose and fatty acids) as is prevalent during obesity and T2D; (ii) ECGAPP differ in level, type (structure, composition), and biophysical properties (viscoelasticity, rigidity) between distinct subtypes of frank T2D (prerequisite for biomarkers for disease stratification) and/or between obese subjects characterized by varying lifestyles, life stages, and disease states along the pathogenesis of T2D (prerequisite for biomarkers for prediction); and (iii) ECGAPP in serum can be detected and characterized with regard to level, type, and biophysics using biosensors.

For the following reasons ECGAPP are thought to be relevant for the prediction and classification of T2D: (i) Certain GPI-APs, such as CD73 and Gce1, have been reported to be released from metabolically relevant cell types, such as adipocytes, in response to metabolically relevant stress factors, such as high levels of saturated fatty acids, reactive oxygen species, and antidiabetic drugs (Müller et al. 2008, 2011a, b). Likewise, elevated levels for a number of GPI-APs have been measured in plasma from cancer patients (Xu et al. 2012); (ii) GPI-APs rather than typical transmembrane proteins are prone to rapid and efficient release from the plasma membrane of donor cells in course of mechanically or chemically induced stress; (iii) phospholipids in complex with GPI-APs have been detected in the supernatants of cultured cells as well as in rodent and human serum and may represent constituents of mixed phospholipid micelles, nanodisklike structures, surfactant- or lipoprotein-like particles, and vesicular structures (microvesicles, exosomes) released by ill-defined nonclassical (e.g., blebbing, shedding, bicelle formation) or classical (e.g., exocytosis) secretory mechanisms (De Broe et al. 1977; Väkevä et al. 1994; Camussi et al. 2011a, b); (iv) plasma phospholipids analyzed by untargeted lipidomics have been shown to predict early neurodegeneration during preclinical and presymptomatic Alzheimer's disease (Fiandaca et al. 2014); (v) GPI-APs are known to be susceptible for transfer from donor cells to acceptor cells in vitro (Medof et al. 1996; Tykocinski et al. 1996) and in vivo (Kooyman et al. 1995, 1998) in functional state, thereby putatively transmitting biological information within or between tissues (e.g., CD73 as carrier of anti-inflammatory and immune-suppressive message). Interestingly, the level of the GPI-AP, CD73, in plasma has been shown to be correlated with insulin sensitivity in diabetic mice and human probands (Lunkes et al. 2003); and (vi) phospholipids in complex with membrane

proteins (e.g., caveolins) were reported to be released from vascular endothelial cells in culture and into plasma from mice following oxidative stress and high-fat diet (Yuan et al. 2011).

ECGAPP and the Present Analytical Options

Typical conventional and commonly used methods for the analysis of the classical secretome in serum (e.g., ELISA, Western blotting, 2D-PAGE, mass spectrometry) are biased toward the detection of primarily high-abundance proteins. This necessitates complex and tedious fractionation procedures for enrichment of low-abundance components, such as presumably ECGAPP. However, those methods most likely fail for the analysis of ECGAPP for the following reasons: (i) loss (as pelleted or floating materials) in course of sample preparation, (ii) disrupture/dissociation in course of sample solubilization, (iii) inadequate sensitivity, and (iv) inadequate throughput due to complex procedures for enrichment. On the basis if pilot experiments (see below), a novel chip-based biosensor technology relying on the principle of surface acoustic waves (SAW) is thought to bypass these issues and to enable monitoring of both the presence of ECGAPP and their biophysical properties.

Principle

ECGAPP consisting of GPI-APs and phospholipids (and additional membrane proteins and lipids) in plasma samples which differ between obese and obese diabetic rodents and humans and vary within the population of diabetic subjects with regard to amount, composition, and biophysical properties are used for the prediction and classification of T2D. Those differences will be reflected in the 1D- and 2D-signatures specific for the corresponding ECGAPP as revealed by SAW biosensing. Ideally, reliable and reproducible differences in the signatures, i.e., statistically significant correlations in the SAW signals between obese and obese diabetics, will be detectable using moderate sample volumes at early time points during the pathogenesis rather than at the onset of frank T2D only. Introduction of novel T2D subtypes will depend on clustering of the individual 1D- and 2D-signatures deduced from the SAW signals of ECGAPP from obese diabetic rodents and humans into distinct well-separated patterns.

Procedure

GPI-APs have been shown (see Rationale) to exhibit high susceptibility for release in ECGAPP from the surface of mammalian cells in vitro and in vivo in response to cellular and metabolic stress, such as high glucose, fatty acids, and reactive oxygen species (Müller et al. 2008, 2011a, b). This is commonly thought to rely on their anchorage in the outer leaflet of the plasma membrane phospholipid bilayer by the glycosylphosphatidylinositol moiety, exclusively, that is covalently attached to the carboxyl-terminus of the polypeptide chain. However, the presence of ECGAPP in the plasma of T2D patients that are faced with elevated blood glucose and free fatty acids levels has not been studied so far. This is presumably due to conceptual restrictions (i.e., reductionistic and holistic thinking) and technological challenges (see above). To overcome these hurdles, a novel type of chip-based biosensor is used for the specific detection and biophysical characterization of ECGAPP. Its principle relies on the generation of horizontal surface acoustic waves (SAW) of defined frequency and amplitude within the gold surface of a microfluidic four-channel chip. Any interaction of (macro)molecules with the gold surface will result in corresponding changes in the shape of the SAW, altering both their frequency and amplitude (Gruhl et al. 2013; Gronewold 2007; Gronewold et al. 2007; Rapp et al. 2010; Länge et al. 2013; Lee et al. 2013). These alterations reflect mass loading (i.e., binding of ECGAPP) to and biophysical properties (i.e., size and shape depending on the ECGAPP protein composition as well as viscoelasticity and rigidity depending on the ECGAPP phospholipid composition, cholesterol content, and open/empty-closed/filled configuration) at the chip surface. The major advantages of the SAW versus the commonly used surface plasmon resonance biosensor rely on the possibility of measurement of large (lipid-containing) macromolecules even in the presence of serum (Voiculescu and Nordin 2012; Liu et al. 2013; Treitz et al. 2008) as well

as on the potential high sensitivity toward alterations in the composition (proteins, phospholipids) and structure (micelles, nanodisks, vesicles, particles) of the ECGAPP. Albeit SAW biosensing per se does not enable the delineation of the type of ECGAPP contained in a given sample, the SAW signatures will be characteristic for the overall contents of all ECGAPP, either as summation signals or as 1D-/2D-signatures of high informative value.

Surface Acoustic Wave (SAW) Summation Signals

For generation of the ECGAPP-specific SAW signal, ECGAPP are specifically bound to the chip surface via interaction of their GPI-AP and/or the phospholipid constituents with the capturing molecules, aerolysin (bacterial toxin which specifically binds to the core glycan of the GPI anchor), and annexin (peripheral membrane protein which specifically binds phosphatidylserine in the presence of Ca2+), respectively. The capturing molecules are covalently coupled to 2D-SAM- or 3D-dextran-coated chips using routine chemical cross-linking techniques prior to measurement. Subsequent time-resolved measurement of phase shift and amplitude reduction as summation signals integrating the effects of all of the various ECGAPP at the various time points reflects the "overall" association kinetics of all ECGAPP contained in the sample and binding to the chip through either their GPI-AP or phospholipid constituents.

SAW 1D-Signatures

Dissociation of the ECGAPP bound to the chip is initiated by the addition of increasing concentrations of GPI core glycans, which compete with the GPI-APs for binding to the aerolysin capturing molecules, or of EGTA, which disrupts the Ca2+-dependent binding of the phospholipids to the annexin capturing molecules, respectively. The time-resolved data for phase shift and amplitude indicate the dissociation kinetics of ECGAPP for their GPI-AP or phospholipid constituents. This leads to one-dimensional (1D) signatures reflecting the sequential dissociation of the various species of ECGAPP in the sample depending on the relative number and accessibility for capturing of their GPI-APs and phospholipids, respectively.

SAW 2D-Signatures

Cycles of sequential association with and dissociation from the chip of the ECGAPP through capturing via their GPI-AP and subsequently phospholipid constituents or vice versa will be run by using chip surfaces coated with both aerolysin and annexin. Alternative changes of the binding conditions enable the simultaneous or successive monitoring of the presence of GPI-APs and phospholipids in the various ECGAPP species along the following cycling protocol: (i) no GPI core glycans plus no Ca2+ for binding via GPI-APs only, (ii) no GPI core glycans plus Ca2+ for binding via both GPI-APs and phospholipids, (iii) GPI core glycans plus Ca2+ for displacement from GPI-APs and (re-) binding via phospholipids only, and (iv) GPI core glycans plus EGTA for release from phospholipids and resulting detachment from the chip. The time-resolved measurements of phase shift and amplitude reduction during each of the four cycles indicate the association and dissociation kinetics of all ECGAPP species contained in the sample via both their GPI-AP and phospholipid constituents. This two-dimensional (2D) analysis leads to signatures reflecting the sequential association and dissociation kinetics of the various ECGAPP species depending on the relative number and accessibility of their GPI-APs and phospholipids for single (cycles 1, 3) and double (cycle 2) capturing and release (cycles 3, 4).

Sample Sources and Preparation

The presence of ECGAPP in plasma will be followed along the pathogenetic path from nondiabetic obesity to obese T2D via the various prediabetic states. Plasma will be collected from Zucker diabetic fatty (ZDF) rats (Charles River) and diet-induced obese (DIO) mice (Tschöp et al. 2011) which both have been fed with high-fat diet and continuously monitored for metabolic parameters for several weeks. This procedure enables the discrimination between obese nondiabetic, obese prediabetic (for prediction),

and obese diabetic (for stratification) animals. ZDF male rats have become a preferred animal model for obesity-linked T2D with predictable progression from the prediabetic to the diabetic state.

Data Evaluation

The information contents are increasing from summation signals via 1D signatures to 2D signatures. For the quantitative evaluation of summations signals, appropriate software has already been successfully introduced and made available by SAW instruments. Corresponding tools for the analysis of 1D and 2D signatures have been developed in parallel with the generation of the primary data. They have to reflect the shapes of the association and dissociation curves as well as the maximal changes in phase and amplitude during each cycle with each concentration of the displacing/releasing agents. The criteria for either summation signals or 1D/2D signatures as the measure of ECGAPP represent reproducibility, reliability, and significance of their association with (pre)diabetic phenotypes.

Evaluation

Biased Detection of Authentic ECGAPP Harboring Specific GPI-APs Using the SAW Biosensor

ECGAPP of the vesicle type harboring the GPI-APs, CD73, and other extracellular nucleotidases (Antonioli et al. 2013a) are prepared from the supernatant of cultured rat adipocytes upon challenge with fatty acids and reactive oxygen species or by homogenization and subsequent differential centrifugation of total rat liver or by detergent solubilization of total rat liver plasma membranes. Subsequently, these ECGAPP were characterized as exosomes/microvesicles, mixed detergent-GPI-APs micelles, or detergent-solubilized GPI-APs using established analytical procedures. These ECGAPP (adjusted to identical CD73 immune reactivity) are detected and differentiated from one another with high sensitivity and reliability by the SAW biosensor equipped with a 2D-SAM-streptavidin-coated chip surface and biotinylated AMP/ADP/ATP as capturing molecules for GPI-anchored extracellular purine-specific nucleotidases (Antonioli et al. 2013a, b), among them CD73, and programmed for the measurement of the phase shifts and amplitude reductions as summation signals.

Subsequent experimentation with cultured primary rat adipocytes revealed that the established SAW biosensing technology is able to resolve the differences in the releasing efficacy of CD73-harboring ECGAPP between the stress stimuli, palmitate, reactive oxygen species, synthetic phosphoinositolglycans, and the antidiabetic sulfonylurea drug, glimepiride, in a precision comparable to the previously used conventional and tedious preparative and analytical methods (Müller et al. 2010a, b). This is also true for the differences in the stress-induced release of ECGAPP between small and large adipocytes prepared from young and old rats, respectively, as well as between insulin-sensitive and insulin-resistant adipocytes generated by primary culture in the absence and presence of high concentrations of glucose and insulin, respectively. Quantitative deviations in relative changes between the phase shifts and amplitude reductions elicited by the various stimuli in the various types of adipocytes confirmed that the two SAW signals monitor different properties of the ECGAPP, mass/size, and viscoelasticity/rigidity.

Unbiased Detection of Artificial ECGAPP Harboring any GPI-APs Using the SAW Biosensor

For capturing of all ECGAPP harboring any GPI-APs and phospholipids, 3D-dextran chips are coated with either aerolysin or annexin, respectively, and then assayed for binding of CD73 (affinity-purified from detergent-solubilized rat liver membranes) and phosphatidylserine liposomes. Analysis of the SAW signals reveals the specific and sensitive detection of CD73 via capturing of its GPI anchor by the aerolysin coat and of the liposomes via capturing of their phosphatidylserine residues by the annexin coat. These successful capturing modes represent a prerequisite for the unbiased detection and of any GPI-APs-harboring ECGAPP. Preliminary data indicate that the

detection is compatible with the presence of unfractionated mouse serum in the sample upon tenfold dilution, at least.

In conclusion, a novel "phenomenological" approach may lead to biomarkers for the prediction and stratification relevant for the individualized prevention and therapy of type 2 diabetes (T2D) with higher informative value than traditional phenotypic, peptidic, and genotypic ones. It relies on the demonstration and biophysical characterization of extracellular complexes in plasma, urine, or saliva which harbor glycosylphosphatidylinositol-anchored proteins (GPI-AofPs) and phospholipids and possibly additional proteins and lipids (ECGAPP) on the basis of the following rationale (Müller 2012):

(i) ECGAPP have been shown to be released from the surface of metabolically relevant cells through nonclassical secretory mechanisms (shedding, blebbing, transfer) in response to metabolic stress (high glucose, fatty acids, reactive oxygen species) as is prevalent during obesity and T2D; (ii) ECGAPP are assumed to differ in level, type (micellar, nanodisklike, vesicular, particular), structure (size, shape, protein, and lipid composition), and biophysical properties (viscoelasticity, rigidity) between distinct subclasses of T2D (for stratification) and between obese subjects with varying lifestyles, life stages, and (pre)diabetic states (for prediction); (iii) ECGAPP in body fluids may be detected and characterized using a novel biosensor; (iv) it relies on the generation of horizontal surface acoustic waves (SAW) within the gold surface of a microfluidic four-channel chip. Specific interaction of ECGAPP via their GPI-APs and phospholipid constituents with the gold surface will result in characteristic changes in the shape, frequency, and amplitude of the SAW.

Aim is the detection of reliable and significant correlations between SAW summation signals or more informative one-/two-dimensional SAW signatures (obtained by successive cycling between association and dissociation of ECGAPP via their GPI-APs and/or phospholipids) and progressive prediabetic states or T2D subclasses of obese rodents and humans.

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Bioimaging

Rapid technical developments in medical imaging, particularly in nuclear magnetic resonance imaging (MRI), positron emission tomography (PET), and computer tomography (CT), have spurred new advances in obesity and T2D research. These techniques have enabled researchers not only to further explore anatomy and morphology of different tissues and organs but also to examine their dynamic functions with increasing accuracy and reduced invasiveness in humans as well as in animals. As a result of these developments, researchers can now use MRI to quantify visceral and subcutaneous adipose subdepots and use MR spectroscopy to investigate the role of ectopic fat in muscle and liver in the pathogenesis of insulin resistance. Functional MRI also has enabled researchers to study brain responses to food stimuli noninvasively and also to extend studies for elucidation of the role of peripheral hormones, such as insulin, in the brain. PET/CT scanning has revealed the presence of significant depots of brown adipose tissue (BAT) in adult humans and suggests that it could play a role in response to metabolic challenges, such as cold exposure. These studies provide a bridge between mechanistic, but invasive, studies in animal models and the analysis of behavior and metabolism in humans.

Targets for Personalized Therapy

Rationale and Purposes

Unfortunately, the current treatment of T2D is usually ineffective in the way that it fails to stop the deterioration of insulin secretion and the development of severe degenerative late diabetic complications. In addition to insulin treatment, combination of insulin sensitizers and sulfonylureas are currently used to correct hyperglycemia in patients with T2D. While such treatment operates efficiently to slow T2D pathogenesis for a while, a large proportion of patients requires insulin injections a few years later, indicative for ß-cell failure that aggravates over time. Unfortunately, apart from glucagon-like peptide-1 analogues that promote insulin secretion, no new classes of antidiabetic drugs have been recently launched. Thus, there is a critical need for novel drug targets. According to the FDA guidelines, any new antidiabetic drug should not only decrease the hyperglycemia but also protect against macro- and microvascular complications. The failure to act on both sides of the T2D metabolic consequences is responsible for the recent withdrawal from the market of rosiglitazone as this drug induces dyslipidemia. It is now suggested that a novel efficient antidiabetic drug should act at different tissue levels and on most of the molecular mechanisms involved in T2D pathophysiology to be efficient in the long term. It should respect the complex mechanisms controlling glucose and energy balance and linking the central nervous system and periphery insulin-sensitive tissues. In this context, the following concepts have recently emerged that may have significant impact on both basic obesity and diabetes research and the development of innovative therapies.

Energy Uptake

Defining the mechanisms that contribute to the onset and maintenance of obesity will require a thorough understanding of the hormonal and neuronal controllers of energy balance. Each constituent of the energy balance system and each mechanism that may act on the energy balance system to promote positive energy balance in obesity represents a potential target for therapeutic intervention. Energy homeostatic circuits exist within a complex and intertwined network, and a myriad of processes regulate each circuit in distinct ways. Adding further complexity, neurons and their networks are capable of adapting organizationally and functionally in the face of changing conditions, engaging in so-called neuronal plasticity. Less is known about the impact of genetic, intrauterine, and acquired factors as well as of cross talk from the gut on these neurocircuits and how they may predispose to childhood obesity. To identify targets for the generation of potential therapies, the nature of these systems and the mechanisms governing their function and dysfunction will need to be more precisely defined.

While the existence of homeostatic systems that maintain body weight and adiposity at near-constant levels has long been appreciated, the mechanisms that underlie energy homeostasis have begun to be elucidated only recently. There is now a better understanding of the role of hormones, such as insulin, leptin, agouti-related peptide (AGRP), neuropeptide Y (NPY), melanocyte-stimulating hormone (MSH), and melanin-concentrating hormone (MCH), that convey information related to energy storage in adipose tissue to homeostatic brain circuits. New evidence is emerging that these brain regulatory centers also modulate glucose production by the liver as part of an integrated response to energy availability. An appetite-stimulating hormone, ghrelin, and other peripheral signals of feeding and energy status have been identified. Within the brain, particularly in the hypothalamus, a number of neural circuits and neurotransmitters that respond to and mediate the effects of leptin and other metabolic signals have been identified. Collectively, these findings reveal that alterations in body weight provoke changes in these neural systems to produce a homeostatic response to defend body weight. In addition to providing new therapeutic targets, these discoveries are opening a new window to the understanding of human motivation to eat.

While the key pathogenic changes are not yet known, a number of processes that may alter the function of the neural circuits that modulate energy balance have been identified. These include (i) molecular/signaling mediators and inflammatory mechanisms that may interfere directly with signaling by appetite-suppressing hormones and/or circuits, (ii) altered early development or later remodeling of the energy homeostatic circuitry in response to dearth or excess of nutrition (and their hormonal surrogates), and (iii) alterations in the access of nutritional or hormonal cues to these circuits. Armed with this knowledge, scientists can now study how these mechanisms go awry and potentially contribute in inappropriate weight gain and/or retention. Identifying key sites of susceptibility to environmental insults and the critical periods when these changes are likely to occur will have a significant impact on the design of successful interventions to prevent obesity.

Low-Grade Systemic Inflammation

The link between immunity and metabolism is increasingly well-documented. Indeed alterations of the immune system, as observed in immunocompromised animals or humans or in chronically infected patients, frequently lead to metabolic disturbances, such as dyslipidemia, T2D, or obesity. On the other hand, diabetic or obese individuals exhibit drastic alterations in their immune system as revealed by the higher incidence of inflammatory diseases, infection, or certain types of cancer.

Understanding the role of obesity in development of diabetes will require further study to understand the contributions of inflammatory processes at multiple levels and increasing complexity. Research suggests that inflammatory mediators produced by activated macrophages

are important factors underlying the synergistic relationship between obesity, insulin resistance, and metabolic dysfunction. First documented in adipose tissue, obesity-associated inflammation and macrophage accumulation have now been demonstrated in diverse tissues, including the liver, skeletal muscle, vasculature, and brain. The macrophage represents a significant new target in developing therapies to break the link between obesity and T2D.

Although chronic consumption of nutrients in amounts that exceed the body's requirements induces deleterious effects on tissues throughout the body, the extent to which these effects are driven by an overall excess of calorie ingestion versus increased exposure to specific nutrients is an open question. Saturated free fatty acids are implicated as having pro-inflammatory effects, and diets in saturated fat content seem to promote systemic inflammation more effectively than diets rich in mono- or polyunsaturated fats. Indeed, foods rich in omega-3 fatty acids may have anti-inflammatory effects, and specific nutrients, such as saturated fatty acids and fructose, may exert pro-inflammatory effects in obesity that are independent of energy balance per se.

Adipose Tissues

The WAT is located at the crossroad between the immune system and the metabolism (see chapter "► Insulin Target Tissues and Cells"). Indeed, WAT is anatomically associated with lymph nodes thereby allowing paracrine interactions between both tissues. For example, adipocyte-derived factors, such as leptin and adiponectin, control the proliferation/activation of immune cells. Moreover, the stromal vascular fraction (SVF) of WAT represents an extramedullary reservoir of functional hematopoietic cells. Furthermore, besides adipose tissue macrophages (ATM), a wide diversity of immune cells, whose contribution to T2D, obesity, and adipose tissue function has long been neglected, appear to play key roles in the induction or maintenance of WAT inflammatory state.

WAT inflammation has been initially associated with the production of Th1 cytokines. However, as for atherosclerosis, IL-17 production by cells, whose development is largely dependent on the nuclear receptor RORα, seems to play a major role. Recent studies have identified WAT-specific lymphocyte subsets. CD4+ Foxp3+ regulatory T cells are found in normal mice, but are virtually absent in WAT from obese mice, where effector CD8+ T cells are recruited and, in turn, induce macrophage recruitment. Surprisingly, innate "natural helper" lymphoid cells, highly expressing the nuclear receptor RORα and producing Th2 cytokines and pro-inflammatory IL-6, a regulator of B1-cell function and IgA production, have been identified in WAT.

The causal associations between lymphocytes and obesity/T2D, however, remain controversial. Indeed, while lymphocytes may contribute to an early inflammatory response and immunotherapy appears to normalize obesity-induced insulin resistance, lymphocyte-associated inflammation does not appear to be the triggering event per se. CD4+ lymphocyte number is however increased in obese patients, illustrating the communication between adipocytes and WAT lymphocytes. Even though the absence of lymphocytes does not appear to affect the development of obesity and insulin resistance in a mouse model of immune deficiency, a major recruitment of natural killer cells (NK) within WAT was observed in this model. Indeed, NK and CD1d+ cells are present in the omentum of healthy individuals, and their number is greatly reduced in severe obesity. Regarding myeloid cells, besides macrophages (see below), the number of CD11c+ dendritic cells (DC), which play an important role in lymphocyte activation, is strongly increased in obese animals and individuals.

As obesity-induced WAT inflammation progresses, (additional) immune cells are recruited, further exacerbating the inflammatory response. A key mediator of obesity-associated WAT inflammation involves the infiltration and activation of macrophages. Adipose tissue macrophages are clearly increased in both number and pro-inflammatory activation in individuals who are obese. Data from both genetic and pharmacological intervention studies hint to a role of these macrophages in obesity-induced insulin

resistance. A key event in this pathological cascade is the induction of a pro-inflammatory phenotype of macrophages in insulin-sensitive tissues (so-called classical activation or M1 phenotype). Yet, macrophages can also be induced to exhibit anti-inflammatory properties ("alternative activation" or M2 phenotype). Available evidence suggests that this macrophage phenotype switch ameliorates obesity-associated inflammation and insulin resistance. Thus, therapeutic interventions that favor the M2 over the M1 macrophage phenotype warrant study as novel strategies for the treatment of obesity-associated metabolic diseases. The mechanism(s) underlying obesity-associated accumulation and subsequent activation of macrophages in insulin-sensitive tissues is an area of intense research. They may encompass the release of chemokines that promote recruitment of circulating monocytes into tissues as well as provoke cellular necrosis as key signals driving this process. Clarifying the mechanisms, whereby macrophages are recruited into different WAT depots during obesity, and whether body fat distribution affects this process, may result in new targets for the therapy of obesity-associated metabolic dysfunction.

Altogether, these data enlighten a previously unsuspected diversity of WAT immune cell populations, in particular lymphocytes and macrophages, and of their roles in the development of metabolic diseases. Qualitative or quantitative variations of some of these populations in obese or T2D patients compared to healthy individuals have been elucidated, but a causal link between their presence and disease progression as well as their exact functions has not been clearly defined so far. In-depth understanding of this newly recognized relationship between inflammation, obesity, insulin resistance, and metabolic syndrome may lead to new approaches to halt progression of T2D and obesity.

Other Peripheral Tissues

Many questions remain regarding the role macrophages play in peripheral tissues in mediating disordered energy intake and storage, especially in response to consumption of a high-fat diet (see chapters "► Insulin Target Tissues and Cells"). Conversely, more needs to be done to understand macrophages and inflammation as an outcome of obesity leading to insulin resistance and other complications, such as atherosclerosis. Clarifying both the mechanisms underlying, and consequences of, peripheral tissue-specific inflammation induced by obesity is a key priority for future research. In this regard, it has to be determined whether inflammatory pathways can be modulated effectively in the prevention and treatment of obesity and its metabolic sequelae, and if so, which specific targets are best suited for therapy. At the cellular level, inflammation induced by nutrient excess and obesity can involve multiple organelles (e.g., mitochondria, endoplasmic reticulum) and signal transduction pathways (e.g., IKKß-NFKB, JNK) depending on the cell type. Optimal strategies for limiting this type of inflammation may therefore vary across tissues and thus will require further studies.

An additional interesting aspect regarding the link between inflammation and the benefit of physical exercise may rely on its need for increased rates of substrate oxidation to meet the energy demand. At the cellular level, this process can favor the mobilization of nutrients that might otherwise accumulate and promote inflammatory responses. Thus, the effect of exercise to mobilize stored nutrients may contribute to its ability to improve metabolic function via attenuation of cellular inflammation induced by nutrient excess. Thus efforts to quantify this effect and its therapeutic potential are warranted.

At the Brain

Like many other tissues, the hypothalamus is susceptible to inflammation induced by nutrient excess. Unlike other tissues, however, this hypothalamic response has the potential to favor weight gain, in addition to simply being its consequence. This hypothesis is based on the evidence that leptin and insulin are "adiposity negative feedback" signals that convey afferent input used by the hypothalamus to control energy balance and that neuronal inflammation causes resistance to both "satiety" hormones. This effect, in turn, is hypothesized to predispose to weight gain until circulating insulin and leptin levels

increase sufficiently to overcome the neuronal resistance. Thus operation of a vicious cycle has been proposed in which nutrient excess itself favors excess weight gain in genetically susceptible individuals. Accordingly, drugs that disrupt this vicious cycle may be effective in obesity treatment and prevention. This necessitates the investigation of mechanisms, whereby hypothalamic inflammation is induced by systemic inflammatory stimuli and the assessment of their consequences for energy homeostasis.

Energy Expenditure

More than just innocent bystanders, adipocytes are present in close physical proximity to all major organ systems and as such can influence neighboring cells (see chapter "► Assays for Insulin and Insulin-Like Activity Based on Adipocytes"). Understanding how to properly manipulate these cells and enhance their metabolic flexibility is key for a successful therapeutic approach of metabolic diseases. Brown adipose tissue (BAT) represents an unappreciated and potentially significant metabolically active tissue that may contribute to overall energy balance. In addition, the variability in BAT mass or function may underlie some aspects of susceptibility to excess caloric intake. Important areas for future research include the study of the mechanisms that determine adipocyte number and cell size and those that determine the relative size and function of different WAT depots as well as the development of improved tools for measuring these endpoints. Furthermore, epidemiological analysis of the effect of fat distribution on metabolic risk and study of the mechanisms governing adipose tissue development in vivo, including determination of the factors that control the adipogenic and mature adipose gene expression profiles in different WAT and BAT depots, and how these factors are affected by metabolic cues have to be performed. Altogether, this will facilitate answering what mechanisms (i) determine adipocyte number and cell size, (ii) link variation in body fat deposition to metabolic sequelae, and (iii) govern adipose tissue development and distribution.

Lipid Storage and Metabolism at the Adipose Tissue

It has been recognized for over 20 years that central obesity produces a high risk of T2D and metabolic syndrome, whereas peripheral obesity is not associated with such a risk and may even be protective (see chapters "► Assays for Insulin and Insulin-Like Activity Based on Adipocytes" and "► Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms"). Both BMI and adipose distribution, as measured clinically by the waist-to-hip ratio, are strongly genetically determined. However, the underlying specific genes remain unknown. Studies have begun to identify the genes involved in fat distribution and indicate that these genes exhibit large difference in expression even in cells in the pre-adipocyte stage, i.e., before differentiation to mature adipocytes. These findings raise the possibility that WAT is more heterogeneous than previously thought. Defining the factors and mechanisms that program and mediate this heterogeneity of WAT in different fat depots may provide new insights into the link between obesity and metabolic disease.

Women differ from men both in regional patterns of fat distribution and in extent of adiposity. Individuals of either gender also show marked differences in pattern of WAT distribution, and this pattern can further change during periods of weight gain or loss and with aging. Delineating the mechanisms involved, as well as the critical periods in which patterns diverge, is essential for a better understanding of how different WAT depots affect metabolic function and diabetes risk as well as of the mechanisms underlying the causes and impact of sexual dimorphism on specific WAT depots. As a better understanding of depot-specific characteristics of WAT emerges, tissue-specific gene expression, cell ablation, or other methods can be utilized to modify those characteristics predisposing to metabolic dysfunction. Modest expansion of certain WAT depots could represent an intervention that ameliorates organ dysfunction by reducing lipid deposition in muscle, liver, and other non-adipose tissues as well as morbidity linked with other depots (see below).

Lipotoxicity

Evidence is increasing over the past years that limited ability of WAT to expand in the face of excess calories drives the accumulation of triglycerides in non-adipose tissues resulting in morbidities associated with obesity (see chapter "► Assays for the Expression and Release of Insulin and Glucose-Regulating Peptide Hormones from Pancreatic β-Cell"). Many of the adverse effects of obesity can be traced to accumulation of triglycerides in organs such as the liver, muscle, pancreas, and blood vessels. It has been demonstrated that mice capable of expanding their WAT depots through adipocyte differentiation and growth without concomitant inflammation become obese, but do not redirect fat deposition to organs other than adipose tissue. These mice appear metabolically healthy with normal blood lipids and remain insulin-sensitive, at least to a certain extent. Similarly, clinical data have highlighted the potentially protective nature of some WAT depots. Moreover, epidemiological studies have revealed correlations between plasma levels of adipokines and systemic insulin sensitivity, diabetes, cardiovascular risk, and many additional disease states. Data from preclinical models further corroborate these correlations and in many instances directly implicate the dysregulated adipokine in the development of insulin resistance (see below). These findings provide new clues that may allow disruption of the link between obesity and T2D and may explain the "fit-fat conundrum" by clarifying how lipid accumulation in some WAT depots is more deleterious than when it is stored in others.

Adipocytes are known to secrete a multitude of factors of diverse nature, such as proteins (i.e., adipokines), acute phase reactants, lipids (the identity of which awaits further study), and small vesicles (i.e., microvesicles and exosomes, see below). Lipidomics, proteomics, and metabolomics can be employed to identify the contributions of WAT to systemic levels of these factors under different conditions. Adipocytes may well signal to other tissues, such as muscle, liver, brain, and other WAT and BAT depots. Conversely, they may receive information from the liver, muscle, brain, gut, and other tissues through as yet undiscovered signaling factors that mediate the link between obesity and T2D through their contribution to the coordinated response to changes in energy availability among tissues.

Furthermore, there is some experimental evidence that the accumulation of triglycerides and/or of biosynthetic intermediates or degradation products in non-adipose tissues may lead to damage and functional impairment of mitochondria over time, especially in an oxidative environment, albeit the underlying molecular mechanisms, which may include the deleterious effects of reactive oxygen species and oxidative stress, remain ill-defined. Thus, mitochondrial dysfunction and impaired oxygen consumption that, in fact, have been reported for adipose and muscle tissues of T2D patients may contribute to the pathological consequences of adiposity. It is therefore conceivable that interventions to stimulate mitochondrial biogenesis and repair may contribute to improved metabolic outcomes. Thus, the investigation of mechanisms for maintaining fully functional, in particular oxidative or partially uncoupled, mitochondria in WAT and the development of technologies and biomarkers, including the use of noninvasive tools, such as magnetic resonance spectroscopy and labeled substrates, for the analysis of adipose tissue biology in vivo, in general, and mitochondrial physiology, in particular, have to be fostered. These tools will be essential both to translate discoveries made in animals into a better understanding of human adipose tissue biology and to monitor the efficacy of novel therapeutics tackling its functionality.

Brown Adipose Tissue (BAT)

The potential importance of BAT in human energy metabolism has resurfaced (see chapter "► Insulin Target Tissues and Cells"). The conventional wisdom that the importance of BAT in energy metabolism is limited to small mammals and human neonates has been challenged by recent evidence. (i) BAT is detectable in a substantial subset of adult humans (although not primarily in the interscapular area typical of rodents and newborn humans), (ii) BAT can be rapidly activated by

cold exposure and other stimuli in many individuals, and (iii) activation of BAT shows gender differences, is attenuated in obese individuals, and is blocked by ß-blockers. Furthermore, obesity-resistant strains of mice have been shown to harbor more BAT in unusual locations, such as embedded in leg muscle. These insights set the stage for studies to determine whether reduced heat production by BAT (non-shivering thermogenesis) contributes to the pathogenesis of obesity and whether pharmacological or other strategies to activate BAT might be therapeutically useful. It has been shown that some BAT depots appear to be derived from precursors that are shared with skeletal muscle, whereas others may be more closely related to the lineage that gives rise to WAT. There is increasing experimental evidence for multiple pathways that control the de novo differentiation of brown adipocytes, the transdifferentiation of white into brown adipocytes, and the "brownishing" of white adipocytes with the common aim to get rid of excessive lipids, fatty acids, and ultimately carbons through mitochondrial oxidation and ATP/heat production. The discovery of the corresponding key genes and components, including growth factors and transcription (co)factors, will provide novel therapeutic targets. Further clinical investigation will be necessary to define the potential role of BAT in pathogenesis, prevention, and treatment of obesity and its comorbidities.

Exosomes and Microvesicles (EMVs)

Biological responses underlying adipose tissue expansion and cell size increase include the process of angiogenesis, as well as recruitment and local proliferation of pre-adipocytes (see section "Communication Between Adipocytes via Exosomes and Microvesicle" in chapter "► Monitoring of Diabetic Late Complication" and section "Detection of Exosomes and Microvesicles (EMVs)"). There is growing experimental evidence for (paracrine) communication between large and small adipocytes within WAT depots not only via adipokines but also involving small membrane vesicles, the so-called exosomes and microvesicles (de Gassart et al. 2003; Del Conde et al. 2005; Scott et al. 1979; Heijnen et al. 1999; Piccin et al. 2007; Denzer et al. 2000), which harbor specific glycosylphosphatidylinositol (GPI)-anchored proteins, mRNAs, and microRNAs (Müller et al. 2011; Cocucci et al. 2009). Interestingly, information transfer via these components in the course of release of the EMVs from large adipocytes and their subsequent fusion with small adipocytes has been shown to shift the burden of lipid loading from the former to the latter with their concomitant size increase (Al-Nedawi et al. 2008; Aoki et al. 2007; Pap et al. 2009; Müller et al. 2008a, b, c, e, 2009a, b). It is tempting to speculate that the modulation of release and fusion of the EMVs could trigger the maintenance of small adipocytes with their characteristics of many small lipid droplets (LDs) and many mitochondria (high oxidative capacity) rather than their conversion into large adipocytes harboring few huge LDs and mitochondria (low oxidative capacity) or even induce the formation of BAT-like white adipocytes (Müller et al. 2010a, b, 2011). The corresponding working model is as follows: white large donor adipocytes release EMVs harboring the GPI-anchored proteins, CD73 and Gce1, mRNAs specific for GPAT3 and FSP27, and the microRNAs, miR-16 and miR-222. The loss of these EMV components from the large donor adipocytes leads to upregulation of lipolysis (in the course of increased levels of cAMP at the LD surface) and downregulation of lipid synthesis (in the course of decreased levels of GPAT3 and FSP27 proteins) that trigger degradation of LDs and eventually induction of the brown adipose tissue-like phenotype. The release of these EMVs from the large donor adipocytes is blocked by modulation of their histone H3 methylation state. The retention of these EMV components in the large donor adipocytes supports the downregulation of lipolysis (in the course of decreased levels of cAMP at the LD surface) and the upregulation of lipid synthesis (in the course of increased levels of GPAT3 and FSP27) that lead to LD biogenesis and elevation of cell size. The EMVs fuse preferentially with the plasma membranes of white small acceptor (pre)adipocytes. Thereby, the EMVs transfer the GPAT3-/FSP27-specific

mRNAs and miR-16/222 microRNAs into the cytoplasm and the CD73/Gce1 GPI-anchored proteins onto the LD surface of the acceptor adipocytes. As a consequence, GPAT3 and FSP27 protein expression becomes upregulated in parallel with the degradation of (c)AMP at the LD surface zone, resulting in direct stimulation of lipid synthesis and inhibition of lipolysis. The consequent promotion of LD biogenesis drives the increase in cell size of the (initially small) adipocytes and contributes to the stabilization of their white adipose tissue-like phenotype. Upon blockade of the release of the EMVs from white large donor adipocytes or of their transfer to and fusion with the plasma membranes of white small acceptor adipocytes, the lack of upregulation of FSP27 and GPAT3 protein expression in concert with elevated levels of cAMP at the LD surface zone fosters lipolysis and impairs lipid synthesis in connection to stimulation and inhibition of the biogenesis of mitochondria and LDs, respectively, as well as induction of the BAT-like phenotype. Studies are needed to identify additional "local triggers" and mechanisms that promote adipocyte expansion versus differentiation and maintain or modulate the functionality of expanding WAT and BAT in rodents and humans.

The Circadian Clock

Many physiological processes involved in the maintenance of energy balance such as feeding behavior, lipid and carbohydrate metabolism, and blood pressure control display daily variation. Modern European lifestyle has dramatically changed the daily rhythm of life. Physical activity, food intake, and light exposure are no longer restricted to daytime hours, and sleep duration has been continuously decreasing over the last century. Strikingly, accumulating evidence points to an increased incidence of the metabolic syndrome, obesity, and cardiovascular diseases in shift workers. Moreover, circadian oscillations in metabolic processes or hormone (e.g., insulin, adipokines) secretion are attenuated in T2D subjects (Spiegel et al. 2009). Even in healthy subjects, acute sleep deprivation results in reduced insulin sensitivity underlining the importance of circadian rhythms and sufficient sleep for a proper metabolic balance. Indeed, epidemiological studies evidence a strong link between short sleep duration and increased risk for T2D and obesity, while restriction of sleep results in increased insulin resistance, increased leptin levels, and increased feeling of hunger in healthy volunteers (Spiegel 2008).

It is now known that the molecular clock, initially thought to be operative only centrally, is also present in peripheral cells and tissues. For example, recent data suggest a direct action of the biological clock on pancreatic ß-cell function (Bass and Takahashi 2010). Evidence from murine animal studies suggests that disturbances of circadian clocks are pivotal in the development of T2D and obesity as well as their cardiovascular disease sequelae. Mutations in the circadian clock genes and bmal1 in mice lead to insulin resistance and phenotypes similar to the metabolic syndrome. Rev-erbα and RORα are two recently deorphanized nuclear receptors which are involved in lipid and glucose metabolism as well as in the modulation of the inflammatory response (Duez and Staels 2010). Both receptors are identified as critical clock components whose expression and activity underlie a circadian rhythm in metabolic tissues and probably contribute to diurnal variation in glucose and lipid metabolism. In contrast, data from human studies are scarce, and translational research that aims to prove causality has not been undertaken yet. Nevertheless, these nuclear receptors appear potentially interesting targets for the development of therapies to reset the molecular clock and correct associated metabolic abnormalities.

Gut Flora

Recent experimental data demonstrate that human intestinal microflora modulates the energy balance through an environment-dependent manner (Cani and Delzenne 2011). Out of the three million genes expressed, 100 times more than in the eukaryotic genome, metabolic inflammation has been first causally linked to the lipopolysaccharide synthesis pathway which is responsible for the triggering of

insulin resistance, adipose tissue development, and hepatic lipid overload. The intestinal microflora represents the major genetic diversity that interferes and programs numerous physiological functions since the first hours of life. Furthermore, specific bacterial fragments may be present in metabolically active tissues, such as the adipose depots, the liver, the blood, the brain, and the blood vessels, and contribute to the regulation of their function. Thus it has been postulated that these "metafactors" are tightly involved in the immunomodulation of energy homeostasis and thus may be causative agents at the early onset of T2D (and complication) development.

In consequence, the identification of tissue metagenomic biomarkers can be extremely helpful for the classification, stratification, and prediction of metabolic abnormalities that precede and are associated with T2D. The access to human tissues (e.g., the gut, adipose tissue, liver) and feces of diabetic morbidly obese subjects receiving obesity surgery (OS) is a key issue to make progress in this area of basic and translational T2D research.

Obesity Surgery (OS)

OS or bariatric surgery is the most effective available treatment for extreme obesity. One frequently performed operation, Roux-en-Y gastric bypass (RYGB), causes profound (and the most efficient) weight loss that, unlike other modalities, does not seem to activate compensatory responses to weight loss that lead to weight regain, at least in some individuals. Importantly, this procedure can also induce a complete normalization of the glycemic state, i.e., euglycemia, via mechanisms that appear, at least in part, to be independent of weight reduction. Potential mechanisms underlying this effect include (i) increased secretion of intestinal hormones (e.g., glucagon-like peptide-1), (ii) neuroendocrine changes induced by excluding ingested nutrients from the upper intestine, (iii) compromised ghrelin secretion, (iv) altered intestinal nutrient sensing, or (v) other so far unidentified processes. These findings have opened a new research avenue that may lead to new options for T2D treatment.

Although T2D is regarded as an incurable disease, it has become clear that some types of OS can bring dramatic improvements in metabolic control or even complete long-term remission in many, but not all, morbidly obese T2D patients. The factors responsible for interindividual heterogeneity of OS outcomes remain unknown, but have to be elucidated as a critical component for the personalization of obesity therapy. OS is the most if not the only effective treatment for morbid obesity currently available. There is also evidence that it should be considered in less obese patients (with BMI 35–40), when they suffer from a comorbidity, such as T2D. For instance, in France, about 500,000 people are morbidly obese (of whom around 1/3 are diabetic), but only 20,000 have received OS in 2009. T2D remission is reported in 70 % of RYGB patients, with improvements in glycemic control often evident within days after surgery, well before major weight loss has occurred.

OS is now beginning to be considered for T2D patients who are only moderately obese in order to resolve their diabetes, even though the surgery carries immediate and (much more significant) long-term risks. Unfortunately, it is currently difficult to assess the balance of risk to benefit of OS in nonobese patients, since at present it is not possible to predict which individuals are likely to experience T2D remission. Elucidation of the molecular basis of T2D remission is, therefore, essential prior to a broader application of OS treatments for T2D patients. Furthermore, OS is now considered as an experimental model for the study of the mechanisms of development and cure of T2D. It is likely that factors able to predict the outcome of OS will also help predict response to these treatments and develop new ones, together fostering the development of personalized diagnosis and therapy for metabolic diseases.

Strategies for Personalized Therapy

Which strategic options for personalized therapy are currently in our hands? (i) The abnormal function or synthesis of a polymorphic/mutant gene product encoded by the susceptibility gene may

be corrected (i.e., inhibited/activated, down-/upregulated) by (orally available) chemicals of small size. (ii) The polymorphic/mutant variant of the susceptibility gene may be replaced by the corresponding wild-type genes (gene therapy). (iii) The abnormal tissues/cells suffering from expression of the polymorphic/mutant variant of the susceptibility gene may be replaced by normal tissues/cells grown in culture (regenerative medicine and tissue engineering). (iv) The missing, insufficient, or excessive function or synthesis of a polymorphic/mutant gene product encoded by the susceptibility gene may be compensated for or counteracted, respectively, by delivery of the wild-type gene product or neutralizing antibodies, respectively, against the corresponding polymorphic/mutant gene product (protein therapeutics and therapeutic antibodies (protein therapeutics and therapeutic antibodies). Each of these strategies could lead to personalized therapy of complex common diseases, i.e., tailored to the individual genetic profile and individual complete pathogenic mechanism. In case of T2D, the targeted susceptibility genes may encompass components of the glucose-sensing system in pancreatic ß-cells and intestinal L-cells, insulin production and secretion in the ß-cells, or insulin action in brain, muscle, adipose, and/or liver cells or combinations thereof. By nature, there are advantages and disadvantages associated with each of these therapeutic strategies.

Gene Therapy and Regenerative Medicine

Gene therapy and regenerative medicine are believed to harbor the greatest potential and application field for the future, but unfortunately have recently experienced serious doubts and issues concerning safety, production, and realization. The success of gene therapy is highly dependent on the quality of the delivery vector, which can be generally categorized into viral and nonviral origin (Li and Huang 2007). Viral vectors are highly efficient. They are currently still the most powerful tools for gene transfection. However, some viral vectors show limited loading capacity, are difficult to produce in large scale, and, most importantly, pose severe safety risks due to their oncogenic potential and their inflammatory and immunogenic effects, which prevent them from repeated administration. To overcome these limitations, nonviral vectors and the oral delivery route have emerged as a promising alternative for gene therapy, such as nanoparticle-based delivery systems (see below).

Chemicals

Small chemicals (synthesized and natural molecules), in general, are easily produced in small and large scale by modern synthesis methods and can often be delivered as a pill via the oral route into the circulation and gain access to the abnormal tissue (in most cases by diffusion) as well as to the cytoplasm of the target cell. However, the past two decades of enormous biomedical and biotechnological expenditure worldwide starting from "traditional" drug discovery, that was based on "pharmacological screening," and now being managing "target- and pathway-driven" modern drug discovery that is focused on state-of-the-art high-throughput screening, structural biology, bioinformatics, and rational drug design finally resulted in a rather disappointing outcome of approved small molecule drugs. This clearly demonstrates the considerable difficulties inherent in the discrimination of "chemicals" and "drug-like" molecules, i.e., in the identification of those compounds in libraries containing millions of chemical substances which have the potential for subsequent successful step-by-step optimization via lead structures to drug candidates in the course of several rounds of structural variation/modification with the help of rational design (leading to quantitative structure-activity relationships) and structural biology along the paradigm of modern drug discovery. The reasons for these difficulties are complex and multifaceted and, in part, based on the complex criteria and intrinsic challenges associated with identification and validation of novel drug targets and their use in drug discovery (Billingsley 2008). In fact, there are fundamental differences in the rationale and processes

underlying the "traditional" and "modern" drug discovery approaches. Albeit "modern" drug discovery apparently offers a number of very important advantages in comparison to the "traditional" one, the novel knowledge-based approach excludes the potential benefit of chance and scientific creativity and intuition. However, in any case, small chemicals intrinsically suffer from limited selectivity between the desired cellular target and unwanted off-targets, in contrast to large macromolecules, such as proteins and nucleic acids. This selectivity issue is intimately associated with safety problems and considerably hampers multiparameter optimization. Therefore, it is not too surprising that during the past decade the identification of susceptibility genes for common diseases has overrun the development of novel small molecule drugs which target and correct the corresponding gene products. This apparently huge problem with the identification of small drug molecules is in contrast to the need to obtain drugs for each defective/missing or hyperactive/overproduced gene product encoded by the multiple susceptibility genes and underlying the common diseases.

Oral Delivery of Protein Therapeutics

General Considerations

By nature, the protein products encoded by susceptibility genes are characterized by exquisite potency and selectivity, as exemplified best by receptor-ligand or enzyme-substrate interactions. In consequence, protein therapeutics in general have the huge advantage of operating "intrinsically" safely as well as of being efficiently discovered. Their wild-type versions represent "natural" cell components. Therefore, upon their identification, they may be used for therapy either directly as substitution of defective or missing gene products or indirectly by blockade of hyperactive or overproduced gene products using neutralizing antibodies. However, therapeutic proteins and antibodies have to overcome a number of critical hurdles prior to beneficial systemic action. One of the most important one is the passage along the gastrointestinal tract and the transport across intestinal epithelial cells into the circulation. For susceptibility gene products with the major site of action in the serum (e.g., plasma enzymes) or interstitial spaces (e.g., polypeptide hormones), this so-called oral bioavailability may represent the only barrier for targeting their primary mode of action. Gene products with primary mode of action in the cytoplasm (e.g., transcription factors, signaling proteins, enzymes) have to be transported from the circulation via the extracellular matrix across the plasma membrane of the target tissue/cells, in addition. This is typically not managed by therapeutic proteins and antibodies, and therefore they have to be injected according to their site of action (e.g., subcutaneously, intravenously, intramuscularly, intraperitoneally).

Consequently, personalized prevention and therapy based on the injection of gene products into the circulation is per se prevented by their inability to be transported into the target cells or/and (in addition) considerably hampered by the known limited patient's compliance toward their non-oral administration, in general. In particular, the latter holds true for common diseases, such as T2D and obesity, which are often characterized during the initial stages by only low to moderate consequences for the individual life quality. However, metabolic diseases underlie vicious cycles of their pathogenesis finally leading to irreversible damages (see above), which resist improvement by late onset of non-oral administration of therapeutic proteins and antibodies. In former times, the motivation for oral delivery of therapeutic proteins and antibodies relied on convenience and avoidance of needles. With the current understanding of the pathogenesis of T2D, additional emphasis has to be put onto the physiological route of delivery of the protein therapeutic to its site(s) of action in the target tissues and cells and in case of T2D, the hepatic route (Arbit 2004). Currently available insulin therapies including the newer injectable short- and long-acting analogues introduce insulin at the periphery, but do not mimic the physiological portal-to-peripheral gradient. These therapies continue to over-insulinize the periphery resulting in undesirable side effects, such as peripheral hyperinsulinemia with resulting insulin

resistance, weight gain, and hypoglycemia. In contrast, hepatically delivered oral insulin would result in high portal vein concentrations without sustained peripheral hyperinsulinemia (Iyer et al. 2010; Kapitza et al. 2010; Heinemann and Jacques 2009). The physiological rationale and therapeutic advantage for oral insulin have meanwhile been widely acknowledged by diabetologists (Arbit 2004). Taken together, the pathogenic mechanisms for T2D and obesity, which are based on complex patterns of multiple susceptibility genes presumably differing from patient to patient, may be interrupted by substitution with (combinations of) the relevant gene products or introduction of (combinations of) the relevant neutralizing antibodies in the circulation and, if required, in the target cells in course of their oral delivery.

Purpose and Rationale

So far the long path from the mouth to the drug target within the body across the intestinal and cellular barriers has remained the major hurdle for the use of protein therapeutics in humans. The intestine is lined by a monolayer of epithelial cells, the so-called enterocytes, which are interconnected to each other by specific protein complexes, the so-called tight junctions. Macromolecules, such as polypeptide hormones, antibodies, and nucleic acids, typically cannot cross the tight junctions for the so-called paracellular passage from the intestinal lumen directly into the circulation. Rather, they are first broken down to their constituent free amino acids, peptides, and nucleotides by various enzymes located throughout the gastrointestinal tract. These constituents are then absorbed by the gastrointestinal epithelium. Of course, this route of absorption destroys almost all physiological activity of the original protein or nucleic acid and explains why typical oral bioavailabilities of proteins are usually less than 1–2 % (Iyer et al. 2010; Kapitza et al. 2010; Heinemann and Jacques 2009; Lasch and Schönbrunner 1936; Pauletti et al. 1996; Wang 1996). Therefore, despite the fact that recombinant DNA technology has allowed for ever increasing numbers of therapeutical proteins and antibodies, delivery of these drugs is still generally through injection. For the past eight decades, a great deal of work has focused on attempts to develop noninvasive methods of delivering protein drugs with the oral route clearly being the most convenient and desired one.

Protease Inhibitors

The prevention of the enzymatic degradation in the gastrointestinal tract in the course of delivery of the protein drug along with a protease inhibitor or inhibitor cocktail could represent a useful strategy. In one pilot study, five different protease inhibitors were tested individually along with insulin (Woodley 1994; Bai and Chang 1995; Yamamoto et al. 1994). It was found that bacitracin, sodium glycocholate, and camostat mesilate promoted the absorption of insulin, while soya bean trypsin inhibitor has very little effect on absorption. Thus coadministration of protease inhibitors may be one possible approach to improve the absorption of protein drugs, such as insulin, from the gastrointestinal tract, presumably due to prolongation of the survival period of the intact protein drug at the intestinal epithelium which raises the probability of its absorption. A general limitation of this approach is that the long-term effect of chronic administration of protease inhibitors is unknown and may result in protein malabsorption and other adverse side effects. This may be one reason why this approach has not reached clinical trials so far for the most attractive candidates for oral protein delivery in the long-term, such as insulin. Alternatively, derivatization of polypeptide drugs by using polyethylene glycol (Abuchowski et al. 1977) and their encapsulation in pH-responsive gels and films (Lowman et al. 1999) or in enteric-coated capsules containing sodium salicylate (Hosny et al. 2002), which become gradually dissolved or leaky along passage in the gastrointestinal tract, have been reported to prevent their enzymatic degradation and to enhance their absorption (Clement et al. 2002). A number of these promising approaches have been taken into the clinics.

Permeation Enhancers

Bypassing of the intestinal epithelial transport barrier has been tackled by several approaches

(Ziv et al. 1987; Carino and Mathiowitz 1999). The most common strategy which has been followed is the use of permeation enhancers that have been demonstrated to increase the efficacy of oral protein delivery, among them bile salt or fatty acid derivatives for increasing the permeability across the intestinal cell walls, presumably via the transcellular route. For instance, salts of fatty acids like caprate, caprylate, laurate, or palmitate have been tested for oral delivery of insulin and other macromolecules with variable success (Palin et al. 1986; Mesiha et al. 1994). The coadministration of other penetration enhancers based on detergent-like molecules and micelles, such as pluronic acid (Wang et al. 2007), seems to destabilize or weaken the tight junctions facilitating the paracellular routing of proteins (Matsumara 2008). Moreover, zonula occludens toxin is currently being assessed as permeation enhancer for insulin and has been demonstrated to be effective in reducing blood glucose levels in animal models of diabetes (Fasano and Uzzau 1997). In fact, this toxin apparently opens up the tight junctions in effective and reversible fashion and thereby manages to improve the paracellular epithelial transport of protein drugs, such as insulin. However, these permeation enhancers could potentially damage or even dissolve the gastrointestinal barrier and thereby facilitate the access of undesired small and large molecules, eventually derived from the food, from the intestinal lumen into the circulation with increased risks for local inflammations and gastrointestinal infections. Consequently, prior to routine therapeutic application, the general loss of the permeability barrier and protective function formed by the enterocytes and long-term toxicity has to be excluded for each penetration enhancer. Taking this into consideration, the encapsulation of the therapeutic proteins, antibodies, and nucleic acids into nanoparticles (NPs) seems to be more promising (Uhrich et al. 1999; Soussan et al. 2009).

Nanoparticles (NPs)

According to the definition by the Royal Society of London in July 2004, nanotechnologies are the design, characterization, production, and application of structures, devices, and systems by controlling shape and size at the nanometer scale (Ebbesen and Jensen 2006; Schwendeman et al. 1996; Mathiowitz et al. 1997; Damge et al. 1988; Takka and Acarturk 1999; Rowsen et al. 2000; Dapergolas and Gregoriadis 1976) (see section "Detection of Exosomes and Microvesicles (EMVs)"). In recent years, in-depth understanding of nanostructured substances has been provided by the use of new sophisticated tools, such as atomic force microscopy. The recent advances in NPs and nanotechnologies make them very promising tools in the delivery of therapeutics, drug discovery, and diagnostics. NPs with a hydrophilic surface are especially desirable to transport therapeutics to the target tissues or cells because they can escape the uptake by mononuclear phagocytes, macrophages, and reticuloendothelial systems (RES) in the blood and organs. In addition, a biological signal can be chemically conjugated onto the surface of NPs to recognize specific tissues or cells. Although NPs as a drug carrier have their own drawbacks, such as low-loading capacity and wide size distribution, they have attracted increasing attention from chemists, biologists, engineers, and pharmacologists since they provide the possibility of transporting bioactive compounds to specific tissues, cells, and cell compartments (Gullotti and Yeo 2009; Yokoyama 2005).

NPs offer numerous advantages over conventional dosage forms, including the ability to protect drugs from biodegradation, for targeting drugs to the site of action. They are made by forming drug-polymer complexes in which the drug is uniformly dispersed or by creating nanoscale vesicles (such as liposomes and micelles) to entrap the drug molecules. The surface characteristics of NPs are important in determining their susceptibility to uptake by the RES. Modification of surface properties affects both the circulation time and ultimate fate of the NPs. It has been reported that NPs with more hydrophobic surfaces tend to be taken up rapidly by the liver, spleen, and lungs (Schwendeman et al. 1996; Mathiowitz et al. 1997), whereas liposomes coated with hydrophilic surfaces exhibit significantly

prolonged circulation times in vivo (Dapergolas and Gregoriadis 1976). It is believed that the dense surface concentration of hydrated polymer chains sterically hinders protein absorption and opsonization of the liposomes. The synthetic hydrophilic polymer PEG is the most common polymer used for NP recognition by the RES. Surface charge as well as the presence of targeting signals at the NP surface is another important parameter in determining how NPs interact with cells, whose membranes are usually negatively charged.

In recent years, the paracellular transport of protein drugs by encapsulation into NPs assembled from various biomaterials, such as chitosan, alginate, mannans, and γ-polyglutamic acid at specific ratios, has been demonstrated (Soussan et al. 2009; Ebbesen and Jensen 2006; Schwendeman et al. 1996; Mathiowitz et al. 1997; Damge et al. 1988). These NPs apparently adhere to the negatively charged mucus and glycocalyx layers of the small intestine, which is achieved by their positive net surface charge. Following infiltration of these layers, the NPs trigger opening of the tight junctions, presumably by causing the redistribution of some of their constituent proteins, e.g., F-actin, occludin, and ZO-1, for their subsequent passage between neighboring enterocytes. However, immediately thereafter, the tight junctions reseal again in order to maintain the intestinal permeability barrier.

Polymeric NPs not only improve the water solubility and bioavailability of drugs but also allow for drug targeting by conjugating biological signals onto their surface or by using stimuli-sensitive polymers. Although the introduction of targeting signals to the NPs leads to a higher concentration of the drug at target sites, such as tumors, considerable portions of drug molecules are still found entrapped in the liver, possibly due to the leakage of the drug molecules from the NPs or the sophisticated liver structure rich in RES. Therefore future research in the field of drug delivery may be focused on enhancing drug stability in the NPs before reaching the target site and drug accumulation at the target sites by using stable polymer core-shell NPs and specific targeting signals.

Structures

Many different macromolecular NP structures, such as drug-polymer conjugates, micelles, liposome, and dendrimers, have been designed to transport drugs to their intended target tissues and cells. Micelles can be made from amphiphilic block copolymers that self-assembly into small spherical structures (Matsumara 2008; Uhrich et al. 1999; Damge et al. 1988; Takka and Acarturk 1999; Rowsen et al. 2000; Dapergolas and Gregoriadis 1976). Liposomes are vesicles made of phospholipid bilayers that can encapsulate drugs in their luminal cores or interfaces between the bilayers (Uhrich et al. 1999). NPs are generally polymeric matrices in the form of nanosized colloids that can encapsulate a drug through physical entrapment (association between the drug and polymer) or chemical conjugation (creating a chemical bond between the drug and polymer) (Soussan et al. 2009).

Typically, NPs with size smaller than 100 nm in diameter accumulate in solid tumors, which are characterized by extensive angiogenesis, defective vascular architecture, impaired lymphatic drainage, and an increased production of permeability factors (Gullotti and Yeo 2009; Yokoyama 2005). This phenomenon is known as the enhanced permeability and retention (EPR) effect. It can be exploited to improve drug delivery by NPs to tumors and other sites, including inflammations and infarcts, which possess similar pathological characteristics. The EPR effect is considered a passive targeting mechanism, but more specific drug targeting can be achieved by binding targeting ligands to the surface of the NPs, such as monoclonal antibodies, peptides, and sugar moieties. Drug targeting can also be achieved by introducing stimuli-sensitive materials into the NPs (Mathiowitz et al. 1997). Temperature- (Lin et al. 2007) and pH- (Mathiowitz 2008; Yang et al. 2007) sensitive polymers have been used in drug targeting since many pathological processes are known to be accompanied by local temperature increases and/or acidosis. Poly (*N*-isopropylacrylamide) and its copolymers are the most extensively studied thermosensitive polymers. One of the drawbacks of using thermosensitive NPs is that heating is inaccessible

to deep tissues or organs. Recently polymer core-shell NPs with both temperature and pH sensitivity were developed with pH changes inducing the temperature sensitivity (Kirpotin et al. 2006). These NPs were stable in the normal physiological environment, but deformed and precipitated in acidic environments, such as the tumor interstitium and intracellular compartments. They are superior to carriers that are either thermosensitive or pH-sensitive due to their potential to target deep tissues where it may not be possible to trigger drug release by hyperthermia. In addition, their phase alternation triggered by external pH changes is much more abrupt than that of pH-sensitive NPs (Mamot et al. 2005; Dagar et al. 2003; Yang et al. 2006; Maeda et al. 2000; Langer 2001; Chaw et al. 2004).

Materials

Polymers used for the fabrication of NPs include polylactide (Liu et al. 2005), poly(lactide-co-glycolide) (Liu et al. 2005), PLA/PLGA-b PEG (Lee et al. 2005; Potineni et al. 2003), PEG-poly (ß-benzyl-L-aspartate) block copolymers (Kataoka et al. 2000), PEG-poly(€-caprolactone)-PEG (Zhang et al. 2000), and poly(trimethylene carbonate)-PEG-poly(trimethylene carbonate) triblock copolymers (Zhang and Zhuo 2005a). A drug can be loaded into the NPs by various methods, such as by an oil-in-water emulsion method if the drug is water-insoluble (Potineni et al. 2003; Dong and Feng 2005) or a water-in-oil-in-water double-emulsion process if the drug is water soluble (Zambaux et al. 1998). Membrane dialysis is a widely used process for the production of drug-loaded micellar NPs (Kataoka et al. 2000; Zhang and Zhuo 2005b; Yoo and Park 2004; Wei et al. 2005). The drug-loading capacity of the NPs depends on the properties of the drug, production parameters, and the structural compatibility between the drug and the hydrophobic segments of the amphiphilic polymers (Allen et al. 1999). It has always been a challenge to incorporate water-soluble drugs into polymeric NPs. Recently, ionic complexation was used to load drugs, such as doxorubicin and verapamil into dextran sulfate, an anionic polymer with relatively high loading levels (Wong et al. 2004).

NP formulations using mucoadhesive polymers, such as chitosan, poly(lactic-)co-glycolic acid, poly-γ-glutamic acid and alginate, poly(alkyl-)cyanoacrylate microparticles, and ß-cyclodextrin liposomes, have been studied extensively (Wong et al. 2004; Borghouts et al. 2005; Pavlou and Reichert 2004; Kompella and Lee 1991; Rabkin and Dahl 1993; Zhang et al. 2000) and found to encapsulate the protein drugs apparently physically protected from enzymatic degradation. Furthermore, it has been shown clearly that such NPs cross the epithelial layer through Peyer's patches (De Campos et al. 2001; McLendon et al. 2010; Bhavsar and Amiji 2007; Singh and Lillard 2009; Agueros et al. 2011; Bimbo et al. 2011; Woitiski et al. 2010, 2011; Yang et al. 2008). While some of the encapsulated protein drugs have been demonstrated to exert the desired physiological effect in appropriate animal models, such as lowering of blood glucose in Streptozotocin-diabetic rats in the case of insulin, further development has not been reported. Presumably, these nanomaterials "actively" induce the transient opening of the tight junctions in the course of their direct binding to protein components of the zona occludens and thereby enable the paracellular passage of the encapsulated therapeutic proteins into the circulation. One critical disadvantage of this "site-specific" delivery and "colonic absorption" could be the inability to adequately correct and compensate for all physiological aspects of the missing or defective endogenous susceptibility gene product, such as the loss of the first-phase secretion of insulin during the pathogenesis of T2D.

Glycosylphosphatidylinositol (GPI)-Anchored Proteins

Very recent experimental results hint to the exciting possibility of a novel strategy for both oral delivery and tissue targeting in parallel. It is based on a "natural" co-translational modification of the therapeutic protein with a glycolipid structure, the covalent coupling to glycosylphosphatidylinositol (GPI) (Nosjean et al. 1997; Brewis et al. 1995; Ikezawa 2002; Tiede et al. 1999; Orlean and Menon 2007). In analogy to adipocytes for which the uptake and transcellular

transport of GPI-anchored proteins via plasma membrane lipid rafts, LD, and EMVs have been amply documented (Müller et al. 1994, 2001, 2005, 2008a, b, c, 2011; Cocucci et al. 2009; Al-Nedawi et al. 2008; Aoki et al. 2007; Pap et al. 2009; Müller 2010a, b, 2011a, b), the multistep oral delivery of therapeutic proteins and antibodies in the course of transcellular transport and/or translocation onto the surface of cytoplasmic LD and subsequent release via EMVs and translocation into the cytoplasm of enterocytes has been proposed. It is based on the unique property of GPI-anchored proteins to associate through their GPI anchor with detergent micelles, plasma membrane lipid rafts, LD, and EMVs in a fashion compatible with inter- and intracellular trafficking as well as physiological function (Premkumar et al. 2001; Kooyman et al. 1998; Medof et al. 1996; Civenni et al. 1998; McHugh et al. 1995, 1999; Ilangumaran et al. 1996; Zhang et al. 1992; Olschewski et al. 2007).

Initial experiments with mixed micelles consisting of phosphatidic acid, cholesterol, negatively charge glycosphingolipids, and GPI-anchored therapeutic proteins, such as human insulin, and encapsulated together with some of the above biomaterials could represent a major advantage in oral protein delivery. When assayed in vitro for paracellular transport (Müller 2010a, b), these NPs were shown to trigger the opening and closure of the tight junctions in reversible and efficient fashion, presumably due to the amphiphilic overall nature exerted by the GPI anchor. Moreover, when enwrapped by an acid-stable film, these NPs have demonstrated their potential to increase plasma insulin and lower blood glucose in insulin-free diabetic rats. Importantly, during these preliminary studies, accumulation of these NPs or of their degradation products and other safety issues were not observed, possibly due to efficient elimination and excretion of all the biomaterials used, including the GPI anchor. The pharmacokinetic/dynamic challenges, such as the adjustment of the time-action profile and the control of the interindividual variability of the anchor removal, will be similar for the paracellular and transcellular (see above) routes of oral protein delivery on the basis of GPI-anchored therapeutic proteins, but do not seem to represent insurmountable hurdles for the future (Müller 2010a, b).

The amphiphilic nature of the GPI glycolipid structure, which typically embeds the attached protein moiety in the outer leaflet of the eukaryotic plasma membrane, imposes novel biophysical characteristics to the carboxyterminally coupled protein moiety with regard to the adherence to hydrophobic and amphiphilic surfaces and materials. As a consequence, purified GPI-anchored proteins can be reconstituted into mixed micelles, liposomes (Nosjean and Roux 1999, 2003; Morandat et al. 2002; Ronzon et al. 2004), model lipid rafts (Milhiet et al. 2002), natural membranes (Medof et al. 1996; McHugh 1995, 1999), or LD harboring a core of neutral triglycerides (Müller et al. 2008b, c, 2011) as well as be adsorbed to natural and artificial polymers. In addition, GPI-anchored proteins can be cross-linked to each other by chemical means or covalently linked to biomaterials, which constitute the shell of the NPs. These assemblies, aggregates and "bubbles" with grossly differing properties are currently being evaluated for a number of different purposes, among them the transport across intestinal enterocytes.

Tissue Targeting

The size of the NPs and the thickness and type of the nanomaterials used apparently determine their systemic trafficking in the circulation following their transit across the intestinal barrier (see chapter "▶ Monitoring of Diabetic Late Complication"). This could open the possibility for specific targeting of therapeutic proteins and antibodies to the affected tissues, such as muscle, fat, liver, and pancreas for T2D and obesity therapy. The strategy of "passive targeting" relies on the relatively selective extravasation and retention of long-circulating NPs on the basis of specific structural features of the diseased tissue. The strategy of "active targeting" is based on the modification of the surface of NPs with ligands that can specifically recognize the diseased cells. These

two clinically relevant and NP-based targeting strategies rely on the specific interaction between the ligands (antibodies, peptide mimics, or nucleic acids) on the carrier surface and the corresponding receptors expressed on the diseased cells. For example, human epidermal growth factor receptor-2 (HER-2) (Yang et al. 2007; Kirpotin et al. 2006; Mamot et al. 2005), folic acid receptor (Wang et al. 2007), and vasoactive intestinal peptide receptors (VIP-R) (Dagar et al. 2003) have been investigated as biomarkers for NPs targeted to breast tumors. Additional new targeting strategies have emerged as a way of improving the targeting efficiency of NPs.

The recently reported observations with adipocytes (Müller 2008, 2010a, b, 2011b) raised the possibility for the operation of similar mechanisms of inter- and intracellular trafficking of GPI-anchored proteins between and in other tissue cells (Kooyman et al. 1995; Suzuki and Okumura 2000), such as pancreatic ß, muscle, or liver cells, with their transfer from EMVs to plasma membrane lipid rafts and subsequent translocation to the surface of cytoplasmic LD. Both lipid rafts and LD are present in virtually all mammalian cell types studied so far, albeit at considerably varying amounts (Brown and London 1998; Brown 1992; Varma and Mayor 1998; Simons and Ikonen 1997; Rajendran and Simons 2005; Brown and Rose 1992; Munro 2003; Simons and Toomre 2000; Ducharme and Bickel 2008; Fujimoto et al. 2008; Ohsaki et al. 2009; Müller et al. 2002; Müller and Petry 2005; Danielsen and Hansen 2006, 2008). The subcellular location at the LD surface enables the therapeutic protein/antibody to substitute for missing or subnormal activity or to dampen hyperactivity, respectively, of a susceptibility gene product encoded. The apparent role of LD in the specific retention and release of regulatory and structural proteins, such as those involved in the intracellular replication of several infectious agents, including chlamydia and hepatitis C virus (Boulant et al. 2007; Cocchiaro et al. 2008; Cermelli et al. 2006), suggests new therapeutic options for a variety of common diseases, including obesity and T2D.

Nucleic Acid Therapeutics

Gene therapy refers to the transmission of DNA encoding a therapeutic gene of interest into targeted cells or organs with consequent temporary or permanent expression of the transgene to compensate for the missing or inadequate expression of a susceptibility gene, for instance, causative for the development of obesity and T2D. Although gene therapy has been extensively studied, the FDA has not yet approved any human gene therapy product for clinical application. Little progress has been made since the first gene therapy clinical trial began in 1990. This is because there are limited safe and efficient gene carriers available. Basically, there are two types of gene delivery vectors, viral and nonviral vectors. Nonviral vectors have recently received increasing attention because they are easier to produce, transport, and store and induce less of an immune response. Many natural and synthetic materials have been explored as nonviral gene vectors, including cationic liposomes, cationic polymer NPs, and inorganic NPs (e.g., silica NPs, carbon nanotubes, and metal nanorods). Of these nonviral gene vectors, cationic polymer NPs are the most attractive because they can be easily tailored and synthesized to suit the special recruitments encountered by gene delivery. The cationic polymers also include natural polymers carrying positive charges, and the most commonly used ones consist of branched and linear polyethylenimine (PEI), copolymers thereof, poly(L-lysine) and its copolymers, chitosan, and dendrimers. Nanoscaled complexes of cationic polymer and DNA can be fabricated by simply mixing these cationic polymer solutions with DNA. In particular, branched PEI with a molecular weight of 25 kDa is often used as a control to evaluate the gene expression efficiently to other nonviral vectors because it provides high gene transfection efficacy in various cell lines. The PEI polymers can effectively condense DNA molecules to form homogenous spherical particles with a size of approximately 100 nm, enabling efficient in vitro gene transfection. Conversely, PEI offers significantly more efficient protection against nuclease degradation than other cationic polymers, such as

poly(L-lysine), probably because of its high charge density and efficient complexation with DNA. The amino groups of PEI may also provide significant buffering capacity to the polymer over a wide range of pH values. This property known as proton sponge is essential for escape of the DNA complexes from the endosome (Bhavsar and Amiji 2007).

Small interfering RNA (siRNA) can be used as a method to silence target genes, which has recently become a powerful tool in drug development. However, in vivo siRNA delivery is typically challenging, and delivery methods effective for other nucleic acids may not be suitable for siRNA. The commonly used method to deliver siRNA in vivo is to incorporate siRNA into a viral vector (Davis 2009). Recently, successful attempts to deliver siRNA using nonviral polymer-based NPs were reported. The siRNA with the target sequence 5′-AATGACATGCCGATCTACATG-3′o; for downregulating EphA2 was loaded into liposomes composed of neutral lipid 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine. The successful delivery of the siRNA decreased protein expression in tumors established in female athymic nude mice by i.p. injection of ovarian cancer cells and significantly suppressed tumor growth when combined with the anticancer drug paclitaxel. In addition, vascular endothelial growth factor receptor 2-targeted siRNA was designed and complexed with PEGylated-PEI with an RGD peptide ligand attached at the distal end of PEG, a signal to target tumor neovasculature expressing integrins. The i.v. injection of RGD-PEG-PEI/siRNA complexes into female nude mice bearing N2A tumors induced sequence-specific inhibition of the target gene, reduction in angiogenesis, and inhibition of tumor growth (Xie et al. 2006; Martin and Caplen 2007; Leung and Whittaker 2005; Kim and Rossi 2007; Suda et al. 2008; Gao and Huang 2009). In summary, cationic polymeric NPs provide a strong gene-binding ability, high gene transfection efficacy in various cell lines, and relatively low toxicity. Successful in vivo gene transfection has also been achieved, which may have potential for clinical immunization. However, for metabolic diseases, gene transfection induced by cationic polymeric NPs is still not efficient enough. Targeting signals may be conjugated to the NPs or DNA to improve cell uptake and nuclear targeting.

On the basis of the demonstrated translocation of GPI-anchored proteins onto the cytoplasmic surface of LD of adipocytes upon their insertion into plasma membrane lipid rafts, the possibility of nucleic acids covalently coupled to GPI anchors undertaking the same route of targeting across target cell plasma membranes has been suggested recently (Müller 2010a, b). The hypothetical model for the transport of therapeutic RNA's in course of a special type of oral nonviral gene therapy encompasses the association of the therapeutic nucleic acid (i.e., siRNA, microRNA, DNA) coupled to GPI anchors with lipid rafts of the apical plasma membrane of enterocytes following their oral administration and their subsequent transport via LD further to the cytoplasm or nucleus, where gene expression is modified in highly efficient and specific fashion.

Evaluation

Albeit these initial findings are promising, many critical requirements for (chronic) use in humans, predominantly concerning safety, non-immunogenicity, and non-accumulation in the body due to their proper degradation and excretion, have to be met by NPs (des Rieux et al. 2006; Alexis et al. 2008; Li and Huang 2008; Rajendran et al. 2010). In particular, for chronic therapeutic application of NPs, the possibility and extent of their systemic entry has to be investigated, including the relevant degrading and eliminating pathways. Most importantly, the accumulation of the NP materials in tissues and cells, which either harbor the target of the orally delivered therapeutic protein or serve as unspecific sink for the NPs after their systemic distribution, has to be excluded in the long term. So far, materials fulfilling all these criteria are not available, and consequently, there is an urgent need for the discovery and development of novel biomaterials.

Common or civilization diseases, such as T2D and obesity, are caused by a complex multifactorial interplay of genes and environmental factors.

Recent developments in GWAS have led to the discovery of numerous disease and susceptibility genes. This nourished great hope in personalized medicine with its promise of reliable prognosis, in-time diagnosis, as well as efficient and safe therapy of common diseases in the near future on basis of the determination of the individual genetic profile. However, association and clinical studies have also revealed the rather limited predictive value of the individual risk for T2D and obesity of the currently available genetic markers, also in comparison to and even in combination with the classical biomarkers and risk factors, such as fasting blood glucose, BMI, and age. However, there are good reasons for the assumption that the detection of an adequate number of additional susceptibility genes in combination with the analysis of proteins, lipids, and metabolites in large scale will improve the predictive value to the degree necessary and sufficient for personalized prognosis and diagnosis of metabolic diseases. Nevertheless, the considerable expenditure for personalized prognosis and diagnosis will be justified only in combination with the availability of adequate personalized therapeutic options.

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Part XIII

Anti-Obesity Activity

Methods to Induce Experimental Obesity

Andreas W. Herling

Contents

General Considerations

Influence of the central nervous system, in particular of the hypothalamus, on development of obesity has been suspected since the early clinical observations of Babinski (1900), Fröhlich (1901), and Biedl (1916). Experiments reported by Smith (1927) showed that injections of chromic acid into the suprasellar region of rats with lesion of the hypothalamus induced obesity in rats (Bomskov 1939). Hetherington and Ranson (1939) found that electrolytic lesions, restricted to the ventromedial region of the hypothalamus, could be associated with the development of obesity.

A virally induced obesity syndrome in mice was described by Lyons et al. (1982).

Chan (1995) gave a review on β-cell stimulus-secretion coupling defects in rodent models of obesity.

Leiter and Herberg (1997) reviewed the advances in understanding the molecular bases for monogenic obesity mutations capable of producing obesity-induced diabetes, or diabesity, in mice.

Astrup and Lundsgaard (1998) discussed pharmacological mechanisms of anti-obesity drugs.

Research with animal models of obesity has become of great interest in the last two decades. The increasing interest is the result of the increase in the obesity incidence worldwide and the importance of obesity and its comorbidities (http://www.idf.org/diabetesatlas): type 2 diabetes and diabetes late complications (macrovascular

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(stroke, cardiac infarction, peripheral occlusion disease) and microvascular (nephropathy, retinopathy, neuropathy)), as well as dyslipidemia, fatty liver, etc.

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Diet-Induced Obesity (DIO) in Mice

Purpose and Rationale

Obesity can be induced in mice by offering a high-fat diet. This is the most often used obesity model worldwide.

Procedure

Male or female mice (e.g., C57Bl6) can be used for this method. Male mice have to be housed individually due to their increased aggression behavior at older ages, while female mice can be group housed. Mostly the mice are kept at normal animal house temperature at 22–23 °C with 12-h light–dark cycles. At the age of 8 weeks, mice are offered a high-fat diet (>10 % fat vs. about 3 % fat of the standard chow) for 12–16 weeks until mice have gained a body weight of about 40–45 g. A typical experiment consists of two (age- and sex-matched) control groups, one on normal chow and one on the high-fat diet. The treatment groups consist of mice on high-fat diet. Each group should have a size of 8–10 animals. Treatment with a test article can be started either (1) with the start of the high-fat diet (prevention mode) or (2) when the animals on the high-fat diet have stabilized on their elevated body weight of above 40 g (intervention mode). Route of administration (orally or subcutaneously) and dosing frequency depend on the characteristics of the test article (small molecule or peptides) and should be based on the pharmacokinetic characteristics (half-life time, Cmax, exposure) of the test article(s). The typical experiment lasts minimally 4 weeks, but can also last up to 12 weeks and even longer. Primary parameters, which are continuously measured during the experiment daily or at least twice weekly, are body weight and food and water intake. Body fat mass, measured by DEXA or Medispec (whole-body magnetic resonance

spectroscopy), and glucose tolerance or insulin tolerance are measured prior to the start of the treatment (baseline value) and near the end of the treatment period (treatment value). At the study end, animals are sacrificed in deep isoflurane anesthesia by terminal blood collection from the aorta or vena cava after laparotomy; appropriate tissues (liver, fat, muscle, brain) are collected for biochemical (e.g., liver, fat) or genetic analysis. Appropriate clinical chemistry parameters are measured in the serum (e.g., serum triglycerides, free fatty acids, ketone bodies, total cholesterol, LDL, HDL, VLDL, etc.) as well as other serum biomarkers of interest (hormones, cytokines, etc.).

Evaluation

Intergroup comparisons are made between lean and obese control groups to demonstrate the effect of the high-fat diet on the various parameters, as well as between obese control and obese treatment groups to assess the efficacy of the test article (s) by applying appropriate statistical methods (*t*-test, ANOVA, etc.).

Critical Assessment

The C57Bl6 mouse are the most commonly used strain in obesity and metabolism research because it is prone to diet-induced obesity with high-fat diets and develop severe insulin resistance. In contrast, A/J, FVB, C3HeB/FJ, and 129v strains are relatively resistant to diet-induced obesity and exhibit lower weight gain on a high-fat diet (Almind and Kahn 2004; Champy et al. 2008). For some studies, it is important to determine the effect of these background genes, and in these cases, comparative studies on different genetic backgrounds, mixed genetic background, or outbred backgrounds can provide additional information (Tschöp et al. 2012).

Mice are the most unfavorable animal species with respect to the relationship between body mass and body surface. Therefore, any direct or indirect activity of the test article on the thermoregulation of the mice, as well as housing conditions as confounding factors, can have a pronounced effect on energy balance and subsequently on metabolic serum parameters (Nedergaard and Cannon 2014). Institutional and governmental guidelines define housing conditions for laboratory animals, and these conditions can affect energy balance particularly in mice due to their unfavorable relationship between body mass and body surface. Mice are most frequently housed at 20–23 °C and a light–dark cycle of 12 h. Because thermoneutrality is for mice at around 28–30 °C, this means in most cases mice are housed under mild thermoregulatory stress, which may not be the ideal ambient temperature for energy metabolism studies aiming to mimic the situation in humans, who normally exist much closer to thermoneutral conditions (Nedergaard and Cannon 2014). The temperature in a cage may be substantially higher than the ambient temperature outside the cage, and in this temperature gradient increases from singly housed to multiply housed mice (Tschöp et al. 2012).

Modifications of the Method

Many modifications are published in the literature. These consist of (1) different diet compositions, (2) functional tests performed during the study, and (3) inclusion of an additional pair-fed control group:

(ad 1): Different diet compositions with respect to the amount of fat content, fatty acid types, as well as the addition of sucrose (disaccharide: glucose–fructose) are reported. Fructose can be directly converted to fatty acids by hepatic lipogenesis, resulting in liver steatosis with subsequent hepatic insulin resistance.

(ad 2): Different functional tests, which are included in the study, e.g., glucose tolerance, insulin tolerance, and glucose clamp study, are applied, when the focus of the study is more on the treatment-induced improvement of insulin resistance, while indirect calorimetry is used when there is the focus more on energy expenditure.

(ad 3): There is also often an additional pair-fed control group included. This group received the amount of food, which was consumed by the treatment group the day before. By applying this method, it is possible to discriminate whether the reduction of body weight in the treatment group is caused exclusively by the reduced food intake or by additional factors, e.g., increased energy expenditure.

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Diet-Induced Obesity (DIO) in Rats

Purpose and Rationale

Obesity can be induced in rats by offering a diet containing high fat or high fat plus high-sugar/high-carbohydrate (cafeteria diet) diets. DIO rat studies are much less reported in the literature compared to DIO mice studies.

Procedure

Rats (e.g., Sprague Dawley, Wistar) at 6–8 weeks of age were made diet-induced obese by offering a high-fat diet. The rats included in the DIO study had ad libitum access to the diet and water throughout the study and were used for experimentation after 17 weeks on the two-choice diet. The animals were housed in a standard 12-h light–dark cycle at a room temperature of 20–22 °C and a relative humidity of 50–60 %. All animals had free access to water.

One week prior to drug dosing, the rats were single-housed and randomized into individual treatment groups ($n = 8$ per group) based on whole-body fat mass and body weight, respectively, measured 3 days prior to initiation of drug treatment.

The typical experiment consists of one control group on normal rat chow and several groups on high-fat or high-fat/high-carbohydrate diets, from which one group serves as obese control group. Duration of treatment lasts minimally 4 weeks, but can also last up to 12 weeks and even longer. Primary parameters, which are continuously measured during the experiment daily or at least twice weekly, are body weight and food and water intake. Body fat mass, measured by DEXA or Medispec (whole-body magnetic resonance spectroscopy), and glucose tolerance or insulin tolerance are measured prior to the start of the treatment (baseline value) and near the end of the treatment period (treatment value). At the study end, animals are sacrificed in deep isoflurane anesthesia by terminal blood collection from the aorta or vena cava after laparotomy; appropriate tissues (liver, fat, muscle, brain) are collected for biochemical (e.g., liver, fat) or genetic analysis. Appropriate clinical chemistry parameters are measured in the serum (e.g., serum triglycerides, free fatty acids, ketone bodies, total cholesterol, LDL, HDL, VLDL, etc.) as well as other serum biomarkers of interest (hormones, cytokines, etc.).

Evaluation

Intergroup comparisons are made between lean and obese control groups to demonstrate the effect of the high-fat diet or high-fat/high-carbohydrate diet on the various parameters, as well as between obese

control and obese treatment groups to assess the efficacy of the test article(s) by applying appropriate statistical methods (*t*-test, ANOVA, etc.).

Critical Assessment

DIO rat studies are much less reported in the literature compared to DIO mouse studies, although the translatability to the human situation is much higher to that of mouse studies. The influence of confounding factors like ambient temperature of the housing conditions and thermoregulation is much less compared to mice, as the relationship of body mass and body surface in rats is not as unfavorable as it is in mice.

Time of treatment with respect to light–dark cycle is also of importance. As rodents are night active and take up the majority of food during the dark phase, a test article with a primary effect on inhibition of food intake should be administered at the start of the dark phase. Therefore, several scientific groups housed their animals on a reversed light–dark cycle (e.g., dark phase/light off at 08:00; light phase/light on at 20:00) to match the rodent behavior to the normal human working hours (e.g., treatment between 07:00 and 08:00 at the end of the light phase).

Modifications of the Method

Hansen et al. (2014) used a two-choice diet consisting of standard rodent chow or a high-palatable high-fat/high-sugar (HFHS) diet made up of a paste (1:1:1) of chocolate spread (Nutella, Ferrero, Italy) and peanut butter (Skippy, Unilever, USA) and powdered regular rodent chow.

We (Herling et al. 2008a, b) used also a two-choice diet. Female Wistar rats were fed a high-fat, high-carbohydrate (candy) diet for 12 weeks plus standard rat chow ad libitum. During the last 6 weeks, rats were treated with rimonabant. Food intake and body weight development were investigated, as well as effects on total body fat, especially visceral fat and ectopic lipid accumulation in the skeletal muscle and liver, determined by in vivo magnetic resonance imaging/magnetic resonance spectroscopy (2008a), as well as energy expenditure was determined by indirect calorimetry (2008b).

Scalfani and Springer (1976) induced obesity in adult female rats by adding a variety of supermarket foods to lab chow ("cafeteria diet"). In behavioral tests, the authors found similarities to hypothalamic and human obesity syndromes.

Rothwell et al. (1982) compared the effects of feeding a cafeteria diet on energy balance and diet-induced thermogenesis in four strains of rats.

Stock and Rothwell (1979) discussed the influence of various forms of feeding, high-fat diets, insulin injections, tube-feeding, and cafeteria feeding on energy balance in laboratory animals.

Rolls et al. (1980) found persistent obesity in rats following a period of consumption of a mixed, high-energy diet. When the high-energy foods were withdrawn after 90 days and just chow was available, the obese rats maintained their elevated body weights.

Hill et al. (1992) studied the influence of amount and composition of dietary fat on development of obesity in rats. Adult male Wistar rats were fed high-fat (HF, 60 % of calories) or low-fat (LF, 20 % of calories) diets for 28 weeks. Half of the rats in each condition received diets with saturated fat (lard) or with polyunsaturated fat (corn oil). There was some indication that unsaturated fat diets were associated with greater accumulation of fat in subcutaneous tissue depots than saturated fat diets. The effects of the type of fat were less than those of the amount of dietary fat.

Wade and Gray (1979) reviewed the gonadal effects on food intake and adiposity in rats. Estradiol and testosterone decrease adiposity, while progesterone increases carcass fat content.

Thermogenesis induced by various diets has been discussed by Rothwell and Stock (1986).

Harris (1993) studied the impact of high- or low-fat cafeteria foods on nutrient intake and growth of rats consuming a diet containing 30 % energy as fat.

Segues et al. (1994) studied long-term effects of cafeteria diet feeding on young female Wistar rats by comparing the circulating levels of

glucose, lactate, glycerol, 3-hydroxybutyrate and urea, and liver glycogen.

Llado et al. (1997) studied fatty acid composition of brown adipose tissue in dietary obese rats. Long time exposure to a hypercaloric high-fat diet such as the cafeteria diet induced an important fatty acid accumulation in brown adipose tissue, mainly for the major saturated and monounsaturated fatty acids.

LeBlanc and Labrie (1997) investigated the role of palatability of the food in diet-induced thermogenesis.

Selective breeding for diet-induced obesity and resistance in Sprague Dawley rats was reported by Levin et al. (1997).

Herberg et al. (1974) reported the effects of either a high-carbohydrate diet or a high-fat diet over prolonged periods in metabolically intact and in obese NZO **mice**.

West et al. (1992) evaluated the effects of a 7-week consumption of a diet containing 32.6 % of kilocalories as fat (condensed milk) on body composition and energy intake in nine strains of inbred mice. Relative to chow diet controls, the condensed milk diet significantly increased carcass lipid content in six strains (AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2 J, and C57BL/6 J), but had no or a marginal effect on adiposity in three strains of mice (SJL/J, I/STN, and SWR/J).

West et al. (1994) studied the genetics of dietary obesity in AKR/J x RWR/J mice. Pups were weaned between the ages of 29 and 34 days onto Purina Chow ad libitum until they were switched to a high-fat, condensed milk diet containing 32.6 %, 15.0 %, and 52.4 % of kilocalories as fat, protein, and carbohydrate, respectively.

Strain-specific response to β_3-adrenergic receptor agonist treatment of diet-induced obesity in mice was reported by Collins et al. (1997).

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Hypothalamic Obesity

Purpose and Rationale

The hypothalamus is particularly regarded as a critical platform that regulates hunger and satiety in relation to energy stores and nutrient availability and thereby controls food intake and energy balance. Hyperphagia in rats has been reported after hypothalamic lesions (Liu and Yin 1974). Surgical techniques were described by Leibowitz et al. (1981) and Vander Tuig et al. (1985).

Procedure

Female Sprague Dawley rats, weighing about 190 g body weight, receive during a 5–9 days initial period of adjustment a high-fat diet. They are then fasted overnight and anesthetized with 35 mg/kg pentobarbital sodium and additionally 1 mg atropine methyl nitrate intraperitoneally. Bilateral wire knife cuts or electrolytic lesions are stereotaxically positioned in the hypothalamus (David Kopf Instruments, CA). With the incision bar positioned at −3.0 mm, parasagittal wire knife cuts are placed between the medial and lateral hypothalamus using a retractable wire knife according to Gold et al. (1973). The cuts are made 1.0 mm lateral to the midline and extended from 8.5 to 5.5 mm anterior to the ear bars and from the base of the brain dorsally 3.0 mm.

To produce electrolytic lesions in the ventromedial hypothalamus, the incision bar is positioned at +5.0 mm, and a stainless steel electrode, insulated except for 0.5 mm at the tip, is lowered 0.6 mm lateral to the midline and 5.8 mm anterior to the ear bars. With the tip of the electrode 0.7 mm above the base of the brain, lesions are made by passing 2.0 mA of anodal current for 20 s to a rectal electrode.

Sham-operated rats serve as controls. Separate rats that are fasted overnight are killed at the time of surgery to provide data on initial body composition.

Histological verification of placement of knife cuts and lesions is made in brains fixed in 10 % buffered formaldehyde solution and embedded in paraffin. Serial sections through the hypothalamic area of the brain are examined histologically.

Evaluation

Not only food consumption and increase of body weight can be determined after hypothalamic lesions but also brown adipose tissue enzymes and guanosine diphosphate binding to brown adipose tissue mitochondria and noradrenaline turnover in various organs.

Modifications of the Method

A survey on hypothalamic and genetic obesity in experimental animals was given by Bray and York (1979).

Caloric compensation to gastric loads in rats with hypothalamic hyperphagia was reported by Liu and Yin (1974).

Sclafani and Aravich (1983) adapted adult female Sprague Dawley rats to a macronutrient self-selection regimen which allowed them ad libitum access to separate sources of protein, carbohydrate, and fat. The rats were then given either ventromedial hypothalamic lesions, paraventricular hypothalamic lesions, parasagittal knife cuts through medial hypothalamus, or sham lesions. Following surgery, all lesioned rats overate and became obese as compared to sham-operated controls. The group with parasagittal knife cuts through medial hypothalamus gained more weight than the groups with ventromedial hypothalamic lesions and paraventricular hypothalamic lesions.

Enhanced expression of rat obese (ob) gene in adipose tissue of ventromedial hypothalamus (VMH)-lesioned rats was described by Funahashi et al. (1995).

Elmquist et al. (1999) discussed the role of leptin and other peptide hormones in different areas of the hypothalamus in controlling food intake and body weight.

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Chemically Induced Obesity

Purpose and Rationale

Chemicals like gold thioglucose or monosodium-L-glutamate can induce obesity in mice by destruction of hypothalamic areas involved in food intake and energy balance.

Procedure

Intraperitoneal or intramuscular injection of gold thioglucose induces obesity in mice (Perry and Liebelt 1961). Swiss albino mice of either sex are fed with commercial mouse chow (Altromin R) ad libitum. At the age of 6 weeks, the animals receive a single intraperitoneal injection of 30–40 mg/kg gold thioglucose.

Adiposity can be induced in mice by repeated subcutaneous injections of monosodium-L-glutamate at an early stage of life (Olney 1969). Male mice are treated immediately after birth with daily subcutaneous injections of 2 g/kg monosodium-L-glutamate for 5 consecutive days. Control mice are treated with physiological saline. The animals are weaned at 3 weeks of age, housed under controlled temperature and artificial light–dark cycle, and provided with commercial powdered chow and tap water ad libitum.

Evaluation

Body weight and food intake are registered for a period of 3 months and compared with untreated controls by applying appropriate statistical methods (*t*-test, ANOVA, etc.).

Modifications of the Method

Debons et al. (1962, 1968, 1977) administered 800 mg/kg gold thioglucose by a single intraperitoneal injection to female CBA mice.

Obesity was induced in rats by implants of gold thioglucose in the hypothalamus (Smith and Britt 1971; Smith 1972).

Several other compounds produce obesity concomitantly with brain lesions.

Single intraperitoneal injection of bipiperidyl mustard (N,N′-bis-[β-chloroethyl]-4,4′-bipiperidine) in doses between 5 and 50 mg/kg induced obesity in mice (Rutman et al. 1966). Brain lesions and obesity by bipiperidyl mustard could also be produced in rats (Laughton and Powley 1981).

Massive and persistent obesity was induced in mice by a single intracerebral injection with 4-nitroquinoline 1-oxide (Mizutani 1977).

Tokuyama and Himms-Hagen (1986) studied brown adipose tissue thermogenesis, torpor, and obesity in glutamate-treated C57B1/6 J mice offered either normal chow or a cafeteria diet and found that the high metabolic efficiency and obesity of the glutamate-obese animals are principally a consequence of their maintenance of a hypothermic torpid state for more than 50 % of the time.

Seress (1982) injected young albino rats with either single or repeated doses of 0.1–6.0 mg/g body weight of monosodium-L-glutamate between the ages of 2 and 40 days. The smallest, single effective dose was 0.25 mg/g body weight administered during the first week of life. The sensitivity to monosodium-L-glutamate decreased with age. In adult rats, an 80–90 % loss of neurons in the anterior part of the arcuate nucleus was found.

Remke et al. (1988) reported on development of hypothalamic obesity after subcutaneous administration of monosodium-L-glutamate to neonate rats.

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Genetically Obese Animals

Andreas W. Herling

Contents

General Considerations

A long history of research in both rodents and humans strongly argues that energy, stored in the form of fat in adipose tissue, is homeostatically conserved. Energy homeostasis is a very complex long-term process composed of multiple interacting homeostatic and behavioral pathways, including glucose homeostasis, lipid homeostasis, the hypothalamic-pituitary-adrenal axis, short-term satiety, and other macronutrient pathways that together act to maintain constant levels of energy stores. Obesity, anorexia, cachexia, and failure to thrive are some of the syndromes that result from mutations in genes critical to energy homeostasis.

Historically, five such mutations were identified in the mouse: obese (*ob*) and diabetes (*db*), as well as agouti (A^y), fat (*fat*), and tubby (*tubby*) gene. The cloning and characterization of these mutant genes led to the discovery of leptin, the key adipocyte hormone encoded by the obese (*ob*) gene that communicates to the brain information regarding the level of energy stored in the form of fat. Discovery of the leptin receptor, encoded by the diabetes (*db*) gene, and the discovery that the product of the agouti (A^y) gene caused obesity by antagonizing the melanocortin-4 receptor (MC4-R) led to the identification of key neural circuits involved in the regulation of energy homeostasis. Since the characterization of these first obesity genes, however, a very large number of transgenic and knockout models with obesity, anorexia, cachexia, or obesity resistance have been created (Robinson et al. 2000).

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Obesity and diabetes are syndromes quite often linked in patients (maturity-onset diabetes) and hereditary animal models. The regulation of body weight in animals by leptin was reviewed by Friedman and Halaas (1998).

Symptoms of diabetes and obesity are overlapping in many animal models (see also chapter "► Genetically Diabetic Animals").

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Spontaneously Obese Mice

Obese-Hyperglycemic (ob/ob) Mice

Ingalls et al. (1950), Mayer et al. (1951), and Bleisch et al. (1952) observed hereditary diabetes in genetically obese mice. The obese-hyperglycemic mice were glycosuric, the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma was observed. One of the most interesting features was insulin resistance; doses as high as 400 IU/kg had little effect on blood sugar. The serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased, and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese-hyperglycemic mice is different from that of the human diabetic patient.

Three working groups identified in 1995 the OB protein (Pelleymounter et al. 1995; Halaas et al. 1995; Campfield et al. 1995), which was later named leptin and which is missing in ob/ob mice. Leptin is a hormone expressed in and secreted from adipose tissue. It signals to the hypothalamus the size of the fat stores and thereby regulates food intake. As ob/ob mice are leptin deficient, there is no break signal to the hypothalamus for food intake with the result of increased food uptake and with subsequent increased adiposity. In addition leptin regulates sympathetic outflow from the brain. Therefore, at normal animal house temperature of about 20–22 °C, ob/ob mice feel cold and subsequently increase food intake for compensation. Since 1977 it has been already known that ob/ob mice have a thermogenic defect and lower body temperature compared to wild-type littermates (Trayhurn et al. 1977).

Pelleymounter et al. (1995) investigated the effects of the *obese* gene product on body weight regulation in ob/ob mice. The OB protein was expressed in *E. coli* and purified to homogeneity as a 16-kDa monomer. Daily intraperitoneal injections of the recombinant OB protein to ob/ob mice lowered their body weight, percent body fat, food intake, and serum concentrations of glucose and insulin.

Halaas et al. (1995) reported that daily intraperitoneal injections of either mouse or human recombinant OB protein reduced the body weight of ob/ob mice but had no effect on *db*/*db* mice.

Campfield et al. (1995) found that peripheral and central administration of microgram doses of recombinant mouse OB protein reduced food intake and body weight of ob/ob and diet-induced obese mice but not in *db*/*db* obese mice.

Reduced oxygen consumption has been noted as early as 10–18 days of age in ob/ob mice (Boissenault et al. 1976; Trayhurn et al. 1977).

Other strains or substrains of mice with obesity and hyperglycemia have been described by Dickie (1962), Westman (1968), Stein et al. (1970), Coleman and Hummel (1973), and Herberg and Coleman (1977).

Strautz (1970) implanted ob/ob mice with Millipore diffusion chambers containing islets isolated from the pancreas of normal littermates.

Trayhurn et al. (1977) found a thermogenic defect in pre-obese ob/ob mice. Rectal temperature of 17-day-old pre-obese mice in response to an environmental temperature of 4 °C fell much more than in lean controls.

Chlouverakis (1972) performed parabiotic experiments of obese-hyperglycemic mice

(ob/ob) with lean littermates and determined body weight, glucose, serum insulin, and triglycerides as well as insulin sensitivity of diaphragm muscle and epididymal fat pad.

Parabiosis of obese (ob/ob) with diabetes (*db*/*db*) mice caused the obese partner to become hypoglycemic, to lose weight, and to die of starvation, while no abnormal changes were observed in the diabetic partner (Coleman 1973).

Cresto et al. (1977) compared the rate of insulin degradation in normal and in ob/ob mice.

Zhang et al. (1994) succeeded in positional cloning of the mouse *obese* gene and its human homologue.

Trayhurn et al. (1996) studied the effects of fasting and refeeding on *ob* gene expression in white adipose tissue of lean and obese (ob/ob) mice using a 33-mer antisense oligonucleotide as a probe for the rapid chemiluminescence-based detection of *ob* mRNA.

Sterility defect in homozygous obese female mice could be corrected by treatment with the human recombinant OB protein leptin (Chehab et al. 1996).

Roupas et al. (1990) used isolated adipocytes from ob/ob mice to study the diabetogenic action of growth hormone.

Rafael and Herling (2000) investigated the effect of leptin on energy balance in leptin-deficient ob/ob mice under conditions of thermoneutrality. It was found that food intake was reduced as long as body weight was above that of lean littermates. The closer the body weight of the obese mice came to that of lean mice, the obese mice increased their food intake gradually. It was concluded that leptin does not inhibit food intake per se but that leptin redirects energy from endogenous stores as long as they are present for energy expenditure.

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Yellow Obese (A^YA) Mouse

The yellow obese mouse is the only example of obesity inherited through a dominant gene and was described as early as 1883 by Lataste and in 1905 by Cuenot. It is located on chromosome 2 at linkage group 5, the agouti locus (Bateson 1903). Since the genes controlling obesity and the agouti coat colors are so closely linked, the obesity is associated with a change of pigmentation from black to yellow. Such an association allows the early identification of pre-obese mice as soon as the coat hair begins to grow.

Since the original description of the yellow (A^ya) mouse, a number of additional alleles have appeared at the agouti locus. The homozygous dominant yellow mutation (A^y/A^y) is lethal in utero (Robertson 1942; Eaton and Green 1962) with approximately 25 % of any litter from A^ya matings dying from an abnormal development after the trophoblast stage (Pedersen 1974).

Yellow (A^ya) mice develop a moderate form of obesity and diabetes. Increased body weight first appears at the time of puberty (8–12 weeks) (Dickie and Wooley 1946; Carpenter and Mayer 1958), after which body weight increases slowly to reach values of approximately 40 g. In contrast to other forms of obesity, yellow mice are characterized by increased linear growth. Plasma insulin concentrations are increased and food is stored more efficiently than in lean mice (Dickerson and Gowan 1967). Food intake returns to normal in older A^ya mice and the animals lose body weight (Hollifield and Parson 1957). The obesity may be exaggerated by being fed high-fat diets (Fenton and Chase 1951; Silberberg and Silberberg 1957; Carpenter and Mayer 1958). Food restriction may normalize body weight but

the animals still remain obese (Fenton and Chase 1951; Hollifield and Parson 1957). Metabolic rate of A^ya mice is depressed when related to body surface, although oxygen consumption per animal is identical to the homozygous recessive agouti (a/a) mouse (Bartke and Gorecki 1968).

Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide (amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-A^{vy}/a).

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KK-A^Y Mouse

Iwatsuka et al. (1970) reported on yellow KK mice (also named KK-A^y mice), carrying the yellow obese gene (A^y). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA_{1c} levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

Sohda et al. (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones for hypoglycemic and hypolipemic activities in yellow KK mice.

Hofmann et al. (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KKA^Y mice after treatment with the oral antidiabetic agent pioglitazone.

Yoshida et al. (1991) compared brown adipose tissue thermogenesis, resting metabolic rate, insulin receptors in adipocytes, and blood glucose and serum insulin levels during a glucose overloading test in yellow KK mice with C57B1 control mice after a β_3-adrenoceptor agonist.

Yoshida et al. (1996) determined body weight, food intake, white adipose tissue weight, brown adipose tissue weight and its thermogenesis,

noradrenaline turnover, blood glucose and serum insulin levels, and GLUT4 in diabetic yellow KK mice compared with C57B1 mice after mazindol.

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Fat/Fat Mice

Fat mice carry an autosomal recessive mutation and display a range of abnormalities, including progressive adult-onset obesity, hyperinsulinemia, and infertility (Coleman and Eicher 1990). The mutant allele of *fat* was identified and shown to be a missense (serine → proline) mutation in carboxypeptidase E which abolishes enzyme activity in a variety of neuroendocrine tissues (Naggert et al. 1995). Carboxypeptidase E is required for both sorting and proteolytic processing of a variety of prohormones including proinsulin and POMC (Cool et al. 1997). As carboxypeptidase E is expressed in the CNS, defective processing of a variety of hypothalamic neuropeptides – such as POMC and MCH – may trigger obesity in these animals (Rovere et al. 1996).

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Tubby Mice

Tub is an autosomal recessive mutation in mice (Coleman and Eicher 1990) which display a tripartite phenotype of blindness, deafness, and

maturity-onset obesity. In response to weight gain, these mice gradually increase their food intake in proportion to body weight and increase plasma insulin levels thereby maintaining normoglycemia. The progressive retinal degeneration in *tubby* mice results from apoptotic loss of photoreceptor cells, with abnormal electroretinograms detected as early as 3 weeks of age (Heckenlively et al. 1995). The mouse obesity gene *tub* has been identified and characterized (Noben-Trauth et al. 1996; Kleyn et al. 1996).

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NZO Mouse

The New Zealand obese (NZO) mouse was first described in 1953 by Bielschowsky and Bielschowsky. The strain was developed by selective inbreeding of obese mice from a mixed colony, beginning from a pair of agouti mice, which also gave rise to the NZB black strain (Melez et al. 1980). NZO mice of both sexes exhibit high birth weights and are significantly heavier at weaning age. Severe obesity (including both visceral and subcutaneous fat depots) develops even when mice are maintained on a standard diet containing 4.5 % fat. NZO mice develop obesity, mild hyperglycemia, glucose intolerance, hyperinsulinemia, and insulin resistance. The adult NZO mouse normally attains a body weight of 50–70 g by 6–8 months, although weight gain continues slowly after this age (Cofford and Davis 1965; Herberg et al. 1970). Hyperglycemia and glucose intolerance increase continuously with advancing age of the animals.

Renal disease in NZO mice is seen by 6 months of age. NZO mice have a high prevalence of autoimmune disorders.

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Diabetes Obesity Syndrome in CBA/Ca Mice

CBA/Ca mice are commonly used for leukemogenesis research because this strain has a low spontaneous incidence of leukemia but has a relatively high inducibility of myeloid leukemia in response to benzene and radiation exposure. A mild spontaneous maturity-onset diabetes obesity syndrome occurs in a small proportion (10–20 %) of male CBA/Ca mice. Inbreeding can increase the incidence to 80 %. It occurs at 12–16 weeks of age and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except for a slight increase in serum insulin. The male obese-diabetic mice have a normal life expectancy.

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Transgenic Animals

Purpose and Rationale

Transgenic animals offer a new approach to study the development of obesity and therapeutic possibilities.

The potential for inserting new genetic material into mammals has produced numerous transgenic mice with increased or decreased quantities of body fat (Bray and Bouchard 1997).

Reduced body weight is a common effect of gene knockout mice (Reed et al. 2008). During a search for obesity candidate genes in a small region of the mouse genome, it was noticed that many genes when knocked out influence body weight. To determine whether this was a general feature of gene knockout or a chance occurrence, the Jackson Laboratory Mouse Genome Database for knockout mouse strains and their phenotypes was surveyed. Based on a data set of 1977 knockout strains, it was found that 31 % of viable knockout mouse strains weighed less and an additional 3 % weighed more than did controls. Assuming that the surveyed knockout genes are representative, then upward of 6,000 genes are predicted to influence the size of a mouse.

For the characterization of the specificity of a candidate compound to a specific target involved in body weight regulation, the use of respective knockout mouse (in which the specific target is knocked out) vs. wild-type mouse might be helpful: the compounds should only be active in wild-type mice but inactive in the respective knockout mouse model.

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Spontaneously Obese Rats

The occurrence of spontaneous obesity has been reported in several strains of rats:

Zucker-Fatty (ZF) Rat

The Zucker-fatty rat is a classic model of obesity combined with insulin resistance and hyperinsulinemia (Zucker 1965). Obesity is due to a simple autosomal recessive (*fa*) gene

mutation (Fa gene encodes the leptin receptor) and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human prediabetes. However, their blood sugar level is usually normal throughout life (Bray 1977; Clark et al. 1983; McCaleb and Sredy 1992; Abadie et al. 1993; Alamzadeh et al. 1993; Kasim et al. 1993; Galante et al. 1994).

Truett et al. (1991) found evidence that the rat obesity gene fatty (*fa*) has homology with the mouse gene diabetes (*db*). Both genes determine a leptin receptor defect.

Triscari and Sullivan (1987) reported a normalizing effect of an inhibitor of thromboxane synthase on the hyperinsulinemic state of obese Zucker rats and diet-induced obese rats.

Rouru et al. (1993) described the effect of chronic treatment with a 5-HT_1 receptor agonist on food intake, weight gain, plasma insulin, and neuropeptide Y mRNA expression in obese Zucker rats.

Santti et al. (1994) studied the potentiation of the anti-obesity effect of a β_3-adrenoceptor agonist in obese Zucker rats by exercise.

Savontaus et al. (1997) investigated the anti-obesity effect of an imidazoline derivative in genetically obese (*fa*/*fa*) Zucker rats.

Lynch et al. (1992) identified several adipocyte proteins, among them pyruvate decarboxylase contributing to the increased lipogenic capacity of young obese Zucker adipocytes.

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Zucker-Diabetic-Fatty (ZDF) Rat

The obese Zucker-diabetic-fatty (ZDF) rat derived from inbreeding of hyperglycemic Zucker obese rats. Male ZDF rats are obese and insulin resistant and progress spontaneously to overt diabetes (hyperglycemia: around 20 mmol/l blood glucose) at the age of around 8–10 weeks. Female ZDF rats are obese and insulin resistant and remain normoglycemic as long as they are fed with standard rat chow (low fat). On a high-fat diet, female ZDF rats experience the slow progression to overt diabetes similar to their male littermates. This transition to overt diabetes appears due to a progressive loss of pancreatic ß-cells. Body weight development is above that of lean littermates as long as they are young and normoglycemic; this reflects their fa genetic background. When they become overt diabetic, body weight development stops and in later diabetic stages declines due to the energy loss via glucosuria.

The phenotype of ZDF rat is due to (1) the autosomal recessive (*fa*) gene identical to that of ZF rats but (2) with an additional ß-cell gene defect (Griffen et al. 2001).

Zhang et al. (1996) reported downregulation of the expression of the obese gene by an antidiabetic thiazolidinedione in Zucker-diabetic-fatty rats and db/db mice.

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WDF/fa-fa Rat

The WDF/Ta-fa rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty (*fa*) gene from the Zucker rat to the Wistar-Kyoto rat (Ikeda et al. 1981; Kava et al. 1989; Velasquez et al. 1990). The Wistar fatty rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia

similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by pioglitazone in Wistar fatty rats (*fa*/*fa*).

Mazusaki et al. (1996) found an augmented expression of the *obese* (*ob*) gene during the process of obesity in genetically obese-hyperglycemic Wistar fatty (*fa*/*fa*) rats.

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JCR:LA-cp Rat

Several substrains were developed from obese SHR rats, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia, and hyperinsulinemia with impaired glucose tolerance and is susceptible to vascular arteriosclerotic lesions (Russell and Amy 1986a, b; Russell et al. 1994).

Cp mutation encodes a leptin receptor defect, which is different to those defects encoded by *fa* mutation in rats or *db* mutation in mice. Compared to Zucker-fatty rats, the JCR:LA-cp (corpulent) rats have higher levels of the insulin-releasing hormone GIP (glucose-dependent insulinotropic polypeptide = gastric inhibitory polypeptide) and higher insulin levels (Pederson et al. 1991).

Vydelingum et al. (1995) found an overexpression of the obese gene in the JCR:LA-corpulent rat.

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OLETF Rat

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM.

Administration of diazoxide (0.2 % in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4–12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995).

Ishida et al. (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

Umekawa et al. (1997) determined induction of uncoupling protein and activation of GLUT4 in OLETF rats after administration of a specific β_3-adrenoceptor agonist.

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WBN/Kob Rat

Spontaneous hyperglycemia, glucosuria, and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985; Tsichitani et al. 1985; Koizumi et al. 1989). These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16-week-old males. Recent publications on this obese rat strain focuses on spontaneous development of chronic pancreatitis (Ohashi et al. 1990; Mori et al. 2009).

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Obese SHR Rat

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Kyoto-Wistar strain with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets.

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Assays of Anti-Obesity Activity

Andreas W. Herling

Contents

Anorectic Activity

Food Consumption in Mice and Rats

Purpose and Rationale

Food intake is measured in acute experiments in normal or obese rats. Additionally, in subchronic or chronic experiments, body weight gain is recorded.

Procedure

Male or female mice (normal mice, ob/ob, db/db, DIO mice, etc.) or rats (normal rats, ZF, ZDF, DIO rats, etc.) are maintained under standard conditions (temperature, light–dark cycle, ground rodent pellet chow, tap water). Measurements of food intake begin on day −5 to generate stable baseline results. The food is offered in special dishes to reduce spillage. Food intake and body weight are measured daily between 8:00 and 9:00 A.M. At this time, any spilled food from the collecting paper under the cage is gathered, air-dried if necessary, and weighed. Individual food intakes in grams are recorded. The test compounds are either administered with the food or administered orally or parenterally. Groups of 8–10 animals are used for control or treatment with various doses of the test compound or the standard. Acute effect of a test article is studied on day+1. Prolonged treatment for several days (e.g., up to day+7 or even longer) can be performed to evaluate the durability of the anorectic activity.

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For several purposes, it might be appropriate to include an additional pair-fed control group. This group received the amount of food, which was consumed by the treatment group the day before. By applying this method, it is possible to discriminate whether the reduction of body weight in the treatment group is caused exclusively by the reduced food intake or by additional factors, e.g., increased energy expenditure or modified food efficiency.

Evaluation

Average food intake and body weight are recorded for each day. Average values of test drugs are compared statistically for each day with the control group.

Critical Assessment of the Method

As mice and rats are night-active rodents, which took up food predominantly during the dark phase, administration of the test article should appear at the end of the light phase to monitor the anorectic activity during C_{max} of the compound. This can be achieved either by administration in the late afternoon (when the animals are on a normal light–dark cycle: light on at 06:00; light off at 18:00) or to reverse the light–dark-cycle accordingly for a more convenient human working day (e.g., compound administration between 08:00 and 09:00; light off at 10:00; light on at 22:00).

Modifications of the Method

Hull and Maher (1990) and Maher and Hull (1990) used male Sprague Dawley rats placed on a mush diet composed of equal parts of ground rodent chow and of 4 % nutrient agar solution. The agar-based chow allows a more accurate measurement of food intake and has been shown to be sufficient for maintaining normal growth in rats. The rats were made hyperphagic by food deprivation for 4 h at the beginning of the dark cycle.

Mennini et al. (1991) and Anelli et al. (1992) studied the anorectic activity of various compounds in different species, such as mice, rats, and guinea pigs.

Bowden et al. (1988) used metabolism cages equipped with automated feeding monitors. Food was provided as 45 mg pellets which were singly delivered to a feeding trough. A photodetector sensed the removal of the pellet, and the number of pellets delivered over a specified time interval was recorded.

Samanin et al. (1979) described anorexia in rats induced by the central serotonin agonist m-chlorophenylpiperazine.

Blavet et al. (1982) studied food intake in fasted rats after treatment with several typical anorectic agents.

Dourish et al. (1985) investigated the effects of the serotonin agonist 8-OH-DPAT on food intake in non-deprived male rats. This effect was prevented by p-chlorophenylalanine (Dourish et al. 1986).

The anxiolytics gespirone, buspirone, and ipsapirone increased free feeding in rats and did not inhibit feeding induced by 8-OH-DPAT (Gilbert and Dourish 1987).

Jackson et al. (1997) investigated the mechanisms underlying the hypophagic effects of the 5-HT and norepinephrine reuptake inhibitor, sibutramine, in the rat.

Simansky and Vaidya (1990) tested the anorectic action of a serotonin uptake inhibitor by measuring the volume of milk consumed by food-deprived rats.

Stevens and Edwards (1996) induced anorexia by subcutaneous injection of 5 mg/kg 5-hydroxytryptamine in Wistar rats habituated to a restricted feeding regime and tested the effects of a 5-HT_3 antagonist.

Rouru et al. (1992) investigated in genetically obese male Zucker rats the effect of subchronic metformin treatment on food intake, weight gain, and plasma insulin and corticosterone levels and somatostatin concentrations in the pancreas.

Robert et al. (1989) found an enhanced food intake after intracerebroventricular administration of the tetrapeptide FMRF-amide (Phe-Met-Arg-Phe-NH_2) in obese "cafeteria" rats.

Cooper et al. (1990a, b) used non-deprived rats to study anorectic effects in a test of palatable food consumption and in nocturnal free feeding.

Cooper et al. (1990c) tested not only food consumption but also the frequency of feeding bouts and duration of individual feeding episodes.

Eberle-Wang and Simansky (1992) studied the influence on the anorectic action of CCK and serotonin by measuring the uptake of sweetened mash mixture in rats.

Voigt et al. (1995) studied the involvement of the 5-HT_{1A} receptor in CCK-induced satiety by recording food intake during a 2-h test meal in food-deprived and in freely feeding rats.

Influence on postprandial satiety in rats was tested by Rosofsky and Geary (1989). Rats were given pelleted chow and water ad libitum. Near the middle of the bright phase of the light–dark cycle, pellets were removed, the animals treated, and condensed milk presented 30 min later. Milk consumption was measured at 4-min intervals for 40 min.

Rats show a dramatic and reliable reduction of food intake if they are prefed a low-protein basal diet and then offered a diet that is imbalanced in any of the essential amino acids (Leung and Rogers 1969). This anorectic response has been used by Hammer et al. (1990) to test $serotonin_3$ receptor antagonists.

Tail pinch is an effective stimulant of eating in rats (Antelman and Szechtman 1975; Fray et al. 1982). Clark et al. (1992) reported that N-methyl-D-aspartate lesions of the lateral hypothalamus do not reduce amphetamine or fenfluramine anorexia but enhance the acquisition of eating in response to tail pinch in the rat.

Thurlby and Samanin (1981) studied the effect of anorectic drugs on food-rewarded runway behavior.

Ferrari et al. (1992) studied the effects on anorexia induced by ACTH and immobilization in rats in an X-maze with alternate open and covered arms, each baited with laboratory chow.

Cooper et al. (1993) studied dopamine D-1 receptor antagonists in rats with chronic gastric fistula which were trained to sham-feed a 10 % sucrose solution in a 60-min test.

Bickel et al. (2004) analyzed anorectic effects in rodents by measurement of food consumption. Mice or rats were individually placed in Makrolon cages equipped with a device for continuous monitoring of food consumption consisting of a container filled with feed, hanging on an electronic balance. The balance transmits the weight changes of the feed container continuously to a central unit. Data were stored and processed at the end of the experiment. This system, obtained from TSE, Bad Homburg, Germany, can measure food consumption for up to 3 weeks from more than 199 feeding sensors simultaneously. Similar equipment was used by Bauhofer et al. (2002) and Gaetani et al. (2003).

We (Herling et al. 2008a, b) used a two-choice diet. Female Wistar rats were fed a high-fat, high-carbohydrate (candy-) diet for 12 weeks plus standard rat chow ad libitum. During the last 6 weeks, rats were treated with rimonabant. An additional pair-fed group was included. Food intake and body weight development were investigated. By comparing the body weight developments of the treated group with that of the pair-fed group, it became obvious that additional factors than reduced food intake alone must be effective to explain the different body weight reductions as well as body fat distribution (subcutaneous vs. visceral fat) of the two groups. Body fat distribution (total body fat, visceral fat, and ectopic lipid accumulation in the skeletal muscle and liver) was determined by in vivo magnetic resonance imaging/magnetic resonance spectroscopy (2008a). Energy expenditure was determined by indirect calorimetry (2008b).

In wild rodents, **hoarding of food** covers the long-term alimentary need. In the laboratory, hoarding behavior does not occur in ad libitum fed rats. On the contrary, rats whose energy balance is threatened by previous food restriction hoard as soon as experimental conditions allow to do so. When such a rat gets free access to a food stock (placed outside its usual territory), it carries food into its shelter and accumulates an amount proportionate to its body weight. Fantino and Cabanac (1980), Fantino et al. (1986, 1988), and Nishida et al. (1990) used the reduction of the amount of food hoarded during a period of 3 h as parameter for anorectic activity of drugs.

Caccia et al. (1993) studied the anorectic effect of D-fenfluramine in the **marmoset** (*Callithrix jacchus*).

Knoll (1979, 1984) described satietin, an anorectic substance which has been isolated from serum of humans and several animal species.

Satietin has been reported to be a 50,000–70,000 dalton MW glycoprotein, containing 14–15 % amino acids and 70–75 % carbohydrates, surviving digestion with proteases and boiling. Purification of bovine serum satietin was reported by Nagy (1994). These factors suppressed feeding in rats after peripheral and after intracerebroventricular administration.

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Computer-Assisted Measurement of Food Consumption in Mice and Rats

Purpose and Rationale

Techniques have been developed for continuous measurement of food consumption in mice and rats.

Procedure

Animals are placed individually in cages equipped with a device for continuous monitoring of feed consumption. This device is a container filled with feed and hanging on an electronic balance in the cage. The balance transmits the weight changes of the feed container continuously to a central unit. The data are stored and processed at the end of the experiment. This system can measure feed consumption continuously for up to 2 weeks from up to 64 feeding sensors (animals) simultaneously. The feeding monitoring system and data processing hardware and software (Release V3.07) can be obtained from TSE Systems, Bad Homburg, Germany. Software options: the data are presented in the form of a matrix. The matrix delivers cumulatively or sequentially feed consumption in grams (g) for given time intervals for each individual animal. The advantages of this system are that the animals are kept in their home cages, the feed monitoring period is long, and, most of all, the animals are left undisturbed during the experimental session. An additional program for microstructural analysis calculates the parameters for feed consumption over a given time (24 h). Parameters delivered are number of meals (Nm/time), inter-meal breaks (IMB) (min), average meal size (g), and average meal duration (min). In the present rat study, a meal was defined to be $\geq$0.5 g and the IMB $\geq$15 min. Using this system, we have determined the anorectic activity in rats of the centrally acting anorectic agents amphetamine, sibutramine, and dexfenfluramine (Bickel et al. 2000), as well as the peripherally acting anorectic SR146131, a CCK_A-receptor antagonist (Bickel et al. 2002), and HMR1426, an anorectic drug that powerfully inhibits gastric emptying rate (Bickel et al. 2004).

A 2-week treatment with HMR1426 in Zucker fatty rats fed a normal standard rat diet (1 g feed = 12.1 kJ) reduced the body weight increase significantly by 5 % (Gossel et al. 2001). In Zucker fatty rats fed a fat-rich diet (1 g feed = 20.2 kJ), 3 weeks of treatment with HMR1426 caused a significant loss of body weight of 33 % (Bickel et al. 2003).

Hansen et al. (2014) used a MANI Feedwin system connected to an online database system

(ES-LabBASE, Ellegaard System, Faaborg, Denmark) for individual online feeding monitoring of DIO rats.

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Metabolic Activity

Indirect Calorimetry

Purpose and Rationale

Assessment of energy balance is essential for the characterization of anti-obese drugs. Body weight remains constant if energy intake (food intake) equals total energy expenditure (Fig. 1). Energy balance is disturbed in case of body weight gain, disbalance between (increased) energy intake and

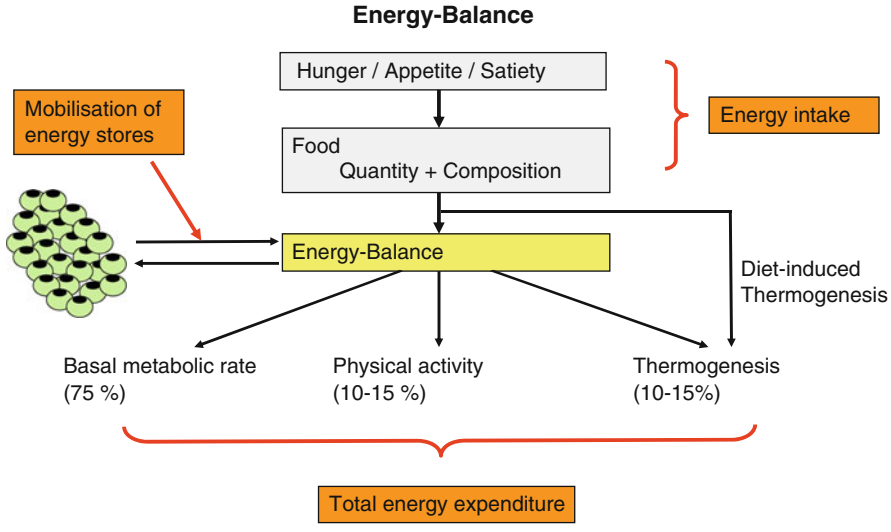

Fig. 1 Factors of energy-balance: energy intake, mobilisation of stored energy, and energy expenditure

(reduced) energy expenditure, as well as of body weight loss, disbalance between (reduced) energy intake and (increased) energy expenditure. Energy expenditure depends on the metabolic rate.

Indirect calorimetry is the method by which metabolic rate is estimated from measurements of oxygen (O_2) consumption and carbon dioxide (CO_2) production. It is the method by which the type and rate of substrate utilization (predominantly fatty acids and carbohydrates) and energy metabolism are estimated in vivo starting from gas exchange measurements (O_2 consumption and CO_2 production). This technique provides unique information, is noninvasive, and can be advantageously combined with other experimental methods to investigate numerous aspects of nutrient assimilation, thermogenesis, the energetics of physical exercise, and the pathogenesis of metabolic diseases.

Procedure

Female yellow KK mice and female C57B1 mice at the age of 12 weeks are housed under controlled temperature and artificial light–dark conditions and are fed with commercial powdered chow and tap water ad libitum. Groups of each strain are treated with doses of test drug or solvent by daily intramuscular injections for 2 weeks. Daily food intake and body weight are measured.

Resting metabolic rate is estimated by means of a closed-circuit metabolic system (Molnar et al. 1986). The system consists of a chamber, circulating pump, desiccant and CO_2-absorbent canisters, solenoid valve, regulated gas source, and modified liquid crystal display calculator. The internal volume of a chamber for a single rat should be 4.5 l. Food cups are placed on the door for convenient access when refilling. Water is supplied in a tray filled intermittently by means of a stopcock in the chamber wall. A shallow stainless steel funnel-shaped tray for urine collection is located in the bottom of the chamber.

Animals are placed in the chamber, and the door is sealed airtight. Chamber air circulates by means of a peristaltic pump at a rate of 2.3 l/min through an external loop containing canisters of desiccant and CO_2 absorbent. In this manner, water vapor pressure remains constant, and the respiration by-product, CO_2, is removed. Consumption of O_2 produces a pressure drop in the sealed system which actuates the pressure sensor for O_2 replacement.

The pressure sensor has two input ports, low and high, producing a simple contact closure whenever the high pressure exceeds the low by ~1 Torr. The low port is connected to the interior of the metabolic chamber by a length of tubing attached to a threaded connector, whereas the high port is open to the atmosphere. Thus, the contact closes whenever the pressure within the chamber falls to more than 1 Torr below atmospheric pressure.

The contact closure is routed to the control unit where a precise pulse of fixed duration is triggered. This pulse activates a solid-state relay through an emitter-follower transistor circuit. The relay output is in series with a solenoid valve and line voltage, so that contact closure results in opening of the valve for a fixed duration. Since the input port of the valve is at a constant pressure, a predictable, highly repeatable volume of gas flows into the chamber. This raises the chamber pressure and opens the pressure sensor contacts. The entire cycle repeats after sufficient metabolism to again decrease the chamber pressure and close the sensor contacts.

After a stable baseline has been achieved after 30 min, drugs are administered, and resting metabolic rate is measured for one hour at an ambient temperature of 22 °C.

Evaluation

Data are presented as means ± SEM. Statistical analysis is performed by Student's *t*-test.

Critical Assessment

Given that obese and "normal" mice basically differ only in the amount of chemical triglycerides they have accumulated, the amount of metabolically active "lean" tissue is generally not different between slim and obese mice. This means that using total body weight as the divisor will result in an erroneous contribution to metabolism from chemically inert triglycerides, and this means that, with actual unchanged metabolism between obese

and slim animals, the slim mice would appear to demonstrate an increased metabolism. This pseudophenomenon has been commented repeatedly over the years (Butler and Kozak 2010; Cannon and Nedergaard 2011; Himms-Hagen 1997; Tschoep et al. 2012). The simplest way to avoid this problem is to express metabolism per total mouse (not dividing by anything).

Modifications of the Method

Poon and Cameron (1978) measured oxygen consumption utilizing a standard Machlett manometer connected to a desiccator that accommodated a single mouse. The desiccator was placed in a 20 °C water bath to ensure constant temperature throughout, and CO_2 was absorbed by means of soda lime UPS. After a 5-min equilibration period, measurements of oxygen consumption were obtained over a 5-min period. The procedure was repeated twice within 1 h for each mouse.

In order to study differential antagonism to amphetamine-induced oxygen consumption and agitation by psychoactive drugs, Niemegeers and Janssen (1979) placed groups of 3 rats with a total weight of 235 ± 5 g consisting of 2 parts, a bottom and a cover, both made up from clear glass. The cover, containing a thermometer, was used to seal the chamber airtight. Inside the chamber, a perforate plate was placed as an animal platform. Containers filled with calcium oxide were placed below and above the animal holder. The chamber was flushed with oxygen for 3 min at an overpressure of 50 units as measured by a manometer connected to one outlet of the chamber. Test drugs were administered subcutaneously 60 min before the animals were placed in the test chambers and 45 min before 2.5 mg/kg amphetamine were injected. Oxygen consumption was measured as decrease of manometer pressure over one hour.

Rothwell (1989) measured central activation of thermogenesis by prostaglandins by resting oxygen consumption in individual closed-circuit calorimeters (Stock 1975) and by registration of colonic temperature in conscious rats.

Jensen et al. (2001) described a self-correcting indirect calorimeter system for the measurement of energy balance in small animals.

Himms-Hagen et al. (1994) tested the effect of a thermogenic β_3-agonist on energy balance in rats. Twenty-four-hour energy expenditure, resting metabolic rate, and thermic effect of food were measured using open-circuit indirect calorimetry. The rat was placed in a respiration chamber (44 × 22 × 18 cm), airflow was measured with a Brooks thermal mass flowmeter (Brooks Instrument Division, Emerson Electric, Hatfield, PA), and the flow of outside air through the chamber was controlled at a variable rate by two adjustable peristaltic pumps that maintained CO_2 concentration at <1 %. Temperature was maintained at 23 ± 1.0 °C. Humidity was measured by an electronic psychrometer, barometric pressure was recorded, and feeding activity was monitored by an infrared detector. The rat was placed into the chamber at 09:30 and removed at 08:30 at the following morning. Food was available to the rat only between 16:30 and 07:30. Total energy expenditure was measured for 23. Resting metabolic rate was determined from the lowest energy expenditure at rest between 11:00 and 16:00. Thermic effect of food was determined from the difference between resting metabolic rate and the lowest energy expenditure at rest between 24:00 and 07:00. For the measurement of minimum metabolic rate, the rat was anesthetized (pentobarbital sodium, 60 mg/kg) and then placed in a small chamber filled with warm circulating water in which it was submerged except for the head. Core temperature was measured with a digital thermometer immediately after induction of anesthesia, and this temperature was maintained (± 0.1 °C) by adjusting the temperature of the circulating water bath. The chamber was sealed and minimum metabolic rate determined with the same equipment as was used for the 24-h energy expenditure. Measurements of minimum metabolic rate were made for 5–15 min between 08:30 and 09:30 after a stabilization period of 5–10 min.

Ghorbani et al. (1997) tested the effect of a β_3-adrenoreceptor agonist on resting metabolic rate in rats by placing the animal in a water-jacketed chamber at 28 °C, volume 1 l, through which air, also at 28 °C, was drawn at a rate of 1 l/min. Oxygen entering and leaving the chamber was measured with an oxygen analyzer (Beckman Industrial Oxygen Analyzer, model 755).

Paulik et al. (1998) described a robust technique to measure thermogenesis of **yeast cells** cultured in microtiter plates using infrared thermography. Thermogenesis increased after exposing yeast to uncoupling protein-2, or troglitazone or β_3-adrenoreceptor agonists.

We (Herling et al. 2008) used a setting consisted of 17 cages, of which 16 cages were used for the individual housing of the animals during the study, and one cage served as a reference cage for corrections of O_2 and CO_2 measurements. All animals (one rat per cage or 2 mice per cage) were accustomed to the special calorimetry cages, equipped with commercial available grid panels, at least 24 h before the start of the measurements. O_2 consumption and CO_2 production were measured every 16 min/cage for 1 min (gas analyzers: Magnos 16 and Uras 14; ABB, Frankfurt/Main, Germany) and recorded by a computer. Values were expressed as the means of liter/d of VCO_2 and VO_2, respectively. For interpretation of substrate use, the respiratory quotient (RQ) was calculated as the quotient of VCO_2/VO_2. Total energy expenditure (TEE) was calculated according to the formula:

$$\begin{aligned}\mathrm{TEE(kJ)} = {} & 16.17\ \mathrm{VO_2(liter)} \\ & + 5.03\ \mathrm{VCO_2(liter)} - 5.98\ \mathrm{N(g)}\end{aligned}$$

where nitrogen loss was set constantly to 0.2 g/day and expressed as TEE per metabolic body mass ($kg^{0.75}$) per day. Fat oxidation and CH oxidation were calculated according to Ferrannini 1988) and modified (Boschmann et al. (1994) by applying the following formulas:

$$\begin{aligned}\text{Fat oxidation (g)} = {} & 1.72\ \mathrm{VO_2(liter)} \\ & - 1.72\ \mathrm{VCO_2(liter)} \\ & - 1.96\ \mathrm{N(g)}\end{aligned}$$

and

$$\begin{aligned}\text{CH oxidation (g)} = {} & 2.97\ \mathrm{VO_2(liter)} \\ & - 4.17\ \mathrm{VCO_2(liter)} \\ & - 2.44\ \mathrm{N(g)}\end{aligned}$$

and expressed as g/day.

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Bomb Calorimetry

Purpose and Rationale

For precise determination of energy intake (not only "food consumption" in g chow per animal), the energy content of the animal feed/chow, which is taken up, as well as the energy loss by the feces (and urine) can be measured by bomb calorimetry. Energy balance can be precisely calculated together with the results of total energy expenditure measured by indirect calorimetry.

Procedure

Combustion of Food and Feces

Food consumption was corrected by segregating the uneaten or scattered food from feces. Samples of chow and feces (~1 g) were dried at 60 °C for up to 7 days, homogenized in a coffee grinder, and squeezed to a pill for determination of energy content in an oxygen bomb calorimeter (model 6300, Parr Instruments, Frankfurt/Main, Germany). For the analysis of solid feces samples, the bomb calorimeter was calibrated using benzoic acid for calorimetrical determination with a guaranteed caloric value of 26.47 kJ/g (Sigma-Aldrich, Taufkirchen, Germany).

Combustion of Urine

For determination of urinary caloric value, samples were usually freeze-dried, pulverized, and combusted in bomb calorimeters to obtain an estimate of excretory energy losses. Because of low urinary volume of mice and rats for lyophilization, we developed a procedure using cotton coils as combustion aid (Heiland Vet GmbH, Hamburg, Germany). Two sizes of coils were used, depending on sample volumes available, from either 8 mm diameter × 19 mm length (for low volumes) or 10 × 19 mm (for high volumes). The caloric value of the combustion aid was 16.49 kJ/g. Results of urinary samples were corrected automatically for the value of the aid later on. For very low concentrated urine, the loading volume must be increased. The higher the concentration or loading, the more reliable the caloric value of the liquid sample. Taking the loaded dry sample weight into account, a linear relationship between applied concentration and caloric outcome could be measured. In addition to the caloric value of glucose (15.45 kJ/g), lactate (16.09 kJ/g), creatinine (21.12 kJ/g), acetoacetate (7.74 kJ/g), β-hydroxybutyric acid (16.08 kJ/g),

urea (10.96 kJ/g), uric acid (11.45 kJ/g), and albumin (22.98 kJ/g) were analyzed, the most common components in urine.

Evaluation

Energy uptake (E_{up}) was determined as the product of food consumed and the caloric value of the food. To obtain metabolized energy (E_{met}), the energy content of feces and urine was subtracted from energy intake. The energy loss, defined as the sum of fecal ($E_{loss\ fec}$) and urinary caloric loss ($E_{loss\ urine}$), was calculated from the feces/urine produced per day and the energy content. The utilization of food energy was calculated as food assimilation efficiency (F_{ass}) indicating the relation of metabolized energy and energy intake.

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GDP-Binding in Brown Adipose Tissue

Purpose and Rationale

Brown adipose tissue is the major site for non-shivering thermogenesis in rodents (Ricquier and Mory 1984; Foster 1986). Drugs activating brown adipose tissue thermogenesis via β-adrenoreceptors cause uncoupling of oxidative phosphorylation from electron transport (Arch et al. 1984; Nicholls et al. 1986). The binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein or thermogenin (Ricquier and Bouillaud 1986), is an established indicator of the thermogenetic activity of brown adipose tissue (Milner et al. 1988).

Procedure

Obese male fatty Zucker rats at the age of 13 weeks weighing about 450 g receive various doses of the test compound in the drinking water or tap water as control for 21 days. Food intake is measured every day and body weight every other day. At the end of the treatment, the rats are sacrificed by decapitation, and interscapular brown adipose tissue is quickly dissected from surrounding tissue.

According to the method published by Nicholls (1976), brown adipose tissue is minced, diluted with 250 mM sucrose, and homogenized with a Potter S homogenizer (B. Braun Melsungen, Germany). The homogenate is centrifuged for 10 min at 8,500 g. The pellet is diluted with 250 mM sucrose and centrifuged at 700 g for 10 min. The supernatant is collected and centrifuged at 8,500 g for 10 min. Bovine serum albumin at 0.2 % is added to wash the suspension of the pellet, which now consists of mitochondria. After centrifugation (8,500 g), the resulting pellet is suspended in albumin-free sucrose buffer. The binding of [3H]-GDP (DuPont NEN, Boston, MA) to mitochondria of single rats is determined by incubating mitochondria in a basic medium containing 100 mM sucrose, 20 mM TES (*N*-tris-[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid), 1 mM EDTA, 10 mM choline chloride, 2 μM rotenone, [^{14}C]sucrose (0.125 μCi ml^{-1}), and 10 μM [^{3}H]-GDP (0.53 Ci mmol^{-1}) at room temperature. Nonspecific binding is assessed in the presence of unlabeled GDP (1 mM). After 10 min of incubation, the reaction is terminated by filtration of the mixture through Thomas A/E glass fiber filters by using a Brandel cell harvester. The radioactivity is measured with the Optiphase "High Safe" II scintillation cocktail and a scintillation counter enabling the samples to be counted for both ^{3}H and ^{14}C. The protein content of the mitochondrial suspensions is assayed according to the method of Peterson (1977).

Evaluation

GDP binding is assessed from ^{3}H radioactivity with a correction for trapped medium using [^{14}C]-sucrose. Two-way analysis of variance (ANOVA) is performed.

Modifications of the Method

Glucose is a minor substrate for isolated brown adipocytes, fuelling thermogenesis by a maximum of 16 % (Isler et al. 1987).

The mechanism of anti-obesity action of a dihydropyridine calcium antagonist was studied by Yoshida et al. (1994b). Obesity was induced by subcutaneous injection of 2 g/kg monosodium-L-glutamate immediately after birth for 5 consecutive days in ICR female mice. Binding of GDP and cytochrome c oxidase activity in brown adipose tissue mitochondria were significantly increased after 4 weeks' drug treatment incorporated in the diet.

Yoshida et al. (1984, 1985) found a reduced norepinephrine turnover in brown adipose tissue of mice with monosodium glutamate-induced obesity.

Kajita et al. (1994), Takahashi et al. (1994) determined regional blood flow in brown adipose tissue by the microsphere method in anesthetized rats.

Yoshida et al. (1996) determined the effect of mazindol on norepinephrine turnover in brown adipose tissue of yellow KK mice and C57B1 control mice.

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Uncoupling Protein and GLUT4 in Brown Adipose Tissue

Purpose and Rationale

Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane transporters which dissipate the proton gradient, releasing stored energy as heat. UCP1 is expressed exclusively in brown adipocytes, UCP2 is expressed widely, while UCP3 is found in the skeletal muscle and brown adipose tissue (Viadal-Puig et al. 1997; Fleury et al. 1997; Masaki et al. 1997; Matsuda et al. 1997; Boss et al. 1997; Gong et al. 1997; Larkin et al. 1997). They are upregulated by thyroid hormones (Larkin et al. 1997; Masaki et al. 1997; Branco et al. 1999; Lanni et al. 1999). Mao et al. (1999) identified and characterized a novel member of the human uncoupling protein family, termed uncoupling protein-4 (UCP4).

In addition to the binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein itself and the glucose transporter 4 (GLUT4) were determined by RNA (Northern blot) analysis and by protein (Western blot) analysis as indicators of the thermogenetic activity of brown adipose tissue.

Procedure

Male fatty (OLETF fatty) rats at the age of 10 weeks are given subcutaneous injection of test compound or solvent once daily. After 14 weeks treatment, the rats are sacrificed and brown and white adipose tissue samples rapidly removed.

Northern Blot Analysis

Total RNA is extracted from 0.1–1 g of tissue using TRIzol (Gibco) and the concentration determined from the absorbance at 260 nm. Total RNA (20 mg) is separated on a 1.5 % agarose/formaldehyde gel and transferred to and fixed on a nylon membrane. A 488 bp uncoupling protein cDNA probe corresponding to the coding region of rat uncoupling protein is prepared by digesting the whole uncoupling protein cDNA with BamHI. The uncoupling protein probe and GLUT4

cDNA are labeled with α-[^{32}P]dCTP (deoxycytidine triphosphate). The blots are hybridized to the labeled probes at 42 °C for 20 h in the presence of 500 μg/ml salmon sperm DNA and exposed to an X-ray film for autoradiography and an imaging plate of BAS1000 (Fuji Film) for quantitative analysis.

Western Blot Analysis

Each tissue is homogenized in 5–10 volumes of a solution containing 10 mmol/l Tris–HCl and 1 mmol/l EDTA (pH 7.4) for 30 s with a Polytron. After centrifugation at 1,500 *g* for 5 min, the fat cake is discarded, and the infranatant (fat-free extract) is used for protein determination according to Lowry et al. (1951) and cytochrome c oxidase activity (Yonetani and Ray 1965). Uncoupling protein and GLUT4 protein in the fat-free extract is measured by Western blot analysis. The fat-free extracts (10 μg protein of brown adipose tissue, 20 μg of white adipose tissue) are solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. After blocking with 5 % non-fat dry milk, the filter is incubated with rabbit antiserum against rat uncoupling protein or GLUT4. The rabbit antisera against rat uncoupling protein and GLUT4 are prepared by immunizing purified rat uncoupling protein (Lin and Klingenberg 1980) and a 12-amino-acid peptide corresponding to GLUT4 (residues 498–509, TELEYLGPDEND) (Shimizu et al. 1993), respectively, coupled with keyhole limpet hemocyanin. Then, the filter is incubated with [^{125}I]protein A (ICN). The dry blot is exposed to an X-ray film for autoradiography and an imaging plate of BAS1000 (Fuji Film) for quantitative analysis.

Evaluation

Data are expressed as means ± SEM and are analyzed by two-way ANOVA followed by Bonferroni *t*-test.

Modifications of the Method

A radioimmunoassay to measure uncoupling protein was used by Milner et al. (1988).

Scarpace et al. (2000) performed unilateral surgical denervation of the interscapular BAT in rats under pentobarbital anesthesia according to Bartness et al. (1986). A transverse incision was made just anterior to the BAT, separating the BAT from the muscles of the scapulae. The BAT was raised to expose the five intercostal nerve bundles entering each pad. On one side, a section of each nerve bundle was removed with scissors. Denervation can be verified by assessing norepinephrine levels in the innervated compared with the denervated BAT pads (Scarpace and Matheny 1996).

Puigserver et al. (1996) studied the effects of retinoic acid isomers on the appearance of uncoupling protein in primary cultures of brown adipocytes, in the brown adipocyte cell line HIB 1B, and directly in intact mice.

Shimabukuro et al. (1997) reported the induction of uncoupling protein-2 mRNA in the pancreatic islets of Zucker diabetic fatty rats by troglitazone.

Foellmi-Adams et al. (1996) found a synergy between norepinephrine and pioglitazone in induction of uncoupling protein in mice.

Kotz et al. (2000) determined uncoupling protein 1 (UCP1) in brown adipose tissue, UCP2 in white adipose tissue, and UCP3 in the muscle of male Sprague Dawley rats after injection of neuropeptide Y into the hypothalamic paraventricular nucleus.

Great efforts have been devoted to study the influence of **β_3-adrenergic agonists** on formation of uncoupling protein.

Umekawa et al. (1997) found an induction of uncoupling protein and activation of GLUT4 in white fat of Otsuka Long-Evans Tokushima fatty rats after treatment with a specific β_3-adrenoceptor agonist.

Ghorbani and Himms-Hagen (1997) found appearance of abundant densely-stained brown adipocytes expressing uncoupling protein in white adipose tissue during reversal of obesity and diabetes in Zucker fa/fa rats induced by the β_3-adrenoceptor agonist CL316,243.

Nagase et al. (1996) found in yellow obese KK mice after treatment with a β_3-adrenergic agonist a significant reduction of body weight, associated with a marked decrease of white fat pad weight and hypertrophy of the interscapular brown adipose tissue with a sixfold increase in mitochondrial uncoupling protein content.

Liu and Stock (1995), Liu et al. (1996), and Stock (1997) reported an increase of glucose utilization in rat brown adipose tissue after treatment with a β_3-adrenoreceptor agonist or with sibutramine, a serotonin and norepinephrine reuptake inhibitor.

Savontaus et al. (1998) reported an increase of UCP3 and UCP1 in brown adipose tissue of obese fa/fa Zucker rats after chronic administration of a β_3-adrenoreceptor agonist.

The effects of β_3-adrenoceptor agonists on uncoupling protein-1 and leptin in culture-differentiated rat brown fat cells are antagonized by a β_3-adrenoreceptor antagonist (Tonello et al. 1998).

Berraondo et al. (2000) found an upregulation of muscle UCP2 gene expression by a β_3-adrenoreceptor agonist in obese rodents, but downregulation in lean animals.

Paulik and Lenhard (1997) found an increased expression of uncoupling protein in C3H10T1/2 cells, a pluripotent stem cell line, after addition of **thiazolidinediones**.

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Uncoupling Protein and GLUT4 in Brown Adipose Tissue

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Assays of Obesity-Regulating Peptide Hormones

Andreas W. Herling

Contents

Hormonal Regulation of Food Intake

Food intake and fat deposition are regulated by peptide neurotransmitters, most of them located in the brain, particularly in the hypothalamus (Elmquist et al. 1999; Kalra et al. 1999) and in the gut. This includes peptides that are **orexigenic** (appetite stimulating) and **anorectic**. A review was given on investigational anti-obesity agents and obesity therapeutic treatment targets by Bays (2004).

Neuropeptide Y (NPY), orexins A and B, galanin, melanin-concentrating hormone (MCH), and **agouti-related peptide (AgRP)** all act to stimulate feeding, while **alpha-melanocyte-stimulating hormone (αMSH)** (see chapter "▶ Effects on Different Peptide Hormones"), **corticotropin-releasing hormone (CRH)** (see chapter "▶ Hypothalamic Hormones"), **cholecystokinin (CCK)** (see chapter "▶ Pancreatic Function"), **cocaine- and amphetamine-regulated transcript (CART), neurotensin, glucagon-like peptide 1 (GLP1)** (see chapter "▶ Measurement of Insulin and Other Glucose-Regulating Peptide Hormones"), **calcitonin gene-related peptide (CGRP)** (see chapter "▶ Effects on Different Peptide Hormones"), **bombesin** (see chapter "▶ Pharmacological Effects on Gastric Function"), and **ciliary neurotropic factor** (Xu et al. 1998) have anorectic actions (Tritos and Maratos-Flier 1999).

Mutations reducing the functional activity of leptin, the leptin receptor, α-MSH, and the

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melanocortin-4 receptors lead to obesity in animals. *Mc4r*-deficient ($Mc4r^{-/-}$) mice do not respond to the anorectic actions of MTII, an MSH-like antagonist, suggesting that α-MSH inhibits feeding primarily by activating Mc4r (Marsh et al. 1999).

Leptin (see section "Leptin") is a 167-amino-acid protein that is synthesized and secreted primarily by white adipose tissue, circulates in the blood, and acts on receptors in the hypothalamus to decrease food intake and increase energy expenditure (Friedman and Halaas 1998; Trayhurn et al. 1999). Leptin and its derivatives are candidates for treatment of obesity; however, clinical studies (Considine et al. 1996) showed that most of obese humans have higher plasma levels of leptin than nonobese individuals, suggesting that obesity is associated with leptin resistance rather than leptin deficiency.

Neuropeptide Y (NPY) (see section "Neuropeptide Y") is a 36-amino-acid peptide that is widely distributed throughout both the central and peripheral nervous systems and which plays a key role in the control of body weight. Central administration of NPY increases food intake (Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). NPY antagonists are candidates for anti-obesity drugs.

Orexin A and orexin B (see section "Orexin") are 33- and 28-residue peptides, also called hypocrites, which were originally isolated from rat hypothalamus (Sakurai et al. 1998). These peptides are located predominantly in the hypothalamus and locus coeruleus but are also found elsewhere in the brain. The orexins have a broad range of physiological functions, including the control of feeding and energy metabolism. Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats (Jain et al. 2000). Orexin antagonists are potential anti-obesity drugs (Parker 1999).

Galanin (see section "Galanin") is 29-amino-acid C-terminally amidated peptide (30 amino acids in humans) which is localized mainly in the mammalian CNS but also in other organs. Central administration of galanin increases and administration of galanin receptor antagonists (Crawley et al. 1990; Leibowitz and Kim 1992) decreases food intake. These data suggest the use of galanin receptor antagonists as anti-obesity agents.

Agouti-related protein affects pigmentation when its expression is limited to the skin, but ubiquitous expression causes obesity (Ollmann et al. 1997). The hypothalamic expression of agouti-related protein is regulated by leptin, and overexpression of agouti-related protein results in obesity and diabetes (Rosenfeld et al. 1998). Recombinant agouti-related protein is a potent, selective antagonist at MC3R and MC4R, melanocortin receptor subtypes implicated in weight regulation (Fong et al. 1997; Shutter et al. 1997; Tota et al. 1999). Ubiquitous expression of human agouti-related protein complementary DNA in transgenic mice caused obesity without altering pigmentation. Agouti-related protein is a neuropeptide implicated in the normal control of body weight downstream of leptin signaling. Ollmann et al. (1998) used a sensitive bioassay based on *Xenopus* melanophores to characterize pharmacological properties of recombinant agouti protein.

Melanin-concentrating hormone (MCH) is a cyclic 19-amino-acid neuropeptide that was originally found to regulate pigmentation in fish. It plays a role in the central feeding behavior increasing food consumption (Qu et al. 1996; Rossi et al. 1999). Mice carrying a targeted deletion of the MCH gene are hypophagic and lean (Shimada et al. 1998). Among other factors, MCH may be a target of leptin signaling in the hypothalamus (Sahu 1998; Huang et al. 1999). Melanin-concentrating hormone is a functional melanocortin antagonist in the hypothalamus (Ludwig et al. 1998). MCH has been identified as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1) (Bacher et al. 1999; Chambers et al. 1999; Shimomura et al. 1999) which has been characterized by Saito et al. (1999). High binding capacity for MCH was found in human keratinocytes (Burgaud et al. 1997). Radioligands for the mammalian MCH receptor were described by Hintermann et al. (1999).

Cocaine- and amphetamine-regulated transcript (CART), a brain-located peptide, has

potent appetite-suppressing activity and is closely associated with the actions of leptin and neuropeptide Y (Lambert et al. 1998; Kuhar and Dall-Vechia 1999). When injected intracerebroventricularly into rats, recombinant CART peptide inhibits both normal and starvation-induced feeding and completely blocks the feeding response induced by neuropeptide Y (Kristensen et al. 1998). In the rat the CART gene encodes a peptide of either 129 or 116 amino acids whereas only the short form exists in humans (Thim et al. 1998a, b). A role of CART peptides in substance abuse and addiction is suggested by psychomotor-stimulant regulation of CART transcription in the striatum, as well as its localization within neural circuits that mediate reward and reinforcing behaviors (Couceyro and Lambert 1999). CART has been found not only in the hypothalamus and other brain areas (Broberger 1999; Koylu et al. 1998, 1999) but also in other organs, such as the pancreas (Jensen et al. 1999), in the rat sympathoadrenal axis (Dun et al. 2000), or in the vagal afferent neurons sensitive to cholecystokinin (Broberger et al. 1999). CART can cross the blood–brain barrier (Kastin and Akerström 1999). CART receptor agonists are potential anti-obesity drugs (Couceyro and Lambert 1999).

Resistin

Steppan et al. (2001a), Berger (2001), Steppan and Lazar (2002, 2004), and Banerjee and Lazar (2004) showed that adipocytes secrete a signaling molecule with 114 amino acids in length which is called **resistin** (for resistance to insulin) and which links obesity to diabetes (Haluzik and Haluzikova 2006). Circulating resistin levels in mice are increased in diet-induced and genetic forms of obesity and are decreased by thiazolidinediones (see section "K.5.0.6"). Central administration of resistin promotes short-term satiety in rats (Tovar et al. 2005). Administration of anti-resistin antibody improves blood sugar and insulin action in mice with diet-induced obesity. Treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action. Insulin-stimulated glucose uptake by adipocytes is enhanced by neutralization of resistin and is reduced by resistin treatment. Resistin with slightly modified amino acid sequence has also been found in humans.

Steppan et al. (2001b) described a family of tissue-specific **resistin-like molecules (RELMs)**. RELMα is a secreted protein that has a restricted tissue distribution with highest levels in adipose tissue. Another family member, RELMβ, is a secreted protein expressed only in the gastrointestinal tract, particularly in the colon, in both mouse and human. RELMβ gene expression is highest in proliferative epithelial cells and is markedly increased in tumors, suggesting a role in intestinal proliferation.

Banerjee et al. (2004) generated mice deficient in resistin by replacing the coding exons of the resistin gene(*rstn*) with the reporter gene *lacZ*. The mice exhibited low blood glucose levels after fasting due to reduced hepatic glucose production indicating a physiological function of resistin in maintenance of blood glucose during fasting. The lack of resistin diminished the increase in post-fast glucose associated with increased weight, suggesting a role for resistin in mediating hyperglycemia associated with obesity.

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Leptin

General Considerations on the Obese Gene Product Leptin

The obese gene product leptin is a cytokine-like non-glycosylated peptide of 146 amino acids that is secreted from adipose tissue. It is an important circulating hormone for the regulation of body weight (Arch and Beeley 1996; Hamann and Matthaei 1996; Friedman and Halaas 1998; Trayhurn et al. 1999). MacDougald et al. (1995) described the expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes.

The mouse obese gene and its human homologue which show approximately 85 % amino acid homology have been cloned by Zhang et al. (1994). Leptin binding was first reported in rat hypothalamic membranes (Stephens et al. 1995).

A **leptin receptor** was subsequently identified by expression cloning from a mouse choroid plexus cDNA library (Tartaglia et al. 1995). Several transcripts of the leptin receptor as a result of alternative splicing which have different lengths of cytoplasmatic region have been identified in mice and rats (Guan et al. 1997; Igel et al. 1997;

Murakami et al. 1997) as well as leptin receptors in man (Maffei et al. 1995, 1996; Liu et al. 1997; Strosberg and Issad 1999). There are at least five different isoforms of the leptin receptor in mice. Mutations in the leptin receptor lead to massive obesity in *db* mice and *fa* rats (Friedman and Halaas 1998).

Administration of recombinant human leptin to mice increases thermogenesis and lipid oxidation in brown fat coupled with increased lipolysis and decreased fat synthesis in white and brown fat, which lead to a rapid reduction in the body weight (Sarmiento et al. 1997).

Leptin increases uncoupling protein expression in brown adipose tissue and oxygen consumption in mice (Scarpace et al. 1997).

Intracerebroventricular injection of recombinant murine leptin produces a dose-dependent reduction of laboratory diet in normal rats. This effect is attenuated in rats with diet-induced obesity (Widdowson et al. 1997).

The role of leptin in human obesity is controversial. Rare forms of human obesity have been identified where mutations in leptin or its receptor play a major role in the development of the disease. Leptin may be a signal for the onset of puberty in humans (Strosberg and Issad 1999). Leptin activates leptin-receptor-expressing neurons in the arcuate region of the hypothalamus. Projections from these neurons stimulate melanocortin MC_4 receptors and neuropeptide Y-containing neurons to activate the sympathetic nervous system which controls lipolysis in white adipose tissue. The firing of gonadotropin-releasing hormone-containing neurons and secretion of gonadotropin-releasing hormone to the pituitary are elicited by leptin-mediated activation of leptin-receptor-expressing neurons and by other factors that control the reproductive axis (Chebab 2000).

Grasso et al. (1997, 1999) found inhibitory effects of leptin-related synthetic peptides on food intake and body weight gain after intraperitoneal administration in female C57BL/6J *ob/ob* mice which may have potential application to the treatment of human obesity.

Ducy et al. (2000) and Takeda et al. (2002) suggest that leptin inhibits bone formation, probably via the sympathetic nervous system.

Dumond et al. (2003) showed in rats that leptin strongly stimulated anabolic functions of chondrocytes and induced the synthesis of IGF-1 and TGFβ 1 in cartilage at both the messenger RNA and protein levels. A key role of leptin in osteoarthritis was assumed.

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Determination of Leptin mRNA Level in Adipose Tissue

Purpose and Rationale

Leptin mRNA levels in adipose tissue can be determined by Northern blot analysis (Frederich et al. 1995a, b; Harris et al. 1996; Zachwieja et al. 1997; Kochan et al. 1999).

Procedure

Male Wistar rats weighing approximately 230 g treated for 14 days with test drug are sacrificed by decapitation. Liver, epididymal white adipose

tissue, and intracapsular brown adipose tissue are rapidly removed and frozen in liquid nitrogen. Total RNA is extracted from frozen tissues by a guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi 1987). The RNA (10 µg per lane) is fractionated by horizontal gel electrophoresis. The RNA is transferred to a positively charged nylon membrane and fixed with UV light. Pre-hybridization is performed at 42 °C for 45 min in pre-hybridization solution containing 7 % sodium dodecyl sulfate, 50 % formamide, 5× saline–sodium citrate buffer, 2 % blocking reagent (Boehringer Mannheim, Mannheim, Germany), 50 mM sodium phosphate (pH 7.0), and 0.1 % *N*-laurylsarcosine. Hybridization is performed at 42 °C overnight in pre-hybridization solution containing oligonucleotide probe (25 ng/ml) specific to leptin, malic enzyme, or 185 RNA. The following post-hybridization washes are performed: twice for 5 min in 2× saline–sodium citrate buffer/0.1 % sodium dodecyl sulfate (at room temperature) and twice for 15 min in 0.1× saline–sodium citrate buffer/0.1 % sodium dodecyl sulfate (at 48 °C). The membranes are then rinsed briefly with washing buffer containing 0.1 M maleic acid (pH 7.5), 0.15 M NaCl, and 0.3 % Tween 20. They are blocked by incubation for 30 min at room temperature with blocking buffer (1 % blocking agent, 0.1 M maleic acid (pH 7.5), 0.15 M NaCl) and incubated (at the same conditions as described above) with blocking buffer containing a polyclonal antibody against digoxigenin conjugated to alkaline phosphatase. After washing twice for 15 min with washing buffer, the membranes are rinsed for 5 min with detection buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl) and immersed for 5 min in CDP-Star solution (Boehringer Mannheim, Mannheim, Germany). Membranes are exposed to Kodak XAR film for 15 min to 1 h (Karbowska et al. 1999).

Evaluation

Signals are scanned and quantified using the NIH-Image software. The level of mRNA for leptin and malic enzyme as well as for 185 RNA is estimated using PeakFit software (Jandel Scientific). The values for leptin and malic enzyme mRNA are normalized to the corresponding amount of 185 RNA. Results expressed in arbitrary units are presented as means ± SEM of samples of 10 rats. The statistical difference between groups is assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or by Mann–Whitney test.

Modifications of the Method

Richards et al. (2000) described quantitative analysis of leptin mRNA using competitive reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection.

Shintani et al. (2000) determined leptin mRNA levels in primary cultured rat adipocytes.

Li et al. (1999) used an anti-leptin polyclonal antiserum to detect leptin immunoreactivity in the central nervous system in rats.

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Determination of Plasma Leptin

Purpose and Rationale

Plasma leptin levels are determined by radioimmunoassay using a rat-specific antibody (Ciba-Geigy, Basel, Switzerland) (Zachwieja et al. 1997) or with a double-antibody radioimmunoassay kit based on a mouse standard (Linco Research, St. Louis, MO) (Surwit et al. 1997; Van Heek et al. 1997). A radioimmunoassay for leptin in human plasma was described by McGregor et al. (1996).

Procedure

The peptide VPIQKVQDDTKTLIKTIVT representing the first 20 amino acids of the predicted sequence of mature leptin protein (leptins 1–20) was synthesized with 9-fluorenyl-methyloxycarbonyl-(Fmoc)-protected L-amino acids on an Applied Biosystems peptide synthesizer and purified by HPLC on a Dynamax column developed with a 0.1 % trifluoroacetic acid (TFA)/H_2O/acetonitrile (8–40 %) gradient. The peptide is conjugated to thyroglobulin using carbodiimide as coupling reagent. Rabbits are immunized by intradermal injections and boosted at 4-week intervals.

A peptide identical to the one used for antibody generation except for a tyrosine residue added to the C-terminal end is labeled with ^{125}I using the oxidizing agent Iodogen (Pierce, USA) and purified on HPLC. For radioimmunoassay, the buffer consists of Tris–HCl 1 M, pH 7.4; 0.1 % gelatin; 0.1 % Triton X-100; and 0.01 % NaN_3. Unlabeled ob peptide is used for standards. 100 µl of standard or unknown, 200 µl buffer, 100 µl antibody at a final concentration of 1:80,000, and 100 µl ^{125}I-leptin peptide (approximately 5,000 cpm) are added to polystyrene tubes and incubated for 48 h at 4 °C. Antibody-bound and unbound peptide are separated by addition of goat anti-rabbit immunoglobulin antibody followed by centrifugation. The precipitates are counted on a computer-linked gamma-counter, and the leptin concentrations of the samples are obtained using the "RIA-Calc" program.

Evaluation

Results are expressed as means ± SD, and statistical comparisons are made with the use of Student's *t*-test. Linear regression analysis is performed with the method of least squares.

Modifications of the Method

Maffei et al. (1995) used an immunoprecipitation assay to measure plasma leptin levels. A quantitative increase of the signal intensity was seen on Western blots when increasing amounts of recombinant protein were added to *ob* mouse serum, which does not have leptin. Densitometry was used to compare the signal intensity of the native protein in mouse plasma to the standard. The level of leptin-like immunoreactivity was quantified as nanograms per ml.

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Neuropeptide Y

General Considerations on Neuropeptide Y and Related Peptides

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) are endogenous 36-amino-acid peptides belonging to the same family, which is characterized by a tertiary structure termed "PP-fold" (Larhammar 1996; Berglund et al. 2003). They have different tissue distributions (Sundler et al. 1993) and act in different physiological systems, mostly neuronal for NPY and endocrine for PYY and PP (Wettstein et al. 1995; Playford and Cox 1996; Goumain et al. 1998).

Neuropeptide Y plays a key role in the control of body weight (Leibowitz 1994; Kalra and Kalra 2004). Central administration of neuropeptide Y increases food intake and decreases thermogenesis in satiated animals (Billington et al. 1991; Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). Hypothalamic neuropeptide Y and mRNA levels are increased after fasting and in genetically obese mice (Stephens et al. 1995). Neuropeptide Y is required for the maintenance of the obese phenotype of the leptin-deficient *ob/ob* mice (Erickson et al. 1996). Conversely, leptin appears to decrease food intake and body weight in part by decreasing neuropeptide Y synthesis and release (Stephens et al. 1995). These data suggest that neuropeptide Y is a key modulator of body weight and that neuropeptide Y receptor antagonists may be useful anti-obesity agents.

Neuropeptide Y mediates its physiological effects via interaction with at least six distinct G-protein-coupled receptors, designated Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6 (Marsh et al. 1998; Michel et al. 1998; Wyss et al. 1998; Iyengar et al. 1999; Larhammar and Salaneck 2004; Lin et al. 2004).

Central administration of peptide analogues of neuropeptide Y has been used to study the physiological roles of neuropeptide Y, especially the role of the peptide in feeding behavior. Kanatani et al. (1999) demonstrated an anorexogenic effect against NPY-induced feeding in rats by a Y_1 antagonist with high selectivity and potency for the Y_1 receptor. Central administration of peptides that activate the Y_5 receptor, peptide YY (PYY), PYY(3–36), [Leu^{31},Pro^{34}]NPY, and human pancreatic polypeptide elicit feeding, whereas central administration of central administration of peptides that are inactive at the Y_5 receptor do not elicit feeding (Gerald et al. 1996). Fuhlendorff et al. (1990) described [Leu^{31}, Pro^{34}]neuropeptide Y as a specific Y_1 receptor agonist. While other neuropeptide Y analogues or fragments interacts with several neuropeptide Y receptors, [D-Trp^{32}] NPY is a completely selective Y_5 agonist that elicits feeding at relatively high doses (Gerald et al. 1996). Peptides with activity on the Y_1 receptor produce conflicting effects on feeding. DesAA$^{10–17}$[$Cys^{7–21}$, Pro^{34}]NPY is a Y_1 receptor agonist with a potency equivalent to neuropeptide Y, but does not elicit feeding after central administration (Kirby et al. 1995).

Schaffhauser et al. (1997) described inhibition of food intake after intracerebroventricular injection of neuropeptide Y Y_5 receptor antisense oligodeoxynucleotides in rats.

Dumont et al. (2004) recommended receptor autoradiography as means to explore the possible functional relevance of neuropeptides such as neuropeptide Y.

Criscione et al. (1998) reported inhibition of food intake in rats by a Y_5 receptor antagonist under various conditions. Several neuropeptide Y antagonists were synthesized which inhibit food intake in rodents and may be used as anorectic agents (Matthews et al. 1997; Kask et al. 1998; Parker et al. 1998; Shigeri et al. 1998; Wieland et al. 1998; Zarrinmayeh et al. 1998).

Besides the stimulation of feeding, NPY and PYY show several other activities: influence on gastric motility (Chen et al. 1997), antisecretory effects in the gastrointestinal tract (Souli et al. 1997), anti-stress activity (Heilig 2004), anti-epileptic activity (Baraban 2004; Vezzani and Sperk 2004), central and peripheral control of the cardiovascular system (Lew et al. 1996; Tadepalli et al. 1996; Hudspith and Munglani 1997; McCloskey et al. 1997), effects on renal function (Blaze et al. 1997; Bischoff et al. 1997), antinociceptive effects (Broqua et al. 1996), or release of ACTH (Small et al. 1997). Kask et al. (2001) described the effects of a neuropeptide Y Y_5 receptor antagonist on food intake and anxiety-related behavior in the rat.

Sajdyk et al. (1999) found that amygdalar neuropeptide Y Y_1 receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test, whereas neuropeptide Y-Y_2 receptors mediate anxiety in the amygdala (Sajdyk et al. 2002).

Neuropeptide Y plays a role in neurobiological responses to ethanol and drugs of abuse (Thiele et al. 2004).

NPY receptor agonists show anxiolytic effects in rat conflict tests (Britton et al. 1997; Kask et al. 2002); neuropeptide Y_1 antagonists have anxiogenic-like effects (Kask et al. 1996).

Goumain et al. (1998) generated a rat Y_5 clone by reverse transcription from rat brain, polymerase chain reaction, and transfection to COS-7 cells. Isolated jejunal crypt and villus cells and colon epithelial cells were analyzed for the Y_5 receptor by reverse transcription and polymerase chain reaction. Inhibition of binding of [^{125}I] peptide YY in these cells by various peptides was studied.

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Receptor Assay of Neuropeptide Y

Purpose and Rationale

At least six distinct G-protein-coupled receptors of neuropeptide Y have been identified, designated Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6 (Doods and Krause 1991; Marsh et al. 1998; Michel et al. 1998; Bischoff and Michel 1999).

Sheikh et al. (1989) described a receptor binding assay for neuropeptide Y.

Procedure

^{125}I-Labeling

^{125}I-labeled NPY is prepared using carrier-free $Na^{125}I$ and 1,3,4,6-tetrachloro-3α,α6-diphenylglycouril (Serva Heidelberg, Germany) as oxidizing agent. Synthetic porcine NPY (7 nmol) in 40 µl of sodium phosphate buffer (pH 7.4, 0.1 M) and 1 mCi of $Na^{125}I$ in 10 µl of dilute NaOH are added to a reaction vial which has been coated with 20 µg of chloroglycouril by evaporation of 20 µl of a solution of glycoluril in methylene chloride. Following incubation in ice for 5 min, 50 µl of 35 % acetonitrile is added prior to HPLC.

Purification by Reverse Phase-High Performance Liquid Chromatography

A prepacked Nucleosil 300-5 C_{18} cartridge is used. The chromatography is performed at 50 °C with a flow rate of 1 ml 35 % acetonitrile/min. Fractions of 0.5 ml are collected into tubes containing 0.1 ml of acetic acid (0.5 M) containing 10 mg/l bovine serum albumin. Aliquots of 10 µl are counted in a γ-counter, and fractions containing the radiolabeled peptide are pooled and stored at −20 °C.

Preparation of Synaptosomal Membranes

Synaptosomal membranes are prepared from the hippocampus of Danish LYY-strain landrace/Yorkshire pigs. The tissue is homogenized in 0.32 M sucrose in a Sorvall Omni-Mixer at 0 °C for 5 min, followed by centrifugation (1,000 g, 10 min). The supernatant is removed and centrifuged (20,000 g, 30 min) to form the crude mitochondrial fraction. After resuspension in 3 ml of sucrose, this fraction is subfractionated on a discontinuous density gradient consisting of 0.8 M and 1.2 M sucrose (3 ml of each) in an SW 27 rotor and a Beckman ultracentrifuge (100,000 g, 90 min). The synaptosomes which concentrate at the interface between the two sucrose concentrations are collected and

recentrifuged (50,000 g, 10 min). The resulting pellet is washed with HEPES buffer (pH 7.4, 25 mM) containing $CaCl_2$ (2.5 mM) and $MgCl_2$ (1 mM), centrifuged (50,000 g, 10 min), and resuspended in HEPES buffer. The protein concentration is determined using the Bio-Rad protein assay with bovine serum albumin as standard. The membrane preparation is diluted in binding buffer to a protein concentration of 2 g/l and stored at −80 °C.

Receptor Binding Assay

The binding buffer is a HEPES buffer (pH 7.4, 25 mM) containing $CaCl_2$ (2.5 mM) and $MgCl_2$ (1 mM), bovine serum albumin (10 g/l), and bacitracin (0.5 g/l). The incubation mixture consists of 0.5 ml of membrane suspension, diluted with binding buffer to a protein concentration of 200 mg/l, 0.05 ml of unlabeled peptide, and 0.5 ml of ^{125}I-labeled peptide (40,000 cpm). After 1 h of incubation, triplicate aliquots of the incubation medium are transferred to polypropylene tubes (0.4 × 4.5 cm), containing 200 µl of ice-cold buffer. Membrane-bound, radiolabeled peptide is separated from the free peptide by centrifugation (7,500 g, 2 min) in a Beckman Microfuge B. The supernatant is aspirated, and the tube and pellet are gently washed with buffer (0.5 ml). The tip of the tube is the cut off and counted in the γ-counter.

Evaluation

The specific binding is calculated as the difference between the amount of ^{125}I-labeled peptide bound in the absence (total binding) and presence (nonspecific binding) if 10 µM unlabeled peptide.

The concentration dependence of the receptor binding is determined by incubating the membranes with increasing concentrations of radiolabeled peptide in the absence or presence of unlabeled peptide.

Modifications of the Method

Rose et al. (1995) reported cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor.

Gehlert et al. (1996) recommended [^{125}I][Leu^{31}, Pro^{34}]peptide YY as selective radioligand for the neuropeptide Y Y_1 receptor and for human pancreatic polypeptide 1 receptors.

Robin-Jagerschmidt et al. (1998) investigated the ligand binding site of neuropeptide Y at the rat Y_1 receptor by construction of mutant receptors and [^{3}H]NPY binding studies.

Dumont et al. (1998) investigated the respective distribution of neuropeptide Y Y_1, Y_2, Y_4, and Y_5 receptor subtypes in rodents (rat and mouse), guinea pig, and primates (marmoset and vervet monkey and human) brains, representing three orders of mammals.

Primus et al. (1998) measured guanyl 5′ (γ[^{35}S]-thio)-triphosphate binding to NPY-receptor-activated G-proteins in adult rat brain sections in order to determine the neuroanatomical distribution of NPY receptor subtypes.

Wyss et al. (1998) administered various doses of synthetic neuropeptide Y agonists intracerebroventricularly to rats in order to establish dose–response curves and to estimate ED_{50} values of feeding. These values were compared with binding affinities (IC_{50}) for rat NPY receptor subtypes Y_1, Y_2, Y_4, and Y_5 in vitro. Mouse fibroblast cell lines (LMTK–) were stably transfected with the rat Y_1, Y_2, and Y_4 receptors, whereas human embryonic kidney HEK-293 cells were used for transfection with the rat Y_5 receptor.

Parker et al. (1998) determined the agonist and antagonist potency of neuropeptide Y and various analogues for cloned human and rat neuropeptide Y receptors expressed in CHO or 293 cells.

Wieland et al. (1998) studied the subtype selectivity of a nonpeptide Y_1 receptor antagonist using membranes from rat hypothalamus, from SK-N-MC (neuroblastoma) cells and from SMS-KAN (neuroblastoma) cells; human Y_1 receptor stably expressed in baby hamster kidney (BHK) cells, rat Y_1 receptor expressing human embryonic kidney (HEK) 293 cells, human Y_2 receptor stably expressed in BHK cells, rat Y_4 stably transfected in BHK cells, and human Y_5 receptor stably transfected in HEK 293 cells.

Several groups reported screening results of neuropeptide Y antagonists mostly using receptor binding experiments (Murakami et al. 1999; Fotsch et al. 2001; Islam et al. 2002;

Poindexter et al. 2002; Andres et al. 2003; Hammond et al. 2003; Blum et al. 2004; Gillman et al. 2006; Kakui et al. 2006).

Dautzenberg et al. (2005) established robust functional assays for the characterization of neuropeptide Y receptors. The human neuropeptide Y (NPY) receptors 1 (hY_1), 2 (hY_2), and 4 (hY_4) and the mouse type 5 (mY_5) receptor were expressed in human embryonic kidney 293 (HEK293) cells. The receptors bound a radioiodinated NPY ligand with high affinity and various NPY analogues competed for binding in a receptor-selective manner. Similarly, cAMP inhibition and GTPγ S binding assays were established. The four NPY receptors were further tested in the fluorimetric imaging plate reader (FLIPR) format, a cellular high-throughput assay, in the absence and presence of chimeric G-proteins, Gq_{o5}, Gq_{i5}, and Gq_{i9}.

Procedure

Cell Transfections

cDNAs of the four NPY receptors inserted into pcDNA3 (5 µg each) or Gq_{i5}, Gq_{o5}, and Gq_{i9} (10 µg each) inserted into pcDNA5 (Invitrogen) were transiently transfected into HEK293 cells with the FUGENE reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. Cell lines stably expressing the various NPY receptors together with Gq_{i9} were generated by transfecting receptor cDNA (0.5 µg each), the Gq_{i9} cDNA (1 µg) and the pOG44 vector (10 µg) encoding the cDNA for the DNA recombinase used in the Flip-in system (Invitrogen) with the FUGENE reagent. Two days after transfection, Geneticin (500 µg/ml) and hygromycin (50 µg/ml) selection was initiated and stable cell clones robustly responding in the FLIPR assay were selected.

Radioreceptor Binding Assays

Membranes from NPY-receptor-expressing HEK cells were prepared according to Dautzenberg et al. 2005. Competition binding assays were performed with 5 (hY_2 or hY_4), 25 (mY_5), and 40 µg (hY_1) of membrane proteins prepared from the four NPY receptors under assay conditions according to Rist et al. (1996), using 96-well plates (Beckmann Instruments, Fullerton, Calif., USA) and a scintillation proximity assay (SPA; Dautzenberg et al. 2000). The membranes, 1 mg wheat-germ agglutinin SPA beads (Amersham Pharmacia Biotech), 100 pM [^{125}I]PYY, and unlabeled peptides (10^{-5} M to 10^{-11} M) were added. The reaction mixture was incubated on a shaker for 120 min at 22 °C and then read in a TopCount (Packard). Nonspecific binding was determined as residual binding in the presence of 10 µM PYY. The dissociation constant K_d, the inhibition constant K_i, and the maximum receptor concentration, B_{max}, were calculated using the interactive curve fitting program Xlfit (Hoffmann-La Roche). Under these conditions less than 10 % of the total radioactivity was specifically bound by the various receptor constructs.

cAMP Inhibition Assay

The inhibition of forskolin-mediated cAMP accumulation by NPY analogues in 96-well plates was determined as previously described (Dautzenberg et al. 2001). Briefly 20,000 cells were incubated in Krebs–Ringer HEPES-buffered solution (KHR; 124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, pH 7.4) in the presence of 1 µM forskolin (Sigma, Munich, Germany) with increasing concentrations of agonists (10 pM to 10 µM) for 60 min at 37 °C. Reactions were stopped by the addition of 0.12 ml ice-cold ethanol and stored at −80 °C for at least 4 h. The cAMP content was determined from the supernatant using the Biotrak nonradioactive cAMP kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

GTPγ S Binding Assay

Agonist-mediated binding of GTPγ^{35}S was investigated in the SPA format using 96-well plates (Rover et al. 2000) and membranes prepared from cells transfected with the hY_1, hY_2, hY_4, and mY_5 receptors. Binding was performed in 200 µl of 20 mM HEPES buffer (pH 7.4, plus 6 mM $MgCl_2$ and 100 mM NaCl), supplemented with 20 µM GDP, 10 µM cold GTPγ S and 0.3 nM

GTPγ^{35}S. Then, 20 µg (hY_2, hY_4, mY_5) to 40 µg (hY_1) membranes, 1 mg wheat-germ agglutinin SPA beads, NPY analogues, or synthetic compounds (10^{-4} to 10^{-11} M) were added. For antagonist assays, a submaximal (EC_{80}) concentration of PYY was added together with increasing antagonist concentrations (10^{-4} to 10^{-11} M). The reaction mixture was incubated on a shaker for 60 min at 22 °C and then centrifuged for 5 min at 1,500 rpm in an Eppendorf 5403 centrifuge. Finally the plates were read in a TopCount reader (Packard).

Calcium Mobilization Assays

HEK293 transiently stably expressing the four NPY receptors with or without chimeric G-proteins were seeded at a density of 100,000 cells into poly-D-lysine-coated 96-well black-walled, clear-bottom microtiter plates (Corning, NY) (Dautzenberg et al. 2004). One day later, the medium was removed and 100 µl loading medium [DMEM high glucose, without serum, supplemented with 10 mM HEPES acid, 0.1 % BSA, 5 mM probenecid, and 2 µM Fluo-3 AM (Molecular Probes, Leiden, The Netherlands)] was added. Cells were loaded for 1 h at 37 °C and washed twice with 100 µl assay buffer (5 mM HEPES acid, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose) and then 150 µl assay buffer was added. Cells were further preincubated at room temperature before adding agonists or agonists plus antagonists in 50 µl assay buffer and then measured on a T-channel fluorimetric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, Calif., USA). Maximum change in fluorescence over baseline was used to determine agonist response.

Evaluation

Statistical analyses of the binding, cAMP, GTPγ S, and calcium mobilization data were performed on a Macintosh PC using StatView software (SAS, Cary, N.C., USA) by one-way analyses of variance (ANOVAs) across experimental groups followed by Dunnett's test with the alpha set at 0.05.

Beauverger et al. (2005) characterized antagonists specific to human neuropeptide Y receptor subtype 5 using a **luciferase reporter gene assay**.

Procedure

CRE-Luciferase Reporter Assay

Standardization of this assay consisted of testing various cell densities and incubation times to optimize the signal-to-background ratio. Briefly, HEK293-hMT1 cells grown to ≈ 80 % confluence were transfected in batches with Lipofectamine Plus (Life Technologies) using 20 ng of pcDOR8, 500 ng of pΔ MC16-Luc, and 1 µg of either pcDNA3.1 or pcDNA3.1-Y5 per million of cells. Then, 24 h after transfection, cells were detached by agitation, centrifuged, and recovered at 10^6 cells/ml in phenol-red-free DMEM medium supplemented with 2 % fetal calf serum. They were then seeded as 50-µl aliquots into white 96-well tissue culture plates containing the drugs previously diluted in the same medium and distributed as 50-µ l aliquots. After a 20-h incubation period, the luciferase activity was measured by adding 100 µl per well of Luclite buffer (Packard) followed by a 30-min incubation in the dark at room temperature and a use of a TopCount (Packard).

Savontaus et al. (1998) measured expression of preproneuropeptide Y mRNA in the arcuate nucleus and preprocorticotropin-releasing factor mRNA in the paraventricular nucleus by in situ hybridization technique after short- and long-term treatment with a β_3-adrenoreceptor agonist.

Bioassays have been used for characterization and classification of neuropeptide Y receptors (Hedge et al. 1995; Pheng and Regoli 1998; Dumont et al. 2000a, b). Some isolated organs appear to be "monoreceptor" systems, e.g., the rat tail artery (Gicquiaux et al. 1996), the rabbit saphenous vein (Pheng and Regoli 1998; Feletou et al. 1999), or the isolated perfused kidney of the rat (Hedge et al. 1995) for the Y_1 receptor. The dog saphenous vein, the rat vas deferens, and the rat colon can be used for the Y_2 receptor, the rat colon for the Y_4 receptor, and the rabbit ileum for the Y_5 receptor (Pheng et al. 1997).

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Orexin

General Considerations on Orexin

Orexin A and orexin B are 33- and 28-residue peptides, also called hypocretins, which were originally isolated from rat hypothalamus (De Lecea et al. 1998; Sakurai et al. 1998). Both peptides are derived from a 130-amino-acid precursor, preproorexin, which is encoded by a gene localized to chromosome 17q21 in humans. These peptides are located predominantly in the hypothalamus and locus coeruleus (Evans et al. 1999), but are also found elsewhere in the brain, and in the spinal cord (Smart 1999; Van den Pol et al. 1999). The orexins have a broad range of physiological functions, including the control of feeding and energy metabolism (Dube et al. 1999; Mondal et al. 1999; Sakurai 1999, 2006), stimulation of insulin secretion (Nowak et al. 2000, 2005), modulation of neuroendocrine function (Date et al. 1999), regulation of the sleep–wake cycle (Piper et al. 2000), narcolepsy (Taheri et al. 2002; Willie et al. 2003; Mieda et al. 2004), stress and anxiety, behavioral activities (Ida et al. 1999; Siegel 2003), cardiovascular function (Shirasaka et al. 1999; Chen et al. 2000), adrenal function (Jöhren et al. 2004), and sexual and reproductive functions (Pu et al. 1998; De Lecea and Sutcliffe 1999; Tamura et al. 1999). Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats which can be suppressed by an NPY Y_1 antagonist (Jain et al. 2000). Food intake could be inhibited by central injection of an anti-orexin antibody in fasted rats (Yamada et al. 2000). Nowak et al. (2000) found an increase of insulin secretion after subcutaneous injection of orexins to rats.

Orexin antagonists are potential anti-obesity drugs (Parker 1999).

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Receptor Assay of Orexin

Purpose and Rationale

The peptides orexin A and orexin B bind both to two receptors, orexin-1 (OX_1) and orexin-2 (OX_2), although orexin B has a low affinity for OX_1 (Sakurai et al. 1998). Differential expression of orexin receptors 1 and 2 in rat brain was reported by Marcus et al. (2001) and distinct recognition of OX_1 and OX_2 receptors by orexin peptides by Ammoun et al. (2005).

Procedure

Orexin OX_1 and OX_2 receptors are produced by polymerase chain reaction from fetal and adult brain cDNA libraries, using primers located across the start and stop codons. The receptors are subcloned into the pCDN vector (with neomycin resistance) and transfected into CHO cells using Lipofectamine (Life Technologies). Clones are selected using 400 μg/ml G418 (Life Technologies) and single clones are produced by limiting dilution cloning.

CHO-OX_1 and CHO-OX_2 cells are routinely grown as monolayers in MEM-Alpha medium supplemented with 10 % fetal calf serum and 400 μg/ml G418 and maintained under 95 % O_2/5 % CO_2 at 37 °C. Cells are passaged every 3–4 days.

Synthetic human orexin A is ^{125}I-labeled at Tyr17 by chloramine-T oxidation in the presence of $Na^{125}I$ (2,000 Ci/mmol). Monoiodinated peptide is purified by C^{18} reverse-phase HPLC (Takigawa et al. 1995). Stable transfectant CHO cell lines expressing human OX_1R or OX_2R are each seeded onto 12-well plates at a density of 3×10^3 cells per well. After an overnight culture, medium is discarded and cells are incubated at 20 °C for 90 min with binding buffer (HEPES-buffered saline/5 % bovine serum albumin) containing 10^{-10} M [^{125}I]orexin A plus designated concentrations of unlabeled competitors. Cells are then washed three times with ice-cold phosphate-buffered saline and lysed in 0.1 N NaOH. Cell-bound radioactivity is determined by a γ-counter.

Evaluation

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment each value is determined in duplicate, and the results are expressed as the means ± standard errors of at least three separate experiments.

Modifications of the Method

Smart et al. (1999) studied the pharmacology of recombinant orexin receptors using the fluorescence imaging plate recorder (FLIPR). CHO-OX_1 or CHO-OX_2 cells were seeded into 96-well black-walled clear-base plates at a density of 20,000 cells per well in MEM-Alpha medium supplemented with 10 % fetal calf serum and 400 μg/ml G418 and cultured overnight. The cells were then incubated with MEM-Alpha medium containing the cytoplasmatic calcium indicator, Fluo-3 AM (4 μM) and 2.5 mM probenecid at 37 °C for 60 min. The cells were washed four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 1 % gelatin, before being incubated for 30 min at 37 °C with either buffer alone (control) or buffer containing various signal transduction modifying agents. The plates were then placed into an FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence ($\lambda = 488$ nM, $\lambda = 540$ nM) (Sullivan et al. 1999) before and after the addition of orexin A or orexin B.

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Radioimmunoassay for Orexin

Purpose and Rationale

A radioimmunoassay for orexin A has been developed by Mitsuma et al. (2000).

Procedure

Synthetic orexin A is conjugated on an equal weight basis to BSA using glutaraldehyde. New Zealand white rabbits are immunized with an emulsion of this conjugate in 1 ml of water and complete Freund's adjuvant (1:2, v/v) which is injected into the foot pad at intervals of 3 weeks. Blood is withdrawn 1 week after each injection, and the presence of anti-orexin is checked by radioimmunoassay.

Radioiodination of orexin A is performed with the chloramine-T method. The radioiodinated materials are chromatographed on Sephadex G-25, eluted with 0.01 M phosphate buffer (pH 7.4), and collected in 1.0 ml fractions. The first peak is orexin A-I^{125}.

For determination of tissue concentrations, brain tissues of rats are dissected into the hypothalamus, cerebral cortex, thalamus, striatum, hippocampal formation, brain stem and cerebellum.

For the extraction of orexin A, the freshly obtained tissues are weighed and placed in 5.0 ml acid-acetone, homogenized, and centrifuged.

For the double-antibody radioimmunoassay, 0.1 ml of standard or samples, 0.1 ml of antibody (1:1,000), 0.1 ml orexin A I^{125}, and 0.5 ml buffer are incubated for 24 h at 4 °C. Then 0.1 ml of the second antibody solution is added and incubated again for 24 h at 4 °C. The samples are centrifuged and the supernatants decanted, and radioactivity is counted in the precipitates.

Evaluation

Bound/total count is calculated and standard curves versus synthetic orexin A are established.

Modifications of the Method

Using separate radioimmunoassays for orexin A and orexin B, Mondal et al. (1999) determined their distribution in microdissected nuclei of the diencephalon and brain stem of rats.

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Galanin

General Considerations on Galanin

Galanin is a neuropeptide of 29 amino acids in length (30 amino acids in humans) which was first isolated from porcine intestine (Tatemoto et al. 1983) and is localized mainly in the mammalian CNS (Skofitsch and Jacobowitz 1985; Melander et al. 1986) but also in other organs (Baltazar et al. 2000). Central administration of galanin increases food intake in satiated rats (Crawley et al. 1990). Conversely, reduction of central galanin levels by antisense oligonucleotide techniques (Akabayashi et al. 1994) or central administration of galanin receptor antagonists (Leibowitz and Kim 1992) decreases food intake. The activity of galanin in the hypothalamus is modulated by metabolic hormones and by the ingestion of nutrients (Wang et al. 1998).

These data suggest the use of galanin receptor antagonists as anti-obesity agents. More recent studies concentrate on pain and CNS effects (Kask et al. 1997; Holmes et al. 2000; O'Meara et al. 2000; Xu et al. 2000; Counts et al. 2003; Elliott-Hunt et al. 2004; Krasnow et al. 2004; Bahdie-Mahdavi et al. 2005a; Lundström et al. 2005).

Physiological actions of peripherally administered galanin include contraction of smooth muscle (Ekblad et al. 1989; Ahtaridis et al. 1998; Korolkiewicz et al. 1998; Niiro et al. 1998), inhibition of glucose-stimulated insulin release (McDonald et al. 1985; Leonharst et al. 1989), influence on learning and memory behavior (McDonald and Crawley 1997; McDonald et al. 1998a, b; Gleason et al. 1999; Ögren et al. 1999; Zachariou et al. 1999), and antinociceptive activity in rats with experimentally induced neuropathy (Burazin and Gundlach 1998; Ma and Bisby 1999; Yu et al. 1999; Kerr et al. 2000; Wang et al. 2000). Based on pharmacological and molecular biological evidence, Mazarati et al. (2001) discussed the hypothesis that galanin works as an endogenous anticonvulsant. A nonpeptide galanin receptor agonist showed anticonvulsant activity (Shi et al. 2002).

Galanin-overexpressing transgenic mice showed no anxiety-related behaviors in three different tests (Holmes et al. 2002), whereas galanin GAL-R1 receptor null mutant mice displayed increased anxiety-like behavior (Holmes et al. 2003).

Galanin has been found to influence secretion of growth hormone (Ottlecz et al. 1988; Murakami et al. 1987), LH (Todd et al. 1998), luteinizing hormone (Finn et al. 1998), and prolactin (Koshiyama et al. 1987; Cai et al. 1998; Wynick et al. 1998), to inhibit dopamine release from the median eminence (Nordström et al. 1987), to influence ACh release from rat brain (Fisone et al. 1989; Kasa et al. 1998), to inhibit norepinephrine release from the hypothalamus (Tsuda et al. 1989), and to modulate 5-HT_{1A} receptors in the ventral limbic cortex of the rat (Diaz-Cabiale et al. 2000).

A fragment of the galanin precursor protein, galanin message-associated peptide (GMAP), is present in dorsal root ganglion cells and influences the spinal nociceptor flexor reflex in rats (Xu et al. 1996).

A galanin-like peptide, named GALP, was isolated by Ohtaki et al. (1999) from porcine hypothalamus.

Some synthetic **galanin receptor agonists** were described, such as galnon (Saar et al. 2002; Bahdie-Mahdavi et al. 2005b) or galmic (Ceide et al. 2004; Bartfai et al. 2004).

Various **galanin receptor antagonists**, such as galantide (Lindskog et al. 1992; Sahu et al. 1994; Arletti et al. 1997; Ceresini et al. 1998), and other compounds (Bartfai et al. 1991; Pramanik and Ögren 1992; Kask et al. 1995; Xu et al. 1995; Kakuyama et al. 1997; Pooga et al. 1998;

Koegler et al. 1999; Park and Baum 1999; Katoh and Ohmori 2000; Kisfalvi et al. 2000; Swanson et al. 2005; Ögren et al. 2005) were synthesized and tested.

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Receptor Assay of Galanin

Purpose and Rationale

Galanin mediates its physiological effects via interaction with at least three G-protein-coupled receptors, designated GAL1, GAL2, and GAL3 receptor (Wang and Parker 1998; Iismaa and Shine 1999; Branchek et al. 2000; Waters and Krause 2000).

The first known galanin receptor GAL1 has been isolated from the human Bowes melanoma cell line (Habert-Ortoli et al. 1994). Human GAL1 contains 349 amino acids with the structure of a G-protein-coupled receptor. A rat GAL1 homologue, cloned from Rin14B cells, contains 346 amino acids (Parker et al. 1995). Human GAL1 mRNA has been detected by Northern blot analysis in fetal brain and small intestinal tissue and also by reverse transcriptase-polymerase chain reaction in the human gastrointestinal tract from the esophagus to the rectum. Rat GAL1 mRNA has been detected by Northern blot analysis in the brain, spinal cord, and Rin14B cells. Human and rat GAL1 share similar binding profiles in [^{125}I] galanin binding assays (Sullivan et al. 1997).

Heuillet et al. (1994) described ligand binding and functional characteristics of the human galanin receptor in the Bowes melanoma cell line.

Jungnickel and Gundlach (2005) studied [^{125}I]-galanin binding in brain of wild-type and galanin- and GALR1-knockout mice.

The rat GAL2 receptor has been cloned and characterized by Wang et al. (1997)and Ahmad et al. (1998), the mouse GAL2 receptor by Pang et al. (1998), and the human GAL2 receptor by Bloomquist et al. (1998) and Fathi et al. (1998). Unlike GAL1, mRNA encoding rat GAL2 is widely distributed in all tissues examined including the brain and peripheral tissues. Likewise, the human GAL2 receptor is detectable by RT-PCR in several central and peripheral tissues. GalR2 plays a key role in neurite outgrowth from adult sensory neurons (Mahoney et al. 2003). Rat GAL1 and GAL2 share similar pharmacological profiles in that they possess high affinity for full-length and N-terminal fragments of galanin.

Rat and human GAL3 receptors were described by Kolakowski et al. (1998) and Smith et al. (1998). Human and rat GAL3 share similar profiles in [^{125}I]galanin receptor binding assays.

Lee et al. (1999) reported the isolation of a cDNA clone named GPR54 which encodes a G-protein-coupled receptor related to the galanin receptors.

Procedure

Preparation of the radioligand ^{125}I-galanin is performed by iodination of galanin at room temperature by the chloramine-T method. Galanin, 10 µg, in 20 µl 0.05 M sodium phosphate buffer (pH 7.5) and 20 µl chloramine-T (5 mg/ml) are added in a batch containing 2 mCi Na^{125}I (245 mCi/ml). The reaction is terminated by adding 100 µl of a solution of sodium metabisulfite (1.2 mg/ml). The reaction mixture is transferred onto a column packed with SP-Sephadex C25, equilibrated with a solution of 100 µg/ml BSA, and then washed and equilibrated with 0.05 M sodium phosphate buffer (pH 5.0). The excess of Na^{125}I is first eluted with 0.05 M sodium phosphate buffer (pH 5.0), while the iodinated galanin is eluted as a single peak with 0.05 M sodium phosphate buffer (pH 8.1). Fractions corresponding to a peak of ^{125}I-galanin are pooled, the pH adjusted to 6.0 with acetic acid, and the aliquots stored at −18 °C until use.

Male Sprague–Dawley rats are sacrificed and their brains quickly removed. The hypothalamus is dissected for **preparation of membranes**. The tissue is homogenized (10 % mass/vol) in 0.32 M sucrose buffered with 5 mM HEPES (pH 7.4). The homogenate is diluted 10-fold and centrifuged at 1000 g for 10 min. The supernatant is centrifuged at 10,000 g for 45 min, and the pellet is resuspended in 5 mM HEPES-buffered Krebs–Ringer solution (pH 7.4).

Displacement experiments are carried out with various test compounds in a final volume of 400 µl HEPES/Krebs–Ringer solution; 0.05 % BSA (pH 7.4), containing 1 mM ^{125}I-galanin; and 70–100 µg membrane preparation. Samples are incubated for 30 min at 37 °C. Incubation is terminated by the addition of 10 ml ice-cold HEPES/Krebs–Ringer solution, followed by rapid filtration over Whatman GF/B filters, precoated for 5–6 h in 0.3 % polyethyleneimine solution. Specific binding is defined as that displaceable by 1 mM rat galanin1–29.

Evaluation

Bound/total count is calculated and displacement curves versus the standard are established.

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Adipsin

General Considerations on Adipsin

Proteins of the alternative complement pathway are secreted by adipose tissue (Choy et al. 1992; Peake et al. 1997). **Adipsin (complement D)** was the first to be cloned from an adipose tissue cell line and shown to be synthesized and secreted by

adipose tissue (Cook et al. 1987; Flier et al. 1987; Johnson et al. 1991; White et al. 1992). Adipsin is markedly suppressed in *ob/ob*, *db/db*, monosodium glutamate-induced obese mice, in obese JCR:LA-cp rats, and in cafeteria-fed rats (Rosen et al. 1989; Dugail et al. 1990; Shillabeer et al. 1992; Spurlock et al. 1996) and is regulated by glucocorticoids, retinoic acid, sympathomimetic drugs, and insulin (Kitagawa et al. 1989; Spiegelman et al. 1989; Lowell and Flier 1990; Lowell et al. 1990; Moustaid et al. 1990; Antras et al. 1991; Miner et al. 1993). In contrast to rodents, adipsin increases with adiposity in humans and in response to feeding and is decreased during fasting, cachexia, and lipoatrophy (Napolitano et al. 1994).

Adipsin is required for the synthesis of **acylation-stimulating protein (ASP)**, a protein implicated in fat metabolism (Sniderman and Cianflone 1994; Cianflone et al. 1999; van Harmelen et al. 1999). ASP is produced by the cleavage of C3a by carboxypeptidase and is highly expressed by mature adipocytes. The synthesis of C3a from C3 requires complement factor B and adipsin. Plasma ASP increases with meals and facilitates the synthesis and storage of triglycerides. Consistent with its role as a mediator of lipogenesis, ASP deficiency increases postprandial fatty acid levels and decreases weight gain and triglyceride synthesis in mice (Murray et al. 1999, 2000).

Several other factors, such as adiponectin (Takahashi et al. 2000), are involved in the function of adipose tissue acting as target as well as an endocrine organ (Ahima and Flier 2000).

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Adipsin Expression in Mice

Purpose and Rationale

Adipsin expression at the protein and mRNA levels was studied in mice (Flier et al. 1987; Spiegelman et al. 1989; Lowell et al. 1990; Dugail et al. 1990) and rats (Miner et al. 1993).

Procedure

Various strains of mice (CD-1 mice, C57BL6 *ob/ob* mice, C57BL/Ks misty diabetes (m db/m db) mice, and mice with monosodium glutamate-induced obesity) are treated with drugs, e.g., ephedrine (1 g/kg chow) and caffeine (1.4 g/kg chow) mixed in the diet. The effects of these regimens on fat pad weight and specific mRNAs in white epididymal and brown adipose tissue and serum adipsin concentrations are assessed.

RNA is extracted from epididymal adipose tissue, brown intercapsular adipose tissue, 3T3-F422A adipocytes, and isolated rat adipocytes by the guanidinium–cesium chloride technique. Total RNA is denatured, electrophoresed in 1.5 % agarose, transferred to nylon filters, and hybridized to random primed adipsin, uncoupling protein (UCP), or actin cDNAs (Flier et al. 1987).

Serum adipsin and adipsin released by 3T3-F442A-cultured adipocytes are assessed by RIA. Mouse adipsin purified from Chinese

hamster ovary cells stably transfected with an adipsin expression vector is iodinated using the Bolton–Hunter reagent. The labeled protein is separated from unincorporated ^{125}I using G-50 Sephadex chromatography. The first rabbit polyclonal antibody is raised to purified mouse adipsin overexpressed using a baculovirus expression system. The assay is carried out in PBS, pH 7.4, supplemented with $CaCl_2$ (0.1 g/l), $MgCl_2$ (0.1 g/l), and BSA (0.1 %). Serum adipsin is assessed in a dilution of 1:1,000–1:5,000 and in culture media at a dilution of 1:100. After adding tracer, standards, and serum, the tubes are mixed and the first antibody is added at a dilution of 1:800. After an overnight incubation, the bound and unbound tracer are separated using goat anti-rabbit immunoglobulin C fixed to heat-killed staphylococcus at a ratio 1:1.2.

Evaluation

Standard curves are generated using mouse adipsin purified from stably transfected CHO cells. Serial dilutions of standards, serum, and culture media are compared.

Modifications of the Method

Dugail et al. (1989) showed that, in sharp contrast with genetically obese mice, adipsin mRNA is not suppressed in genetically obese Zucker rats.

Platt et al. (1994) found that a tissue-specific transcription factor that regulates adipsin expression is less active in the adipose tissue of obese animals.

Napolitano et al. (1994) determined concentrations in blood and rates of adipsin secretion by adipose tissue in humans using a two-monoclonal "sandwich" ELISA assay.

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Ghrelin

Small synthetic molecules called growth hormone secretagogues (GHSs) (Bowers et al. 1980, 1984) stimulate the release of growth hormone (GH) from the pituitary. They act through the GHS-R, a G-protein-coupled receptor (Howard et al. 1996). The natural ligand of this receptor was discovered as a 28-amino-acid-containing peptide, called ghrelin (Kojima et al. 1999, 2000, 2001; Davenport et al. 2005), produced in the XA-like cells of the stomach (Kojima et al. 1999; Date et al. 2000); however, smaller

amounts of ghrelin are also found in the small and large intestine (Hosada et al. 2000). Ghrelin and the growth hormone secretagogue receptor are expressed in the rat adrenal cortex (Andreis et al. 2003). GH, GH receptor, GH secretagogue receptor, and ghrelin are also expressed in human T cells, B cells, and neutrophils (Hattori et al. 2001).

Ghrelin is the first peptide hormone in which the third amino acid serine is modified by a fatty acid; this modification is essential for the peptide's biological activity. Besides ghrelin's GH-releasing effect, it is a powerful appetite-simulating peptide. Plasma ghrelin concentration is increased in fasting conditions and reduced after habitual feeding (Kojima and Kangawa 2005), suggesting that ghrelin may act as an initiation signal for food intake. Chronic systemic or intracerebroventricular application to rats produced hyperphagia and obesity in rats (Wren et al. 2001). The appetite-stimulatory signal of ghrelin is mediated through action on the hypothalamic neuropeptide Y (NPY) and the Y1 receptor (Asakawa et al. 2001).

[^{125}I-His^{9}]-ghrelin was recommended as a radioligand for localizing GHS and GH receptors in human and rat tissue (Katugampola et al. 2001).

Various heterocyclic compounds acting as ghrelin receptor agonists have been developed, which might be useful for the treatment of anorexia nervosa (Palucki et al. 2001). Multiple ghrelin-derived molecules produced by posttranslational processing were identified indicating structural divergence of human ghrelin (Hosoda et al. 2003). Until now, however, no specific ghrelin receptor antagonist has been described, although such a compound might be an attractive approach to reduce feed intake and to decrease obesity.

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Part XIV

Endocrinology

Adrenal Steroid Hormones

Jürgen Sandow

Contents

Adrenalectomy in Rats

Purpose and Rationale

The classical way to evaluate hormone function is to surgically ablate the hormone-producing endocrine gland and substitute it with exogenously administered substances (extracts or synthetic hormones) (Biedl 1916). Many studies on the physiological role of adrenocortical hormones and the pharmacological effects of corticosteroids were performed using adrenalectomized rats.

Procedure

Sprague–Dawley or Wistar rats of either sex weighing 120–150 g are used. The dorsal fur is shaved: the rat is anesthetized and placed on a support in order to elevate the viscera. A transverse incision, about 5 mm long, is made in the mid-line at the costovertebral angle. To remove the left adrenal gland, the skin is retracted to the ventral side and the lumbar muscles are incised just superior and anterior to the splenic shadow. The adrenal gland now appears directly beneath the incision and no exteriorization of the kidney is necessary. The periadrenal tissue is grasped between the kidney and the adrenal by small curved forceps and the intact gland together

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with the periadrenal fat and the mesenteric attachments is removed in toto. The bleeding is negligible in young animals so that no vessels need be tied off.

After ablation of the left adrenal, the animal is turned around and the right gland is removed through the original skin incision. A small incision through the lumbar muscles is made just above and anterior to the prominent lumbocostal arch which is seen near the costal margin. The curved forceps are inserted over the kidney and by elevating the liver, which covers the adrenal on this side; the gland is brought into view and grasped by the forceps, removing again the intact gland with the periadrenal fat and the mesenteric attachments. The incisions made in the lumbar muscles need not exceed 3 mm in length and may be made by spreading the blades of a pair of scissors; hemostasis or closure by sutures is not necessary. The incision is closed by a skin clip. The entire procedure is done in a time sufficiently short to avoid long-acting anesthetics. The animals appear normal in every aspect within a few minutes following the operation.

It is essential to provide good postoperative care for animals after adrenalectomy and to correct the electrolyte imbalance due to deficiency of mineralocorticoid secretion (1 % sodium chloride solution in water).

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Glucocorticoid Activity

In Vitro Methods for Glucocorticoid Hormones

Corticoid Receptor Binding

Purpose and Rationale

Steroid hormone receptors are intracellular metalloproteins which bind steroids with high affinity and high specificity (nuclear receptors). The lipophilic steroids enter the cell by diffusion. The binding of the hormone to its specific receptors results in a series of conformational changes, leading to an increase in affinity for specific DNA regulatory elements (steroid responsive elements). After binding, transcription of target genes is enhanced and mRNAs are produced. These mRNAs are then translocated to the cytoplasm and translated into proteins regulating cellular metabolism and initiating the cellular responses. The structure and functions of the steroid receptor superfamily have been reviewed by Carson-Jurica et al. (1990), Lazar (1991), Barnes and Adcock (1993), Distelhorst (1993), Power et al. (1993), Brinkmann (1994), Ojasoo et al. (1994, 1995), Wittliff and Raffelsberger (1995), Beato et al. (1996), and Jensen (1996). The relative binding affinities for the glucocorticoid receptor of rat liver or thymus cytosol can be measured by competitive displacement of [^{3}H] dexamethasone (Raynaud et al. 1979; Schlechte et al. 1982; Wojnar et al. 1986; Ueno et al. 1991).

Procedure

A steroid receptor preparation may be obtained from rats. Male Wistar rats weighing 140–150 g are adrenalectomized under anesthesia. Two days later, the liver is excised and homogenized in a tenfold buffer containing 50 mM Tris–HCl, 1 mM EDTA, 2 mM dithiothreitol, 10 mM Na_2MoO_4, and 10 % glycerol (pH 7.4). The homogenate is centrifuged for 1 h at 105,000 *g* at 4 °C and collected as the supernatant fraction. The cytosol is mixed with 5 nM [^{3}H]dexamethasone in the presence or absence of appropriate concentrations of competitors and incubated for 2 h at 4 °C.

The reaction is terminated by the addition of hydroxyapatite in order to separate the receptor–steroid complex from the free [^{3}H]dexamethasone. The radioactivity bound to the receptors is determined by liquid scintillation spectrometry.

Evaluation

IC_{50} values for the receptor preparation are calculated by probit analysis.

Modifications of the Method

Numerous modifications of the glucocorticoid receptor assay have been reported, and cloning of receptor subtypes is ongoing (Zeelen 1992; White et al. 1994). Cytosols have been prepared from rat liver, from cultured rat hepatoma cells (Rousseau and Schmit 1977), and from normal human lymphocytes (Steiner and Wittliff 1985); thymocytes, from rat thymus gland (Lefebvre et al. 1988), human leukemic lymphoid cell line, CEM C7 (Srivastava and Thompson 1990), rat lung (Druzgala et al. 1991; Hochhaus et al. 1991), human lung (Rohdewald et al. 1985), rat hippocampus (Jacobson et al. 1993), and other sources.

Differentiation of type I (mineralocorticoid) and type II (glucocorticoid) receptors has been attempted (Spencer et al. 1990; Jacobson et al. 1993).

A new affinity label for glucocorticoid receptors was described by Lopez and Simons (1991).

The solution structure of the glucocorticoid receptor DNA-binding domain has been determined (Härd et al. 1990).

Berger et al. (1992) used transient cotransfection of receptor cDNA and suitable reporter genes to study human glucocorticoid receptor function in a CV-1 mammalian cell line. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the human glucocorticoid receptor and to bind to extracts of cells expressing human glucocorticoid receptor cDNA. A good correlation between both in vitro parameters and in vivo anti-inflammatory activity was reported for most of the steroids tested.

Guo et al. (1995) tested the binding of various steroids to the synaptic plasma membrane, which may be a novel type of glucocorticoid receptor on neuronal membranes that is significantly different from cytosolic glucocorticoid receptors.

A general survey of steroid hormones, receptors and antagonists was given by Jensen (1996).

Teutsch et al. (1995) discussed the structure–activity correlation of hormone antagonists ("antihormones").

Yoshikawa et al. (2002) showed that the pyrazolosteroid cortivazol is a specific ligand for the glucocorticoid receptor.

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Transactivation and Transrepression Assays for Glucocorticoids

General Considerations

Purpose and Rationale

The effects of glucocorticoids are mediated by both transactivation and transrepression mechanisms. Classical glucocorticoids, such as prednisolone or dexamethasone, induce both mechanisms. The desired anti-inflammatory effects are mainly mediated by repression of gene expression, whereas side effects are predominantly mediated via transactivation (Pfahl 1993; Schäcke et al. 2002; Buttgereit et al. 2005).

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Transactivation Assay for Glucocorticoids

Purpose and Rationale

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows the determination of the agonistic and also the antagonistic potency of a given compound, by induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

Procedure

Vector Construct

The expression plasmid pHGO containing the full-length coding sequence of the human glucocorticoid receptor driven from the SV 40 early promoter is prepared according to Hollenberg et al. (1985). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

CV-1 cells and COS-1 cells for transfection are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum, 4 mmol/l L-glutamine, penicillin, and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989), and Felgner et al. (1987). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80 % confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg pHGO (hGR expression plasmid) and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h, the transfection solution is

replaced by 6 ml DMEM supplemented with 10 % fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled, and replated onto 60-mm dishes at a density of 4.5×10^5 per dish, 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in a medium supplemented with 3 % charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1 % ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982). After the cells are spun for 15 min in an Eppendorf microfuge at 4 °C, the supernatants are removed and assayed for enzyme activity. The assay mixture contains (in a final volume of 180 µl) 100 µl of 0.25 M Tris-hydrochloride (pH 7.5), 20 µl of cell extract, 1 µCi of [^{14}C]chloramphenicol (50 µCi/mmol; New England Nuclear), and 20 µl of 4 mM acetyl coenzyme A. Controls contain CAT (0.01 U; P.L. Biochemicals) instead of cell extract. All of the reagents except coenzyme A are preincubated together for 5 min at 37 °C. After equilibration is reached at this temperature, the reaction is started by adding coenzyme A. The reaction is stopped with 2 ml cold ethyl acetate, which is also used to extract the chloramphenicol. The organic layer is dried and taken up in 30 µl of ethyl acetate, spotted on silica gel thin-layer plates, and run with chloroform:methanol (95:5), ascending. After autoradiography of the separated acetyl chloramphenicol forms, the spots are cut out and counted. Data are expressed as the amount of chloramphenicol acetylated by 20 µl of extract.

Evaluation

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to determine the potency of the test hormone. Dexamethasone (10^{-10}–10^{-6} mol/l) serves as standard.

For antiglucocorticoid activity, CAT activity in the presence of 10^{-8} mol/l dexamethasone is set as 100 % and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the steroid hormone antagonist RU 486 as the standard inhibitor.

Critical Assessment of the Method

Hollon and Yoshimura (1989) examined the causes of high variability in data from enzymatic transient gene expression assays. Their results strongly suggested that variation in transfection efficacy is the major cause of data variation and can seriously compromise valid interpretation of data.

Modifications of the Method

White et al. (1994) described a simple and sensitive high-throughput assay for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high levels of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter

wells using a colorimetric chloramphenicol acetyltransferase assay.

Dias et al. (1998) recommended the use of CRE recombinase in mammalian cells for high throughput screening of chemical libraries to identify new receptor ligands. A translational fusion of CRE recombinase and the ligand binding domain of the human glucocorticoid receptor was transfected into mammalian cells with the LOX P/luciferase reporter gene (DeWet 1907). A stable transfected clone was isolated and used to characterize the kinetics, ligand specificity, and dose–response to various receptor ligands.

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Transrepression Assay for Glucocorticoids

Purpose and Rationale

The immunosuppressive and anti-inflammatory actions of glucocorticoid hormones are mediated by transrepression of activating protein-1 (AP-1) and nuclear factor kappa B (NFκB) transcription factors. Since the anti-inflammatory effects of glucocorticoids are thought to be related to repression mechanisms and the side effects mainly to activation mechanisms, a therapeutic benefit of dissociated glucocorticoids is expected (Vayssière et al. 1997; Vanden Berghe et al. 1999; Belvisis et al. 2001; Lin et al. 2002; Tanigawa et al. 2002; Schottelius et al. 2002, Coghlan et al. 2003; Li et al. 2003; Ali et al. 2004; Hochhaus 2004; Schäke et al. 2004; Smith et al. 2005; Thompson et al. 2005). Schäke et al. (2004) investigated

the dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist, leading to separation of therapeutic effects from side effects.

Procedure

Receptor-Binding Assays

Cytosol preparations of Sf9 cells, infected with recombinant baculovirus coding for the human GR, MR, or PR, were used for the binding assays. After centrifugation (15 min, 600 *g*) Sf9 pellets were resuspended in 1/20 volume of 20 mM Tris–HCl, pH 7.5; 0.5 mM EDTA, 2 mM DTT, 20 % glycerol, 400 mM KCl, 20 mM sodium molybdate, 0.3 μM aprotinin, 1 μM pepstatin, 10 μM leupeptin and shock frozen in liquid nitrogen. After three freeze–thaw cycles, the homogenate was centrifuged for 1 h at 100,000 *g*. The protein concentration in the resulting supernatant was between 10 and 15 mg/ml. Aliquots were stored at –40 °C.

For the binding assays for GR, MR, and PR, [^{3}H]dexamethasone (≈20 nM), [^{3}H]aldosterone or [^{3}H]progesterone, SF9 cytosol (100–500 μg protein), test compound, and binding buffer (10 mM Tris–HCl, pH 7.4; 1.5 mM EDTA, 10 % glycerol) were mixed in a total volume of 50 μl and incubated for 1 h at room temperature. Specific binding was defined as the difference between binding of [^{3}H]dexamethasone, [^{3}H]aldosterone, and [^{3}H]progesterone in the absence and presence of 10 μM unlabeled dexamethasone, aldosterone, and progesterone.

After incubation, 50 μl of cold charcoal suspension was added for 5 min, and the mixtures were transferred to microtiter filtration plates. The mixtures were filtered into Picoplates (Canberra Packard, Dreieich, Germany) and mixed with 200 μl of Microszint-40 (Canberra Packard). The bound radioactivity was determined with a Packard Top Count plate reader. The concentration of test compound giving 50 % inhibition of specific binding (IC_{50}) was determined from Hill analysis of the binding curves.

Induction of Tyrosine Aminotransferase (TAT)

Induction of TAT by test compounds was determined in vitro in the rat liver hepatoma cell line H4-II-EC3. Cells were grown in MEM (Life Technologies, Karlsruhe, Germany) supplemented with 10 % FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), and 1 % NEAA (nonessential amino acids) (Life Technologies). For compound testing, cells were seeded in 96-well plates (2×10^4 cells per well) and incubated with test compounds or dexamethasone for 20 h. Cells were then lysed and TAT activity assayed as described below for hepatic TAT induction in vivo.

Inhibition of IL-8 Production

IL-8 synthesis was induced by stimulation of the human promyeloic cell line THP-1 with lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8; Sigma). Cells (2.5×10^4 cells per well) were treated with 10 μg/ml LPS in the absence or presence of test compounds or dexamethasone for 18 h. IL-8 concentration was determined in the supernatant by an IL-8-specific ELISA (Beckman Coulter).

Inhibition of Monokine Secretion

Monocytic secretion of tumor necrosis factor-alpha (TNF-α) and IL-12 p70 was determined after stimulation of peripheral blood mononuclear cells from healthy donors with 1 μg/ml LPS (*E. coli* serotype 0127:B8; Sigma) and 10 ng/ml interferon-gamma 1b (IFN-γ 1b) (Imukin, Boehringer Ingelheim). After 24 h of stimulation at 37 °C, 5 % CO_2, in the absence or presence of different concentrations of the compounds, the concentrations of IL-12 p70 and TNF-α in culture supernatants were determined by using commercial ELISA kits from R and D Systems (IL-12 HS Immunoassays, Nivelles, Belgium) and Bio-Source International (TNF-α EASIA, Wiesbaden, Germany). The calculated IC_{50} value represents the concentration of compound giving 50 % inhibition of maximal TNF-α and IL-12 p70 production.

Modifications of the Method

Vayssière et al. (1997) described synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression as being weak transactivators of tyrosine aminotransferase (TAT) in cultured liver

cells but potent inhibitors of LPS-induced IL-1β secretion in the human monocytic cell line THP1.

González et al. (2000) found that glucocorticoids antagonize AP-1 by inhibiting the activation/phosphorylation of JNK without affecting its subcellular distribution.

Kagoshima et al. (2001) reported that glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation.

Reichardt et al. (2001) studied the repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor using mice with a mutation in the glucocorticoid receptor, which cannot activate glucocorticoid receptor response element promoters.

De Haij et al. (2003) reported dissociation of transrepression and transactivation in the steroid responsiveness of renal epithelial cells.

Schaaf and Cidlowski (2003) investigated the importance of ligand affinity for molecular determinants of glucocorticoid receptor mobility in living cells.

Stevens et al. (2003) investigated the role of tyrosine 735 in the dissociation of steroid receptor coactivators and nuclear receptor recruitment to the human glucocorticoid receptor.

Carballo-Jane et al. (2004) recommended skeletal muscle cells as a dual system to measure glucocorticoid-dependent transactivation and transrepression of gene regulation.

Studying transactivation and transrepression activity, Koubovec et al. (2005) found that synthetic progestins used in hormone replacement therapy have different glucocorticoid agonist properties.

Eberhardt et al. (2005) reported that dissociated glucocorticoids equipotently inhibit cytokine- and cAMP-induced matrix-degrading proteases in rat mesangial cells.

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Induction of Tyrosine Aminotransferase (TAT) in Hepatoma Cells

Purpose and Rationale

The synthesis of tyrosine aminotransferase in hepatoma tissue culture (HTC) cells (Thompson et al. 1966) can be induced by glucocorticoids (Giesen and Beck 1982; Raynaud et al. 1980;

Rousseau and Schmit 1977). This effect can be abolished by glucocorticoid antagonists.

Procedure

Hepatoma tissue culture (HTC) cells are grown in suspension to a density of about 8×18^5 cells/ml. The cells are washed three times at 0 °C in a total volume of buffered saline equivalent to half of the culture medium. The suspension is centrifuged and cell pellets resuspended in serum-free medium containing 0.1 % BSA and 0.1 % $NaHCO_3$. Ethanol solutions of standard (dexamethasone) and test steroids in various concentrations are added to 10-ml aliquots of cell suspension, with the final ethanol concentration not exceeding 0.5 %. After 16 h of incubation in tightly capped flasks on a rotary shaker (100 rev/min) at 37 °C, the cells are harvested and tyrosine aminotransferase determined. The cells are washed twice and then disrupted with an ultrasonicator. The enzyme is assayed at 37 °C by conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde (Diamondstone 1966). One unit of activity represents the conversion of 1 μmol of *p*-hydroxyphenylpyruvate per minute.

Evaluation

Enzyme-specific activity is expressed in milliunits of tyrosine aminotransferase/mg of cell protein. Dose–response curves are established for the standard and the test preparation allowing the calculation of potency ratios with confidence limits.

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Effect on T-Lymphocytes

Purpose and Rationale

Corticosteroids as immunosuppressive agents have suppressive effects on T-lymphocyte function. Snijdewint et al. (1995) determined in vitro dose-dependent inhibition of Th1- and Th2-type cytokine production by various corticosteroids.

Procedure

Culture Media

T-lymphocyte clones are maintained and expanded in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 % pooled c-inactivated human serum and gentamicin (80 μg/ml). Experiments are performed in IMDM supplemented with 10 % fetal calf serum (FCS), human transferrin (35 μg/ml), 2-mercaptoethanol (2-ME, 3.5 μg/l) and human insulin (1.75 IE/ml).

Peripheral Blood Lymphocytes and T-Lymphocyte Clones

Peripheral blood mononuclear cells are prepared from heparinized venous blood of healthy volunteers by flotation on Lymphoprep (Nycomed, 1.077 g/ml) and the peripheral blood lymphocytes are separated by centrifugation on a discontinuous Percoll density gradient. Peripheral blood lymphocytes are collected from the interface between densities 1.977 and 1.061 g/ml, washed three times in HBSS plus 2 % FCS, and suspended in IMDM plus 10 % FCS. Isolated peripheral blood lymphocytes (0.5×10^6 cells/ml) are stimulated

with CD2 mAb plus CD28 mAb (1.1000 final dilution of ascites) in the absence or presence of corticosteroids (Van der Pouw-Kraan et al. 1992). Supernatants are collected after 48 h for IFN-γ and IL-2 measurement, and after 96 h for IL-4 measurement.

T-lymphocyte clones specific to house dust mite (*Dermatophagoides pteronyssinus*) are generated from the peripheral blood and skin of atopic individuals (Van der Heijden et al. 1991). These clones are Th0 typed, capable of producing both IFN-γ and IL-4 (Kapsenberg et al. 1988). T-lymphocyte clone cells (0.5×10^6 cells/ml) are stimulated with CD2 and CD28 mAb (1.1000) plus 1 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce optimal levels of cytokine production. Corticosteroids are added at the start of the stimulation, and then 24 h after stimulation, supernatants are collected and stored at ≈20 °C until cytokine production is analyzed.

Proliferation Assay and Cytokine Measurements

Proliferation by peripheral blood lymphocytes is measured 48 or 96 h after stimulation in 96-well flat-bottom culture plates using 10^4 cells/well, the last 16 h in the presence of 13 kBq (0.33 μC)/well of [^{3}H]TdR (Radiochemical Centre, Amersham, UK). The proliferation of T-lymphocyte clones (10^4 cells/well) is similarly measured after stimulation for 40 h, again the last 16 h in the presence of [^{3}H]TdR. Incorporation of [^{3}H]TdR is determined by liquid scintillation spectroscopy.

Measurement of IFN-γ and IL-4 in peripheral blood lymphocytes and T-lymphocyte clone supernatants is performed with specific solid-phase sandwich ELISA systems (Van der Pouw-Kraan et al. 1992). For quantification of IL-2 production, a commercial ELISA kit is used. IL-2R expression by T cells of peripheral blood lymphocytes is determined 48 h after stimulation by labeling with mouse antihuman CD25 mAb.

Evaluation

Dose–response curves of inhibition of IFN-γ, IL-4 and IL-2 production as a percentage of initial values in peripheral blood lymphocytes and in different T-lymphocyte clones are established for various corticosteroids in the range of 10^{-10} to 10^{-6} M.

All measurements are performed in duplicate or triplicate. Data are expressed as mean ± SEM and differences are assessed using Student's *t*-test.

Modifications of the Method

Mollison et al. (1999) compared a macrolactam inhibitor of T helper type 1 and T helper type 2 cytokine biosynthesis in various animal models with several steroid preparations used for topical treatment of inflammatory skin diseases.

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Inhibition of Cartilage Degradation

Purpose and Rationale

The efficacy of glucocorticosteroids after intra-articular injection has been clearly demonstrated in several animal models of osteoarthritis (Van

den Berg et al. 1992; Pelletier et al. 1995). Augustine and Oleksyszyn (1997) used in vitro experiments to study the inhibition of degradation in bovine cartilage explants stimulated with concomitant plasminogen and interleukin-1α by various glucocorticosteroids.

Procedure

Preparation of Bovine Articular Cartilage Explants

Bovine (calf) radiocarpal joints are acquired from a local abattoir immediately after sacrifice and transported on ice. The specimens are washed thoroughly and placed on ice containing 25 % Povidine (10 % Povidone-iodine topical solution). The specimens are then dissected in a sterile hood using good sterile technique. Media (DMEM containing 4.5 g/l D-glucose and L-glutamine, without sodium pyruvate) is supplemented with HEPES buffer (3.57 g/l) and sodium bicarbonate (3.7 g/l), and the pH adjusted to 7.4. The medium is further supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 50 µg/ml C-ascorbic acid. The articulating cartilage surfaces are exposed, and the synovial fluid is wiped away with sterile gauze. A sterile cork borer with a diameter of 3.5 mm is used to remove uniform cores of cartilage. The cores are placed in a sterile flask, washed four times with sterile medium, and then placed in an incubator (37 °C, 5 % CO_2/95 % air, adequate humidity) and allowed to equilibrate for 1 h. The cartilage disks are then labeled en mass with [^{35}S]sulfate at 10 µC/ml for approximately 72–96 h, with hand-stirring every few hours. After labeling, explants are equilibrated with fresh medium (minimum of two washes before use in experiments).

Inhibition of IL-1-Induced Cartilage Degradation in Bovine Articular Cartilage in the Presence of Human Plasminogen

Individual explants are transferred to 96-well plates containing 250 µl of fresh medium per well, with or without plasminogen and IL-1α, and with or without glucocorticosteroids. A negative control consists of medium alone, while two positive controls are IL-1α alone, and plasminogen with IL-1α. All other groups contain the glucocorticosteroids along with recombinant human IL-1α (0.4 ng/ml) alone or concomitant human plasminogen (0.4 µM) plus IL-1α. Glucocorticosteroids are added in concentrations between 10 and 10,000 pM. Control and experimental explants are incubated for approximately 96 h (4 days) prior to counting a 50-µl sample of supernatant from each well. A 50-µl sample of a papain-digest of each explant is also counted.

Evaluation

The data are expressed as percentage glycosaminoglycan release over the 4 days. The values for all groups with glucocorticosteroids present are then compared to the values for concomitant plasminogen and IL-1α without glucocorticosteroids, and percentage inhibitions are calculated. Dose–response curves are established for each glucocorticosteroid.

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In Vivo Methods for Glucocorticoid Hormones

General Considerations

Muscle work tests (early fatigue) were used in early stages of adrenal gland research (Bomskov 1937; Ingle 1944; Dorfman 1962), and their description is

of historical interest. The method is based upon the fact that muscular responsiveness is lost within a few hours following the removal of the adrenal glands and can be maintained by the administration of corticosteroids. Since the 11-oxygenated steroids are quite active and compounds such as desoxycorticosterone are practically without activity, it appears that the main effect monitored is on carbohydrate metabolism.

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Adrenal and Thymus Involution

Purpose and Rationale

Repeated administration of corticosteroids causes central and peripheral effects on pituitary hormone content and secretion and on adrenal weight and histology. In the immature rat, thymus involution is observed in a dose-related manner.

Procedure

Groups of six to ten immature male Sprague–Dawley rats weighing 60–70 g are injected subcutaneously daily for 6 days with 0.2 ml of a homogenized suspension of different doses of the test compound in 0.5 % aqueous carboxymethyl cellulose solution. The standard, hydrocortisone acetate, is given in daily doses of 0.05 and 0.2 mg per animal. Controls receive the vehicle. On the 7th day, the animals are sacrificed and the adrenal and thymus weights determined.

Evaluation

The involution of the thymus gland is a measure of the catabolic activity of the compound (metabolic effect). Involution of the adrenals is a measure of the ability of the compound to inhibit the secretion of ACTH (negative steroid feedback). Dose–response curves are established for both parameters and compared with hydrocortisone and the potency ratios are calculated.

Modifications of the Method

The numeric test result is improved when the product of thymus and adrenal weights is calculated. Dose–response curves of the product of thymus and adrenal weights are steeper than those of each individual parameter (Laschet and Hohlweg 1960).

Inhibition of uridine incorporation into the ribonucleic acid of thymocytes has been used as a parameter for agonistic and antagonistic glucocorticoid activity (Gaignault et al. 1977).

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Eosinopenia in Adrenalectomized Mice

Purpose and Rationale

Glucocorticoids decrease the eosinophilic cell blood count in laboratory animals as well as in humans. This effect was used to quantitate the

potency of corticosteroids in adrenalectomized mice (Silber and Arcese 1964; Speirs and Meyer 1951; Tolksdorf 1959).

Procedure

Male mice weighing 20–25 g (e.g., of Jax C57 Brown, subline cd strain) are adrenalectomized and maintained at 28 °C with 1 % sodium chloride solution in water. Then, 15-mg pellets of desoxycorticosterone acetate are implanted at the time of operation. Steroids are dissolved in benzyl alcohol and mixed with sesame oil (1:10). Three days after operation, the mice receive 5 μg epinephrine by subcutaneous injection and 4 h later 1–6 μg of hydrocortisone (or the test substance) in 0.03 ml oil. Three doses of the unknown substance and standard are tested, using six animals per dose. Blood samples are taken from the tail before and 3 h after steroid injection. Mice with fewer than 100 eosinophils per cubic millimeter of blood before injection are discarded.

Evaluation

The percentage decrease of eosinophil blood count after 3 h is averaged per group. From dose–response curves, potency ratios are calculated.

Critical Assessment of the Method

The test is relatively simple and has the advantage of requiring little material.

References and Further Reading

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Liver Glycogen Test in Rats

Purpose and Rationale

The liver glycogen deposition test, as described by Stafford et al. (1955), is a simple and specific test for glucocorticoid activity (Dorfman 1962; Ringler 1964; Silber and Arcese 1964).

Procedure

Male Sprague–Dawley rats weighing 140–160 g are adrenalectomized. They are fed stock laboratory diet and 1 % sodium chloride solution. On the morning of the fourth postoperative day, food is withdrawn. On the morning of the fifth day, the drinking fluid is withdrawn and the rats are given the test compound by a single subcutaneous injection in appropriate dosages suspended in 0.5 ml sesame oil. Seven hours later, the rats are sacrificed. The livers are removed and blotted on filter paper to remove blood, weighed, dropped into flasks containing 10 ml hot 30 % potassium hydroxide and digested on a hot plate. The digest is diluted to 100 ml and a 50-fold dilution of an aliquot is used for analysis.

Then, 10 ml 0.2 % anthrone in 95 % sulfuric acid is slowly added to 5 ml of liver digest dilution with cooling. The mixture is heated in a boiling water bath for 10 min and then placed into cold water. Optical density is measured in a spectrophotometer at 620 μm using the anthrone-reagent as blank. Calibration curves are established using glucose as standard. Groups of five animals are used for each dose and for the vehicle controls. Three doses of test compound and of standard are used to find dose–response activities. Standard doses of hydrocortisone are 0.5, 1.0, and 2.0 mg per animal subcutaneously or 1.25, 2.5, and 5.0 mg per animal orally.

Evaluation

The amount of glycogen expressed as glucose is calculated per gram liver weight. Dose–response curves are established for each compound and for the standard in order to calculate potency ratios.

Modifications of the Method

The method can be used to determine the time course and duration of action of derivatives such

as corticosteroid esters compared to the free alcohol (Vogel 1963, 1965) and for evaluation of topical and systemic activity (Alpermann et al. 1982).

The liver glycogen deposition in the adrenalectomized mouse has been used by Dorfman et al. (1946) and by Venning et al. (1946) to evaluate glucocorticoid activity. Liver tryptophan peroxidase activity in the rat is decreased after adrenalectomy and increased after administration of corticosteroids (Knox and Auerbach 1955) indicating an early effect of corticoids.

The enzyme activity of tryptophan pyrrolase in guinea pig liver is increased by systemic administration of corticoids, but this effect is short lived and achieved only with high subcutaneous doses (Albrecht et al. 1979). In contrast, the enzyme is markedly reduced by protracted application of corticoids to the skin ("dermatocorticoids").

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Anti-Inflammatory Activity of Corticoid Hormones

General Considerations

Most of the tests described in chapter "▶ Anti-Inflammatory Activity" have been used for evaluation of corticosteroids.

The effects on acute inflammation are less suitable, [Ultraviolet erythema in guinea pigs (see chapter "▶ Anti-Inflammatory Activity"), Vascular permeability in rats (see chapter "▶ Anti-Inflammatory Activity"), Paw edema in rats (see chapter "▶ Anti-Inflammatory Activity")], whereas the methods measuring subacute inflammation are very well suited [Granuloma pouch test in rats (see chapter "▶ Anti-Inflammatory Activity"), Cotton granuloma test in rats (see chapter "▶ Anti-Inflammatory Activity"), Glass rod granuloma test in rats (see chapter "▶ Anti-Inflammatory Activity"), Sponge implantation technique (see chapter "▶ Anti-Inflammatory Activity")].

The molecular mechanisms of the anti-inflammatory actions of glucocorticoids were reviewed by Barnes (1998). Development of

dissociated steroids which are more active in transrepression (interaction with transcription factors) than transactivation (binding to glucocorticoid response elements) as reported by Vayssiere et al. (1997) was recommended.

References and Further Reading

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Animal Studies for Corticoid Hormone Evaluation

Several examples are presented here for the specific pharmacology of corticoids. The methods applied in some instances are the same methods as for antiphlogistic and anti-inflammatory compounds. Corticosteroids intended for systemic treatment or local application are administered daily for 3–14 days, and the pharmacological effects are determined. NMRI mice (26–28 g) or Wistar rats (140–160 g) are housed according to institutional guidelines with access to food and water ad libitum; 8–11 animals may be randomly allocated to the different treatment groups.

Croton Oil-Induced Ear Inflammation

This test is performed by local application to reduce or prevent the effect of a local irritant. Topical application of the nonspecific contact irritant croton oil, a mixture of several phorbol esters, leads to acute inflammation characterized by edema and a mainly granulocytic cell infiltration into the skin. Swelling of the ear due to inflammation is prevented by the corticoid solution or suspension. For topical application, corticosteroids are dissolved in the same vehicle as used for croton oil and may be applied separately, or together with the irritant croton oil.

Systemic application of corticosteroids (subcutaneously) may also be performed 2 h before croton oil application. At the maximum of the inflammatory reaction, animals are killed, and ears (mice, area $\approx$1 cm^2) or a punch biopsy (rats, 10-mm diameter) of each ear are weighed as an indicator of edema formation. The tissue samples are then snap frozen in liquid nitrogen in polypropylene tubes and kept at −20 °C for up to 24 h (Tonelli et al. 1965; Alpermann et al. 1982; Vayssière et al. 1997).

Peroxidase Activity Assay

Peroxidase activity, as a measure for total granulocyte infiltration, may be assayed in the tissue samples by using a modification of a method described by Schottelius et al. (2002).

Induction of Tyrosine Aminotransferase (TAT) In Vivo

In a similar manner as performed in vitro, the induction of tyrosine aminotransferase (TAT) may be investigated ex vivo in tissue of treated animals. TAT induction is evaluated 6 h after compound administration (subcutaneously) to juvenile rats by determination of TAT activity in liver homogenates. Animals are killed, and biopsy samples (10-mm diameter) are taken from the liver and snap frozen. Biopsy samples are homogenized in 2 ml of homogenizing buffer (140 mM KCl in 20 mM KPO_4 buffer, pH 7.6) and centrifuged at 24,000 *g* for 20 min at 4 °C. Supernatants are assayed for protein content by the BCA (bicinchoninic acid) Protein Assay Kit from Pierce. Then 20 µl of supernatant diluted 1:50 in PBS is incubated for 30 min at 37 °C with 200 µl of TAT-reaction buffer (tyrosine 1.2 mg/ml or 6.6 mM/45 mM KH_2PO_4/0.06 mM pyridoxal-5′-phosphate/12 mM oxoglutaric acid, adjusted to pH 7.6 with KOH). After stopping the reaction with 10 M KOH and further incubation for 30 min, extinction was measured at 340 nm, and TAT activity is calculated in relation to 500 µg/ml total protein content. TAT induction is defined as *x*-fold increase in TAT activity measured as OD at 340 nm in comparison to the TAT activity in vehicle-treated animals (Thompson et al. 1966).

ACTH Suppression in Rats

Animals are housed under conditions of minimum stress before the study, in a quiet laboratory room.

Six hours after application of compounds, animals are killed with minimum stress (decapitation in a separate room), and EDTA-anticoagulated blood is collected from the abdominal aorta. Plasma ACTH content is determined using the ACTH ^{125}I-assay system (ImmuChem Double Antibody hACTH) following the manufacturer's instructions (ICN).

The method requires considerable experience because the isolation of animals and sacrificing with a minimum of stress are difficult to achieve. Refer to pretreatment with lower doses of dexamethasone to improve this test method. Treatment with increasing doses of the corticosteroids and prednisolone as the reference standard will reduce the ACTH concentrations, such tests however have an inherently high variability of the vehicle-treated control groups (Sakakura et al. 1981; Baumann et al. 1985).

Increased Blood Glucose in Rats (Hyperglycemia Induced by Corticoids)

Animals are isolated before the study to avoid stress and fasted for 16 h. Six hours after application of compounds, animals are killed, and EDTA-treated blood is collected from the abdominal aorta to assess the corticoid-induced rise in blood glucose. Glucose in plasma is measured by colorimetric serum glucose determination by using hexokinase and glucose-6-phosphate dehydrogenase with a Hitachi 904 automatic analyzer (Roche Diagnostics).

Skin Atrophy After Chronic Corticoid Application

To assess the effect of local application on collagen composition of the skin, the test compound or the reference compound prednisolone (75 µl on a marked area of 9 cm^2 in 95 % ethanol/5 % isopropyl myristate) was applied daily for 19 days to the dorsal skin of nude rats (strain hr-hr, 120–140 g, Iffa-Credo). Animals were killed on day 20. Skin thickness was determined by using a specifically designed dial thickness gauge. Mean values were derived by measuring two adjacent treated skin areas. To determine skin-breaking strength of treated skin, a dorsal skin patch (5 × 5 cm) was removed. The skin patch was placed on filter paper, and two double-T-piece skin strips (50 mm long, 4 mm wide at the narrowest point) were punched in a caudal–cranial direction out of the patch. The skin strips were covered with moistened filter paper to avoid drying and were fixed with the wider ends into an apparatus developed in-house to measure skin-breaking strength. At a constant rate of stretch (200 mm/min), the force necessary to tear the skin strip was determined with a pressure sensor and was expressed as the skin-breaking strength (Alpermann et al. 1982; Vogel and Petri 1985).

Effect of Chronic Treatment on Thymus, Spleen, Adrenal, and Body Weight

To determine effects of repeated-dose corticoid treatment on body weight, animals were weighed before and after completion of treatment. Thymus, spleen, and adrenal glands were removed from animals killed after systemic or topical treatment and weighed. Organ weights are expressed as absolute values or as a percentage inhibition when compared with untreated animals (Laschet and Hohlweg 1960b; Stoeck et al. 2004).

Evaluation

For the examples given here, group means were calculated and the statistical significance of the effects of TAT, glucose induction, and ACTH suppression was assessed by using Dunnett's test. Differences between test compound and the corresponding prednisolone group (used as the reference standard) were assessed by using the Mann–Whitney test.

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Effects of Steroids on Mechanical Properties of Connective Tissue

Breaking Strength of Bones

Purpose and Rationale

Patients with Cushing's disease or after long-term treatment with corticosteroids have an increased susceptibility for bone fractures. The related animal tests were developed as a model for corticosteroid-induced osteoporosis, and they are of historical interest. The tests are based on changes in the mechanical properties of bone. Surprisingly, in chickens as well as in rats, short-term treatment with low and medium doses of glucocorticosteroids increased the breaking strength of bones dose-dependently whereas very high doses induced a decrease of breaking strength.

Procedure

White Leghorn chicken at an age of 14 days weighing 170 ± 20 g were used. The animals were treated for 14 days by intramuscular injection of 0.2 up to 100 mg/kg cortisol (reference compound) or equivalent doses of other glucocorticoids in oily solution. At autopsy, body weight, and comb weight are recorded and the femur and tibia bones from both sites are weighed. Length of bone (l) and outer diameter (D) of the diaphysis are measured using calipers. The bone is fastened on both ends, supported on edges 5 mm distant from the ends, and broken in the central portion of the diaphysis by a special device attached to an Instron-instrument. Breaking load (P) is recorded. The contralateral bone is cut at the middle of the diaphyseal shaft into rings by a small saw. Inner (d) and outer diameter (D) are measured in two perpendicular directions.

Evaluation

Breaking strength (σ) of the hollow bones is calculated according to the technical formula for hollow cylinders:

$$\sigma = \frac{8}{\pi} \times \frac{P \times 1 \times D}{D^4 - d^4}$$

Doses between 0.2 and 2.0 mg/kg cortisol and 0.05 and 1.0 mg/kg prednisolone show a parallel increase of breaking strength allowing the calculation of potency ratios. With higher doses, the response changes to inhibition of breaking strength. Excessive doses of 50 and 100 mg/kg cortisol, which are in the range of the LD_{50}, induce a decrease of breaking strength (Vogel 1968, 1969, 1990; Vogel and Ther 1962).

Modifications of the Method

In young and adult rats, 10 days of treatment with cortisol and other corticosteroids induce a dose-dependent increase of breaking strength, whereas with longer treatment (30 days) a bell-shaped dose–response (increase with low doses, decrease with high doses) is seen.

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Tensile Strength of Femoral Epiphyseal Cartilage in Rats

Purpose and Rationale

The epiphyseal cartilage of rats is very sensitive to treatment with hormones and other desmotropic drugs. The tensile strength of the distal femoral epiphyseal plate is decreased after adrenalectomy and is restored or increased by treatment with low doses of corticosteroids.

Procedure

Male Sprague–Dawley rats weighing 120 ± 10 g are treated with several doses of corticosteroids or other hormones for periods of between 1 and 14 days. After sacrifice, the hind legs are exarticulated in the hip joint and fastened at the collum femoris. Longitudinal tension always results in rupture of the distal femoral epiphyseal cartilage (Ther et al. 1963; Vogel and Ther 1964; Vogel 1969). The ultimate load of the femoral epiphyseal plate is recorded by an Instron instrument at an extension rate of 5 cm/min.

Single injections of doses between 10 and 100 mg/kg cortisol acetate or 2.5 and 40 mg/kg prednisolone acetate induce a dose-dependent increase of tensile strength up to twice the control levels after 24–48 h, which subsides after 96 h. Repeated administration up to 15 days also results in a dose-dependent increase. There is a maturation-dependent increase of tensile strength in untreated rats.

The effect of other hormones, e.g., gonadal steroids, on cartilage is much smaller than that of glucocorticosteroids.

Evaluation

Dose–response curves for steroids can be established allowing the calculation of potency ratios in experiments with single-dose or repeated-dose administration.

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Tensile Strength of Tail Tendons in Rats

Purpose and Rationale

The thread-like tail tendons of rats are very suitable for studying the mechanical properties of connective tissue. They are easy to prepare and consist predominantly of collagen. Like other organs of connective tissue, such as bone, cartilage, and skin, the tensile strength of tail tendons

is reduced after adrenalectomy and dose-dependently increased after short-term treatment with glucocorticosteroids (Vogel 1965, 1969).

Procedure

Male Sprague–Dawley rats weighing 120 ± 10 g are treated with increasing doses of corticosteroids or other hormones for periods of between 1 and 14 days. After sacrifice, the tail is amputated at its base. The tail skin and the last few coccygeal vertebrae are removed. Single tendons are pulled out from the dorsal and ventral bundles and kept in saline solution. Tendons of the same diameter (0.25 mm) are selected with a stereomicroscope. Since the diameter of the tendons may be influenced by long-term treatment of the animals, tendons of the same vertebral insertion are tested, alternatively. For this purpose, the 10th vertebra is counted from the tail tip. All six tendons (four on the ventral and two on the dorsal side) inserting on this vertebra are removed and tested. The tendons are fixed in special clamps at a distance of 2.0 cm and immersed in physiological saline. Stress–strain curves and ultimate loads are determined with an Instron instrument with an extension rate of 5 cm/min.

A single injection of cortisol acetate (10–100 mg/kg) or prednisolone acetate (2 and 20 mg/kg) causes a dose-dependent increase of tensile strength allowing the calculation of a potency ratio of 1:4 for prednisolone versus cortisol. Repeated injections of cortisol acetate in doses between 1.0 and 50 mg/kg (s.c.) or prednisolone acetate in doses between 0.05 and 50 mg/kg (s.c.) show the same dose dependence and potency ratio. The tensile strength of tail tendons is similarly changed by treatment with gonadal steroids, depending on the dose and duration of treatment, but to a much lesser degree than by corticosteroids.

Evaluation

For changes in mechanical properties, the potency ratios of glucocorticoids versus cortisol as the standard are calculated.

Modifications of the Method

In addition to tensile strength, other biophysical parameters can be determined in tail tendons, such as hysteresis behavior (Vogel 1984). The ratio of energy input versus energy dissipation is calculated. Furthermore, dependence on strain rate and relaxation behavior under the influence of corticosteroids (Vogel 1989) as well as retardation (strain rate under constant load) has been studied (Vogel and Schorning 1990).

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Tensile Strength of Skin Strips in Rats

Purpose and Rationale

Clinical experience shows a decrease of skin thickness after long-term systemic or local treatment with corticosteroids (skin atrophy, thinning). The mechanical properties of human skin after treatment with corticosteroids are difficult to measure. As with other connective tissues, in animal experiments a clear increase of tensile strength of skin has been found after short-term administration of corticosteroids (Vogel 1969, 1971a, b, 1974, 1981,1986, 1993b).

Procedure

Male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated with increasing doses of

corticosteroids or other substances for periods of between 1 and 14 days. After sacrifice, the back of the animals is shaved and a flap of skin measuring about 5 × 5 cm is removed. The skin flap is placed between two pieces of plastic material with known thickness. By these means, the actual thickness of the excised skin is measured by calipers. Two dumbbell-shaped specimens are cut with a special punch in perpendicular direction to the body axis, having in the middle a width of 4 mm. They are fixed between the clamps of an Instron instrument. In the usual experiments, stress–strain curves and ultimate loads are recorded at a strain rate of 5 cm/min. From stress–strain curves, the values of ultimate load and ultimate extension are recorded. The stress–strain curves are almost linear in part, allowing the calculation of the modulus of elasticity. Tensile strength is calculated from ultimate load divided by original cross-sectional area.

Dose–response curves for 10 and 100 mg/kg cortisol acetate or 2 and 20 mg/kg prednisolone acetate show a sharp increase of ultimate load and tensile strength up to 5 days of treatment, a continuous decrease of skin thickness upon longer treatment, and a decrease of ultimate load up to 2 months of treatment, which is below controls for ultimate load, but not for tensile strength (Vogel 1970a).

Evaluation

Dose–response curves are established for corticosteroids versus the standard cortisol and potency ratios with confidence limits are calculated.

Modifications of the Method

With a similar method, the tensile strength of skin wounds after treatment with corticosteroids was studied in rats (Vogel 1970a, b). See also chapter "▶ Acne Models."

Strain rate influences the values of ultimate load, tensile strength, and modulus of elasticity, but not the effect of corticosteroids (Vogel 1972).

In contrast to parameters indicating the strength and elasticity of collagen, the parameters indicating plasticity, such as relaxation, hysteresis, strain at constant load (creep experiments), and isorheological behavior, are less dramatically changed by corticosteroids but indicate a decrease of viscosity or an increase of stiffness.

For a more detailed description of relaxation experiments (Vogel 1973), see chapter "▶ Acne Models"; of hysteresis experiments (Vogel 1976, 1989), see chapter "▶ Acne Models"; of creep experiments (strain at constant load) (Vogel 1977, 1989), see chapter "▶ Acne Models"; of measurement of isorheological behavior (Vogel 1987, 1989), see chapter "▶ Acne Models"; and for in vivo experiments in rat skin (Vogel and Denkel 1985; Vogel 1989, 1993a), see chapter "▶ Acne Models"

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Topical Effects of Glucocorticosteroids on Skin

Skin Thickness and Tensile Strength

Purpose and Rationale

From clinical experience it is known that a decrease of skin thickness occurs after long-term local treatment with corticosteroids. The mechanical properties of human skin after treatment with corticosteroids are difficult to assess. A clear increase of tensile strength of skin after short-term local administration of corticosteroids has been found in animal experiments, whereas skin thickness decreased (Alpermann et al. 1982; Vogel and Petri 1985).

Procedure

Male Sprague–Dawley rats with an initial weight of 150 ± 10 g are used. The animals are shaved before and once again during treatment. The animals are treated once daily over 10 days with various concentrations of the test compound or the standard or with fixed concentrations of the test compound in different galenical preparations. The test material is applied in a volume of 0.2 ml to an area of 4 cm^2 to the shaven back skin. The animals are kept in individual cages to avoid systemic absorption by mutual licking. Two days after the last treatment the animals are sacrificed, a flap of skin measuring about 5 × 5 cm is removed, and skin thickness is determined. The skin flap is placed between two pieces of plastic material with known thickness. The thickness of the excised skin is measured by calipers. Perpendicular to the body axis two dumbbell-shaped specimens are punched out. Stress–strain curves are recorded using an Instron instrument. The following parameters are determined:

- Load at rupture (ultimate load)
- Extension at rupture (ultimate strain)
- Skin thickness
- Tensile strength (ultimate load divided by cross-sectional area)
- Modulus of elasticity (calculated from the straight part of the stress–strain curve).

Evaluation

Dose–response curves are established for the parameters ultimate load, ultimate strain, skin thickness, tensile strength, and modulus of elasticity for the standard (prednisolone acetate) and the test compound allowing the calculation of potency ratios with confidence limits.

Critical Assessment of the Method

In addition to corticosteroid-induced atrophy of the skin after topical application assessed by histology, mechanical parameters can be determined.

Modifications of the Method

Kapp et al. (1977) used specially devised apparatus for rats covering the site of substance application, which guarantees an exclusive dermal absorption and excludes oral ingestion of the steroid.

In addition to skin thickness and breaking strength, Töpert et al. (1990) determined thymus weight, water and glycosaminoglycan content in the skin of rats after topical application of steroids over 30 days.

Iwasaki et al. (1995) measured the skin atrophy in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of simultaneously applied RU 486. Then, 24 h after the last application, the thickness of the skin was measured with a dial skin thickness gauge, with an accuracy of 0.01 mm. The treated skin was pinched lengthwise, and both skin surfaces were held vertically between two plastic discs of the gauge.

Hartop et al. (1978) measured transepithelial water loss (TEWL) in the skin of rats deficient in essential fatty acids (Prottey et al. 1976; Lowe and Stoughton 1977) after local treatment with corticosteroids.

Woodbury and Kligman (1992) recommended the hairless mouse as a model for assaying the atrophogenicity of topical steroids. Epidermal atrophy was determined by the number of cell layers of viable epidermis on five fields under × 400 magnification. Dermal thickness was determined under × 400 magnification. The total number of sebocytes in all visualizable sebaceous glands was counted in ten fields at × 250 magnification.

Van den Hoven et al. (1991) used the hairless mouse as a model to distinguish between local and systemic atrophogenic effects of topical steroids. Male hairless mice (strain h/h-NMRI) were treated on the left flank with test preparations once daily for 3 weeks. During this time, body weight was measured and the skinfold thickness of treated and untreated sides was determined using a graduated micrometer. On day 21, the animals were sacrificed and dermal thymidine uptake and weights of the thymus were determined.

Corticosteroid-induced skin atrophy in hairless mice can be prevented by tretinoin (Lesnik et al. 1989; Schwartz et al. 1994). In these studies, albino Skh-hairless-1 mice were treated topically twice daily with corticosteroids or in combination with tretinoin. At the end of the treatment, all mice were sacrificed and specimens of the dorsal skin were frozen for light microscopy and quantification of glycosaminoglycans, fibronectin, and collagen.

Wrench (1980) applied commercially available topical corticosteroid preparations to the proximal halves of albino mouse tail for 21 days and measured epidermal thickness by histology. All steroids caused epidermal thinning, except clobetasone butyrate.

Altmeyer and Buhles (1981) tested the antiacanthogenic effect of topically applied steroids after long-term treatment of guinea pigs (up to 118 days) by histological measurement of epithelial thickness. For delimitation of the measuring lines, the upper boundary was taken as that between the stratum granulosum and the stratum corneum, and the lower boundary as that between the basal cell layer and the corium.

Kajita et al. (1986) tested epidermal beta-adrenergic adenylate cyclase responses in pigs after topical application of glucocorticoids. A significant increase of this receptor response was observed 24 h following topical application of potent glucocorticoid ointments. Domestic white-haired pigs were anesthetized with 30 mg/kg nembutal i.p. Four 5 × 5 cm areas were chosen and the following treatments were administered to each area: (1) topical administration of glucocorticoid ointments; (2) UVB irradiation alone (230 mJ/cm^2); (3) topical application of glucocorticoid ointments following UVB irradiation; (4) no treatment as a control. After 24 h the treated pigs were anesthetized again and skin specimens were obtained from the four areas by means of a Castroviejo keratome (Storz Instrument, St. Louis, Mo., USA) adjusted to the 0.3 mm setting. Each skin slice obtained by the Castroviejo keratome was cut into 5 × 5 cm squares, which were washed three times in RPMI 1640 medium and preincubated in RPMI 1640 medium for 37 °C to standardize the cAMP

level (Yoshikawa et al. 1975). After the preincubation, two pieces of skin squares were randomly selected and were incubated with various adenylate cyclase stimulators (Iizuka et al. 1985). The concentrations of epinephrine, adenosine, and histamine added to the incubation medium were 50 μM, 2 mM and 1 mM, respectively. The cAMP content in skin squares was measured by radioimmunoassay using a Yamasa cAMP assay kit (Yamasa Shoyu, Tokyo, Japan) after partial purification (Yoshikawa et al. 1975). The cAMP phosphodiesterase activities in skin squares were measured by the method of Adachi et al. (1976) who purified multiple forms of pig epidermal cyclic nucleotide phosphodiesterases by DEAE-cellulose column chromatography.

In vitro experiments measuring the effects of retinoids and glucocorticoids on the beta-adrenergic adenylate cyclase system of pig skin epidermis were performed by Iizuka et al. (1985).

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Assay of Topical Glucocorticoid Activity in Transgenic Mice

Purpose and Rationale

Katchman et al. (1995) proposed a transgenic mouse model as a biological assay of topical glucocorticosteroid potency.

Procedure

A homozygous line of transgenic mice expressing 5.2 kilobases (kb) of the human elastin promoter region linked to the chloramphenicol acetyltransferase (CAT) reporter gene was developed (Hsu-Wong et al. 1994). For this purpose, 5.2 kb of human elastin 5′-flanking DNA is linked to a 0.7-kb CAT gene, followed by 0.3 kb of DNA with a polyadenylation signal. This linearized construct is injected into fertilized oocytes, and a line of transgenic mice expressing the human elastin promoter, as detected by CAT activity, is developed. These transgenic mice have no clinical phenotype and they do not express human elastin protein, as no part of the coding sequence is contained within the transgene. The human elastin promoter/CAT construct is expressed in a tissue-specific and developmentally regulated manner.

Test animals are 4- or 5-day-old hairless pups, homozygous for the transgene. In each experiment, pups of the same litter are used for comparison between glucocorticosteroid-treated and control preparations in parallel. Steroid preparations are tested by applying 0.03 g uniformly on their dorsal surface (≈14 mg/cm^2). Control animals receive the same amount of cream base. The test animals are separated from each other and from their mothers and are sacrificed at different time points. Skin biopsy samples are removed immediately from the treated area.

For the CAT assay, skin is homogenized with a Polytron tissue homogenizer in 0.25 M TRIS hydrochloride (pH 7.5). The homogenates are freeze-thawed three times and centrifuged at 10,000 *g* for 15 min. The protein content of the supernatant is determined by a commercial kit and aliquots, containing 100 mg of protein, are assayed for CAT activity in the linear range of the assay (Gorman et al. 1982) (see section "Transactivation and Transrepression Assays for Glucocorticoids").

Evaluation

The significance of differences between treatment groups is evaluated by Student's *t*-test.

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Effect on Epidermal DNA Synthesis

Purpose and Rationale

Du Vivier et al. (1978), Marshall and Du Vivier (1978), Marshall et al. (1981), and Clement et al. (1983) evaluated the local and systemic effects of topically applied corticosteroids on epidermal DNA synthesis in hairless mice.

Procedure

Hairless mice of either sex, 2–4 months old, are dosed in groups of six with test preparations or control base: 0.03 ml of test drug formulation is

spread over 3 cm^2 of the anterior dorsal skin. The posterior half of the dorsal surface of the animal is left untreated. Animals are injected subcutaneously with 25 mCi (methyl-^{3}H) thymidine, specific activity 5 Ci/mM, in the right thigh 1 h before being sacrificed 6 or 24 h after application of test formulation.

The neck and back skin samples, with at least 2 cm unsampled skin left between them (to avoid inclusion of treated skin in the back sample) are removed. The epidermis is separated from the dermis by placing the sample, dermis downwards, on a stainless steel plate at 57 °C for 25 s. The epidermis can then easily be separated from the dermis with a scalpel blade. The epidermal samples are wrapped in aluminum foil and stored at 20 °C until analysis.

Approximately 30 mg of epidermis in 5 ml of a 0.24 M sodium phosphate buffer solution, containing 8 M urea, 1 % sodium lauryl sulfate and 1 mM EDTA at pH 6.8, is lysed using an ultrasonic disintegrator. DNA is extracted from the cell lysate by column chromatography using hydroxylapatite. The epidermal cell lysate is added to the hydroxylapatite column and washed with a buffer containing 0.24 M sodium phosphate and 8 M urea at pH 6.8 to remove RNA and protein. The urea is then removed with 50 ml of 0.14 M sodium phosphate buffer at pH 6.8. Double-stranded DNA, which binds to hydroxylapatite under low-salt buffer conditions, is eluted with 0.48 M sodium phosphate buffer at pH 6.8, and a 2-ml sample is collected.

The DNA concentration in the sample is determined by UV absorption at 260 nm in a spectrophotometer. Then 0.5-ml aliquots of the sample are added to 12 ml scintillation cocktail and 0.2 ml N HCl in scintillation vials. Each sample is prepared in triplicate and counted for 20 min/vial in a scintillation counter.

Evaluation

The results are initially expressed as mean counts per minute (c.p.m.) for the vial triplets, converted to mean disintegrations per minute (d.p.m.) and the results expressed as d.p.m./μg DNA. The significance of differences between treatment groups is evaluated by Student's *t*-test.

Modifications of the Method

Marks et al. (1973) described a method for the assay of topical corticosteroids in mice based on changes in thymidine incorporation after treatment. Sticky tape was applied five or six times to the dorsal skin of mature male hairless mice of the Harwell strain to remove the horny layer. A similar quantity of topical test preparations (about 70 mg) was applied on the stripped dorsal skin of each mouse. A dressing of cotton gauze lined with a plastic film was applied and secured with strapping, staying for periods of from 5 to 156 h. Then 4 h before sacrifice the mice were intraperitoneally injected with 0.15 ml of a 0.1 % Colcemid solution together with 30 μCi of tritiated thymidine. The dorsal skin area previously treated was removed, fixed in 10 % buffered formalin and stained with hematoxylin and eosin. For estimation of the mitotic index, cells in the prophase, anaphase, and metaphase stages were counted. To quantitate the labeling index, the number of labeled basal and suprabasal cells was estimated as a proportion of the total number of basal cells.

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Induction of Drug-Metabolizing Enzymes

Purpose and Rationale

Finnen et al. (1984, 1985) evaluated the effects of topical application of glucocorticosteroids on the activity of drug-metabolizing enzymes in the skin of adult hairless mice. The ability of steroid preparations to induce enzyme activity was related to their clinical potency.

Procedure

Adult male and female hairless mice are treated with 0.3 g of test creams or cream base applied to the dorsal skin and rubbed in until no visible traces remain. Then 16–18 h after treatment, the animals are sacrificed, the treated area of skin removed, and placed epidermis-down on a curved surface, and subcutaneous fat and muscle are removed using a scalpel blade. Epidermis and dermis are separated by a heating technique (Thompson and Slaga 1976). The tissue is then placed in 0.1 M phosphate buffer (pH 7.4) and minced finely using surgical scissors. Scissors-minced tissue is then homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) using a Polytron homogenizer. Whole-skin homogenates are then centrifuged at 9,000 *g* for 20 min and the resulting supernatant used as the source for the determination of 7-ethoxycoumarin *O*-deethylase (Greenlee and Poland 1978). For the determination of ethoxyresorufin *O*-dealkylation (Pohl and Fouts 1980) the 9,000 *g* supernatants are further centrifuged at 100,000 *g* for 1 h, the resulting microsomal pellet resuspended in 0.1 M phosphate buffer (pH 7.4), and used as the enzyme source. The DNA content of the 9,000 *g* pellet is estimated by the diphenylamine reaction (Burton 1956).

Evaluation

The significance of differences between treatment groups is evaluated by Student's *t*-test.

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Cornea Inflammation in Rabbits

Purpose and Rationale

Leibowitz and Kupferman (1974), Leibowitz et al. (1978, 1992) and Cantrill et al. (1975) studied the anti-inflammatory efficacy of topical corticosteroids for treatment of glaucoma in rabbits. Again, this test is purely of historical interest.

Procedure

New Zealand albino rabbits weighing 1.8–2.3 kg are anesthetized for approximately 5 min with 15 mg/kg i.v. thiamylal sodium. A corneal inflammatory response is produced by intralamellar inoculation of 0.03 ml of laboratory-grade clove oil. Before the induction of corneal inflammation, the rabbits are given three intravenous inoculations of 1.85×10^6 Bq/kg of an aqueous solution of tritiated thymidine (24.79×10^{10} Bq/mol) at 24-h intervals. The intracorneal injection of clove oil is given concomitantly with the third thymidine injection, and 24 h later, therapy is initiated. A standard drop (0.05 ml) of drug is instilled

hourly for a total of six doses, and then, after a lapse of 18 h, one drop is administered hourly for an additional total of seven doses.

Corticosteroid preparations are tested in different concentrations. A control group is run with each experimental trial; control rabbits are handled in the same manner as experimental rabbits, except that the control rabbits receive either prednisolone acetate (positive control) or no treatment (negative control). At autopsy, a 10-mm penetrating corneal button is removed by trephination, tissue samples are placed in a commercially available solubilizing agent (Soluene 350, Packard Instrument), 1 ml per cornea, until dissolved. The samples are counted for a minimum of 10 min, measuring the amount of radioactivity in each cornea sample.

Evaluation

The significance of differences between treatment groups is evaluated by Student's *t*-test.

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Endotoxin-Induced Uveitis in Rats

Purpose and Rationale

Salmonella endotoxin, a lipopolysaccharide, produces uveitis 12–24 h after injection into the footpad of rats. Uveitis is characterized by miosis, iris hyperemia, increase of protein content in aqueous humor, and inflammatory cell accumulation in the anterior uvea and aqueous humor. Glucocorticoids strongly inhibit endotoxin-induced uveitis by suppressing inflammatory mediators (Cousins et al. 1982; Tsuji et al. 1997).

Procedure

Female inbred Lewis rats weighing about 160 g are used. Then 500 μg/kg *Salmonella* endotoxin dissolved in saline is injected into the footpads. Twelve hours later, the animals are sacrificed and both eyes of each animal used in the experiments. The anterior chamber of the eye is punctured, using a 27-gauge needle, to collect the aqueous humor. Then 5-μl aqueous humor samples are placed into 495 μl of phosphate-buffered saline containing 1 % paraformaldehyde. The number of cells in the aqueous humor is then counted using a flow cytometry system. The cell number for both eyes of each animal is averaged for statistical analysis of the results.

In topical applications, glucocorticoids are instilled (5 μl/eye) three times at 1 h before and 3 and 7 h after lipopolysaccharide injection. For systemic application, glucocorticoids are injected subcutaneously 3 h after lipopolysaccharide injection.

Total RNA is isolated from the iris-ciliary body with a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). Each RNA sample is extracted from the pooled iris-ciliary bodies of both eyes of each animal. The extracted RNA is quantified after which 4 μg is used for DNA synthesis via polymerase chain reaction.

Evaluation

Dunnetts's multiple comparison test was applied for statistical analysis.

Further methods for measuring local or topical activity of corticosteroids are very suitable for evaluating local corticosteroid activity, described in chapter "► Anti-Inflammatory Activity," such as oxazolone-induced ear edema in mice (see chapter "► Anti-Inflammatory Activity") and croton-oil-induced ear edema in rats or mice (see chapter "► Anti-Inflammatory Activity").

Furthermore, methods described in chapter "► Methods for Testing Immunological Factors," and section "Delayed Type Hypersensitivity (DTH)" are Recommended as Models for Testing Topical CorTicosteroid Potency.

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Croton Oil-Induced Ear Inflammation

Topical application of the nonspecific contact irritant croton oil, a mixture of several phorbol esters, leads to acute inflammation characterized by edema and a mainly granulocytic cell infiltration into the skin. For topical application, compounds are dissolved in the same vehicle as used for croton oil and are coapplied. Systemic application of compounds (s.c.) is performed 2 h before croton oil application. At the maximum of the inflammatory reaction, animals are killed, and ears (mice, area ≈ 1 cm^2) or a punch biopsy (rats, 10 mm diameter) are weighed as an indicator of edema formation, then snapfrozen in liquid nitrogen in polypropylene tubes and kept at -20 °C for up to 24 h.

Antiglucocorticoid Activity

Adrenal and Thymus Involution

Purpose and Rationale

The involution of thymus and adrenal glands induced by hydrocortisone can be antagonized by compounds with antiglucocorticoid activity (Dorfmann 1962).

Procedure

Groups of six to ten immature male Sprague–Dawley rats weighing 60–70 are injected subcutaneously daily for 6 days with 0.2 ml of the antagonistic test compound in 0.5 % aqueous carboxymethylcellulose solution. Hydrocortisone acetate is used as the agonist in daily doses of 0.2 mg per animal. Controls receive hydrocortisone acetate only. On the seventh day, the animals are sacrificed and the adrenal and thymus weights determined.

Evaluation

The relative weight [quotient of adrenal or thymus weight (mg) and body weight (g)] is calculated for each rat. Means of the antagonist-treated groups are compared with the means of hydrocortisone-only controls. An increase of thymus and adrenal weight relative to the hydrocortisone control indicates antiglucocorticoid activity.

Modifications of the Method

Inhibition of tyrosine aminotransferase induced by corticosterone or dexamethasone was used by Vincent et al. (1997) to characterize the antiglucocorticoid properties of a novel synthetic steroid.

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Mineralocorticoid Activity

In Vivo Methods

General Considerations

Very early bioassays ("Survival tests") have been used to standardize extracts of adrenal cortex for mineralocorticoid activity (Grollman 1941; Ringler 1964; Tolksdorf et al. 1956). For evaluation of mineralocorticoid activity these tests have been replaced by bioassays measuring electrolyte excretion in the urine.

Several animal species do not survive after adrenalectomy. However, they can be kept alive by administration of mineralocorticoids. The first animal species used successfully was the adrenalectomized drake (Bülbring 1937). The methods employing adrenalectomized rats and mice (Dorfman 1962) suffered from the fact that in these species aberrant adrenal cortical tissue may occur, which is not removed by surgical adrenalectomy. In contrast, male Syrian golden hamsters weighing 50–60 g are very suitable for testing adrenocortical activity (Junkmann 1955).

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Electrolyte Excretion

Purpose and Rationale

Mineralocorticosteroids enhance sodium retention and potassium excretion. The sodium excretion in adrenalectomized rats is dose-dependently decreased. This parameter can be used to assess the mineralocorticoid activity of test compounds (Kagawa et al. 1952; Marcus et al. 1952).

Procedure

Male Sprague–Dawley rats weighing 140–160 g are adrenalectomized. They are maintained on 1 % sodium chloride solution. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; 1 h later 5 ml of 0.9 % sodium chloride solution is given orally. Test compounds are injected s.c. in 0.2 ml of vehicle suspension. Desoxycorticosterone acetate (DOCA; 1–40 μg per rat) is used as a standard (reference compound). The rats are placed in metabolic cages, two rats per cage, three cages per dosage group, for 4 h, and then removed from the cages. The use of light, transient ether anesthesia in this method is obsolete. Urine volume is recorded and in addition cages may be rinsed with a distilled water spray. Appropriate dilutions of urine are analyzed for sodium with a flame photometer. The amount of sodium excreted by each rat is calculated, and group means are expressed as percentage of excretion by control animals.

Evaluation

Percent reduction of sodium excretion compared with controls is calculated for each dosage group. Potency ratios are calculated with reference to desoxycorticosterone acetate.

Modifications of the Method

Simpson and Tait (1952) measured both urinary sodium and potassium and used the sodium-to-potassium ratio as an index of the electrolyte activity of corticoids. Nikisch et al. (1991) infused glucocorticoid-substituted adrenalectomized rats with saline-glucose solution containing aldosterone and measured sodium and potassium concentrations in 1-h fractions of urine. The antialdosterone activity was assessed by the ability of test compounds to reverse the aldosterone effect on the urinary Na/K ratio.

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In Vitro Methods

Mineralocorticoid Receptor Binding

Purpose and Rationale

Rat kidney receptor preparations and radioactively labeled aldosterone were used to test affinity for the mineralocorticoid receptor (Raynaud et al. 1975, 1979; Pasqualini and Sumida 1977; Ojasoo and Raynaud 1978; Wambach and Higgins 1971).

Procedure

Kidney homogenates of adrenalectomized rats are centrifuged at 0 °C for 10 min at 800 *g* in a buffer solution (10 mM Tris, 0.25 M saccharose, HCl, pH 7.4). Following addition of RU 28362 at 0.001 mM (to inhibit binding of aldosterone to the glucocorticoid receptor), the supernatant is centrifuged again at 105,000 *g* for 60 min. The supernatant (cytosol) is removed and incubated at 0 °C with ^{3}H-aldosterone (5 nM) and increasing concentrations of test compounds (0–25,000 nM). Nonspecific binding is determined in the presence of 1 μM aldosterone. Free ^{3}H-aldosterone is removed from the incubation medium by charcoal-dextran absorption after 1 h or 24 h of incubation (Raynaud 1978). Following centrifugation, the concentration of receptor-bound ligand is determined in the supernatant by liquid scintillation counting.

Evaluation

The following parameters are calculated: total binding of ^{3}H-aldosterone, nonspecific binding in the presence of 1 μM aldosterone, specific binding (= total binding–non-specific binding), % inhibition with specific binding as a percentage of the control value.

For structure–activity studies, all compounds are first tested at a single high concentration (25,000 nM) in triplicate. For those showing more than 50 % inhibition a displacement curve is determined using seven to eight different concentrations of compound. The binding potency of compounds is expressed as binding affinity (RBA), relative to the standard compound (aldosterone).

Modifications of the Method

Affinity for the mineralocorticoid receptor was tested using the cytosol of rabbit kidneys (Claire et al. 1993) or rat kidney slices (Funder et al. 1974). It is also useful to use overexpressed human mineralocorticoid receptors.

Monkey kidney COS-1 cells were transfected with plasmids containing the human mineralocorticoid receptor – cloned by Arriza et al. (1987) – and the glucocorticoid receptor for binding studies with various steroids (Rupprecht et al. 1993a, b).

A survey of mineralocorticoid receptor ligands has been given by Sutano and de Kloet (1991).

Specific bioluminescent in vitro assays for selecting potential antimineralocorticoids were developed by Jausons-Loffreda et al. (1994).

Wehling (1994) found evidence for a membrane-bound mineralocorticoid receptor in human mononuclear leukocytes.

The human mineralocorticoid receptor was used by Grassy et al. (1997) to study

structure–activity relationships of steroids with antimineralocorticoid activity.

Davioud et al. (1996) described the synthesis and biological activities of new steroidal diazoketones as potential photoaffinity labeling reagents for the mineralocorticoid receptor.

Fagart et al. (1997a) recommended [^{3}H-2]-21-diazoprogesterone as a potent photoaffinity labeling reagent for the mineralocorticoid receptor.

Fagart et al. (1997b) proposed a three-dimensional model for antagonism in the human mineralocorticoid receptor.

The biological and clinical relevance of glucocorticoid and mineralocorticoid receptors has been reviewed by Funder (1997).

Rogerson et al. (1999, 2003) studied the structural determinants of aldosterone-binding selectivity in the mineralocorticoid receptor. To characterize ligand-binding specificity, chimeras were made between the human mineralocorticoid receptor and glucocorticoid receptor ligand-binding domains. Models of the mineralocorticoid receptor ligand-binding domain bound to aldosterone and spironolactone were created based on the crystal structure of the progesterone receptor ligand-binding domain. These techniques have been used by Rogerson et al. (2004) to evaluate the differences in the determinants of eplerenone, spironolactone, and aldosterone binding to the mineralocorticoid receptor.

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Transactivation Assay for Mineralocorticoids

Purpose and Rationale

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also of the antagonistic activity of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

Procedure

Vector Construct

The expression plasmid pRShMR containing the full-length coding sequence of the mineralocorticoid receptor expressed from the long terminal repeat of the Rous sarcoma virus is prepared according to Arriza et al. (1987). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

CV-1 cells and COS-1 cells for transient infections are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum, 4 mmol/l L-glutamine, penicillin, and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80 % confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 μg pRShMR

(hMR expression plasmid) and 5 μg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 μg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml of DMEM supplemented with 10 % fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled, and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3 % charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1 % ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay (Chloramphenicol Acetyltransferase)

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations in the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

Evaluation

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to demonstrate the potency of the test hormone. Aldosterone (10^{-10} to 10^{-6} mol/l) serves as standard.

For antimineralocorticoid activity, CAT activity in the presence of 10^{-8} mol/l aldosterone is set as 100 % and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the antihormone.

Critical Assessment of the Method

See section "Transactivation and Transrepression Assays for Glucocorticoids."

Modifications of the Method

White et al. (1994) described a simple and sensitive high-throughput assay for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector, which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high levels of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase assay.

Rupprecht et al. (1993a, b) examined the functional agonistic and antagonistic activity of several steroids by cotransfecting human mineralocorticoid or human glucocorticoid receptor expression vectors, together with a mouse mammary tumor virus-luciferase (MTV-LUC) reporter gene into the human neuroblastoma cell line SK-N-MC. Transfections were performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, Calif., USA).

Lombès et al. (1994) used a cotransfection assay in CV-1 cells to study the discrimination of aldosterone from natural and synthetic glucocorticoids by the human mineralocorticoid receptor. Cells were transfected by the calcium phosphate method with pRShMR, a plasmid that contains the entire coding sequence of the human mineralocorticoid receptor; pFC31Luc, which contains the mouse mammary tumor virus (MMTV) promoter

driving the luciferase gene; pCH110 encoding the β-galactosidase as an internal transfection control; and pSP72 as plasmid carrier.

Lim-Tio et al. (1997) studied the determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor in three cell lines: CV-1 cells; a porcine renal epithelial cell line, LLC-PK1; a pig kidney cell strain, and RN33B; a neuronal medullary raphe cell line. The reporter gene used was MMTV-LUC (the long terminal repeat of the mouse mammary tumor virus promoter linked to the luciferase reporter gene). RSV-CAT (Rous sarcoma virus promoter-chloramphenicol acetyltransferase gene) was used as an internal control for the transfection efficacy.

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Antimineralocorticoid Activity

Electrolyte Excretion

Purpose and Rationale

The method is based on the mineralocorticoid activity of desoxycorticosterone acetate (agonists) (reference compound) reversed by spironolactone

(reference antagonist) and other antimineralocorticoids to be evaluated (test compounds).

Procedure

Male Sprague–Dawley rats weighing 140–160 g are adrenalectomized and maintained on 1 % NaCl solution as drinking fluid. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; 1 h later it is given 5 ml 0.9 % NaCl orally and injected with 40 μg desoxycorticosterone acetate. At a separate site, the antagonistic test compound is injected s.c. in 0.2 ml of vehicle suspension. Spironolactone, 50 μg or 500 μg, is injected as the reference compound with antimineralocorticoid activity. Rats are placed in metabolic cages, two rats per cage, three cages per dosage group, for 4 h of urine collection. Urine volume is recorded and cages rinsed over the collection cylinders with a distilled water spray. Appropriate dilutions of collected urine are analyzed for sodium and potassium with a flame photometer.

Evaluation

The amount of water, sodium, and potassium excreted per 100 g bodyweight is calculated. The product of water volume and sodium excretion is divided by the potassium excretion. The excretion ratios (quotient) are compared for untreated adrenalectomized rats, animals treated with DOCA, and animals treated with spironolactone.

Modifications of the Method

Antimineralocorticoid activity of spironolactone and its analogs has been tested in adrenalectomized golden hamsters treated simultaneously with daily injections desoxycorticosterone acetate over a period of 3 weeks (Vogel, unpublished data 1965). The mean survival of adrenalectomized animals of 4.7 days was prolonged to 13.5 days by daily subcutaneous injections of 2 mg desoxycorticosterone acetate. Additional injection of 0.5 or 1.0 mg spironolactone reduced the survival time.

Losert et al. (1985) tested the ability of several steroids with progestogenic potency to inhibit the renal actions of aldosterone in adrenalectomized, glucocorticoid-treated rats. The rats were continuously infused with an isotonic solution of low sodium content (0.05 % NaCl + 5.2 % glucose, 3 ml/rat per hour) supplemented with D-aldosterone (1 μg/kg per h) resulting in a long-lasting reduction in renal sodium excretion, an increase in renal potassium excretion, and hence a decrease in the urinary Na/K ratio. The test drugs were administered either subcutaneously or orally 1 h before the start of infusion. The antimineralocorticoid activity was judged by the increase in the aldosterone-lowered Na/K ratio in urine collected at hourly intervals up to 21 h.

Cutler et al. (1978, 1979) described a potent mineralocorticoid receptor antagonist with decreased antiandrogenic activity relative to spironolactone.

De Gasparo et al. (1987) evaluated epoxyspironolactone derivatives for their antimineralocorticoid activity (Kagawa test) and their antiandrogenic and progestogenic side effects in vitro and in vivo.

Gómez-Sánchez et al. (1990) reported the effect of intracerebroventricular infusion of mineralocorticoid antagonists on the hypertension in rats produced by chronic subcutaneous administration of aldosterone.

The evolution of aldosterone antagonists has been reviewed by Garthwaite and McMahon (2004) and Hu et al. (2005) featuring eplerenone, a molecule with improved steroid receptor selectivity and pharmacokinetic properties in humans compared with spironolactone.

The beneficial effects of eplerenone have been demonstrated in rats with myocardial infarction (Masson et al. 2004; Fraccarollo et al. 2005) and in rats with autoimmune myocarditis (Wahed et al. 2005) as well as in mice with chronic heart failure (Wang et al. 2004).

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Ovarian Hormones

Jürgen Sandow

Contents

Ovarian Hormones

Ovariectomy of Rats

Procedure

Ovariectomy is performed in immature female rats weighing 40–60 g (Bomskov 1939; Emmens 1969; May 1971). Animals are anesthetized. A single transverse incision is made in the skin of the back. This incision can be shifted readily from one side to the other by traction on the skin. A small puncture is then made over the site of the ovary, which can be seen through the abdominal wall, embedded in a pad of fat. The top of a pair of fine forceps is introduced, and the fat around the ovary is grasped, care being taken not to rupture the capsule around the ovary. The tip of the uterine horn is crushed with a pair of artery forceps, and the ovary, together with the fallopian tube, is removed with a single cut by a pair of fine scissors. Usually, no bleeding is observed. In older rats, the tip of the uterine horn may be ligated and the ovary removed distally from the ligature. The ovary of the other side is removed in the same way. The skin wound is closed by one or two clips. The animal recovers immediately.

Ovariectomized rats are now obtained from commercial suppliers when needed, the surgical procedure however is easy to learn, and licenses for animal experimentation are readily obtained for qualified laboratory personnel.

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Estrogens

In Vitro Methods

Estrogen Receptor Binding

Purpose and Rationale

The nuclear receptor assays are used to estimate the estrogenic activity of test compounds. Estradiol-17β is used as the reference compound. Estrogen receptors are prepared from mouse uteri or from human endometrium. Measurements of association rates and dissociation rates at different temperatures allow evaluation of relative binding affinities (Bouton and Raynaud 1977, 1978). Binding to the cytosolic and the nuclear fractions is measured.

Procedure

Cytosol Preparation

Uteri from 18-day-old female Swiss mice are removed and homogenized at 0 °C in 1:50 (w/v) of 10 mM Tris–HCl (pH 7.4) and 0.25 M sucrose buffer in a conical homogenizer. Human endometrium from menopausal women is frozen within 2 h of hysterectomy and stored in liquid nitrogen until use. The frozen endometrium is pulverized and homogenized in 1:5 (w/v) Tris-sucrose buffer. Homogenates are centrifuged for 1 h at 105,000 g.

Dextran-Coated Charcoal (DCC) Adsorption Technique

Binding is measured as follows. A 100-µl aliquot of incubated cytosol is stirred for 10 min at 0 °C in a microtiter plate with 100 µl of dextran-coated charcoal (DCC) suspension (0.625 % dextran 80,000, 1.25 % charcoal Norit A) and then centrifuged for 10 min at 800 g. The concentration of bound steroid is determined by measuring the radioactivity in a 100-µl aliquot of supernatant.

Determination of Specific Binding in Mouse Uterus Cytosol as a Function of Steroid Concentration, Incubation Time, and Temperature

Triplicate aliquots of 125 µl of cytosol are incubated with 5 or 25 nM labeled steroid (estradiol-17ß) either for 2 or 24 h at 0 °C or for 2 or 5 h at 25 °C in the absence (total binding) or presence (nonspecific binding) of a 100-fold excess of radio-inert steroid. Bound steroid is measured by DCC adsorption.

Measurement of Association Rate at 0 °C

For this, 1 or 5 nM concentration of labeled steroid is added to cytosol maintained at 0 °C. Every 5 min for 1 h after addition of the labeled steroid, a 100-µl aliquot is transferred into a 5,000 nM radio-inert steroid solution in a microtiter plate to stop the reaction. Bound radioactivity is determined by DCC adsorption.

Measurement of Dissociation Rate at 25 °C

Dissociation rate is measured by the isotopic dilution technique. Radio-inert steroid (2,500 nM) is added to crude cytosol previously incubated with 5 nm labeled steroid for 15 h at 0 °C. After different times of incubation at 25 °C, 100-µl samples are treated with DCC at 0 °C in order to determine bound radioactivity. Specific binding is evaluated by subtracting nonspecific binding from total binding.

Nuclear Uptake

Homogenate samples (0.5 ml; two uteri per 0.5 ml) are incubated with 2.5, 5, or 25 nM labeled steroid for 5 or 60 min at 25 °C, then cooled on ice, and centrifuged at 800 g for 10 min at 0 °C.

The pellet (crude nuclei) is washed three times with 1 ml Tris-sucrose buffer, dissolved in 0.5 ml Soluene (Packard) and counted.

Nuclear Extract Preparation

After incubation with 25 nM labeled steroid for 1 h at 25 °C, the crude nuclei are resuspended in 0.5 ml Tris-sucrose buffer, to which 50 µl of 4 M KCl is added. The suspension is stirred (vortex), left for 30 min at 0 °C, and then centrifuged for 30 min at 105,000 g. The radioactivity in a 100-µl supernatant sample (nuclear extract) is counted.

Evaluation

Relative binding affinity is calculated from the percentage of radioligand bound in the presence of a competitor compared to radioligand bound in its absence, plotted against the concentration of unlabeled competing steroid. A standard curve for the competition of the unlabeled radioligand is constructed with the use of nine to ten concentrations; five or six concentrations of the competitor are tested. These are chosen to provide a linear portion of the semilog plot which crosses the point of 50 % competition. From this plot, the molar concentration of unlabeled radioligand or steroid competitor is determined that reduces radioligand binding by 50 %.

The relative affinity of a test compound is the ratio of unlabeled radioligand concentration to competitor concentration, at 50 % competition. This ratio is multiplied by 100.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}t = (2.3/E_0 - R_0)\log(ER_0/RE_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t = 0$ and time t, respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3\log B/B_0$$

where B_0 and B represent bound steroid at time $t = 0$ and time t, respectively.

Similar studies were reported by Raynaud et al. (1975), Clark et al. (1976), Katzenellenbogen et al. (1977), Ojasoo and Raynaud (1978), Clark et al. (1982), Mukawa et al. (1988), Smanik et al. (1989), Labrie et al. (1990), Chander et al. (1991), and Dhar et al. (1994).

Modifications of the Method

Sheep uteri were used for the preparation of cytosol by Shutt and Cox (1972).

Pons et al. (1990) described receptor binding of estrogens and antiestrogens in the estrogen receptor-positive breast cancer cell line MCF-7, using the firefly luciferase assay (Brasier et al. 1989) as endpoint.

Ludwig et al. (1990) described a microtiter well assay for the quantitative measurement of estrogen receptor binding to estrogen-responsive elements.

Hwang et al. (1992) studied the use of tetrahydrochrysenes, inherently fluorescent, which are high-affinity ligands for the estrogen receptor.

Estrogen receptor binding has also been used to study the mechanism of action of nonsteroidal antiestrogens (Jordan et al. 1977; Wakeling and Slater 1980; Astroff and Safe 1988).

The sequence and expression of human estrogen receptor complementary DNA were reported by Greene et al. (1986).

Solution structure of the DNA-binding domain of the estrogen receptor has been described by Schwabe et al. (1990).

Obourn et al. (1993) studied the hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor overproduced in insect cells using a baculovirus expression system.

Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β are reported by Tremblay et al. (1997).

Nichols et al. (1998) showed that agonists and antagonists differently position the C-terminus of the ligand-binding domain (helix 12) and the F domain of the estrogen receptor.

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Transactivation Assay for Estrogens

Purpose and Rationale

Transient transfection of a suitable cell culture with a reporter gene controlled by an estrogen-responsive element (ERE) has become a convenient assay for estrogens.

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone-responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988).

The assay in the estrogen receptor-positive human breast cancer cell line MCF-7, described by Meyer et al. (1994) as a rapid luciferase transfection assay for transcription activation of estrogenic drugs, has been used for evaluation of synthetic estrogen antagonists by Von Angerer et al. (1994, 1995) and Biberger and Von Angerer (1996).

Procedure

MCF-7 cells used for transfection are grown in Dulbecco's modified essential medium supplemented with 10 % fetal calf serum, 100 U penicillin, 100 µg streptomycin, and 150 mg L-glutamine in 500 ml of medium without phenol red. Shortly before confluence, the cells are washed with 10 ml of PBS. Cells are gently shaken for a few seconds with trypsin-EDTA solution (4 ml) and after removal of the solution incubated for 2 min at 37 °C. After addition of 10 ml of medium, the cell suspension (0.5 ml/well) is transferred to 6-well plates containing 2 ml of medium. Cells are grown until the density of the monolayer is about 50 % (1–2 days) before 2 µg of the luciferase reporter plasmid EREwtc luc, harboring the luciferase gene from *Photinus pyralis*, is added. For a successful transfection, it is necessary to generate a very fine precipitate of the DNA by subsequent dilution with 45 % water, 5 % M $CaCl_2$, and 50 % HBS buffer and continuous shaking. After 20 min at room temperature, an opalescent solution should be obtained.

After addition of the DNA solution, the medium is removed, and the cells are washed with 2 ml of PBS, followed by treatment with glycerol (15 % in PBS) for 2 min. After washing with PBS, fresh medium containing the test substances is added. The maximum of luciferase expression is reached 18 h after addition of the transfection solution. At this time, the medium is removed and cells are washed with PBS. Cell lysis and quantification of luminescence are performed according to the procedure described in the luciferase assay system E1500 of Promega (SERVA, Heidelberg, Germany). Luminescence is measured in a luminometer Lumat LB (Berthold, Wildbad, Germany) as relative light units, which are converted into fg luciferase by a calibration curve.

Evaluation

Dose–response curves of luciferase activity after induction by the test substances and estradiol as control (10^{-14} to 10^{-9} M) are established.

Critical Assessment of the Method

See chapter "► Adrenal Steroid Hormones". Variation in transfection efficacy is the major cause of data variation and needs to be controlled for valid interpretation of data.

Modifications of the Method

With the same method as for MCF-7 cells, Von Angerer et al. (1994) and Biberger and Von Angerer (1996) transfected HeLa cells using the estrogen expression receptors HE0, HEG0, HE15, and HEG19, together with the receptor plasmid EREwtc luc.

Bergmann et al. (1994) determined estrogenic activity by a transient transfection assay in CHO cells deficient in estrogen receptors (ER) transfected with an expression vector encoding the ER using an estrogen-responsive reporter gene construct, $(ERE)_2$-TATA-CAT, containing two estrogen-response elements linked to a TATA promoter and the chloramphenicol transferase reporter gene.

For assessing environmental chemicals for estrogenicity, Shelby et al. (1996) recommended a transcriptional activation assay in ER-transfected HeLa cells using the estrogen-responsive reporter, ERE81CAT, and the pRSV vector containing the mouse ER cDNA without the neomycin resistance cassette. Triplicate samples for each hormone concentration were harvested at 28 h post-transfection and assayed for CAT protein using the CAT-ELISA kit (Boehringer Mannheim).

Several authors used yeast for assays of estrogenicity (McDonnell et al. 1991; Pierrat et al. 1992; Kohno et al. 1994; Bush et al. 1996; Tran et al. 1996; Odum et al. 1997).

Pierrat et al. (1992) constructed yeast strains in which the *Saccharomyces cerevisiae* URA3 gene is induced by the human estrogen receptor. Promoter sequences required for both basal and activated transcription of URA3 were replaced with estrogen-response elements positioned upstream of the native TATA box. These constructs were integrated at the TRP1 locus of a yeast strain in which the natural URA3 gene has been deleted and the integrants were transformed with shuttle plasmids expressing wild-type or truncated derivatives of human estrogen receptor. Transformants were assayed for growth on uracil-deficient medium plus or minus estradiol, for resistance to 5-fluoroorotic acid, and for activity of orotidine-5′-monophosphate decarboxylase, the product of the URA3 gene.

Tran et al. (1996) used the yeast strain ER (wt) expressing human estrogen receptor and an estrogen-sensitive reporter to characterize the estrogenic or antiestrogenic activities of polynuclear aromatic hydrocarbons.

Gaido et al. (1997) described a yeast-based steroid hormone receptor gene transcription assay to evaluate estrogenic and androgenic activity. The yeasts contain two separate plasmids: (1) an expression plasmid which contains the CUP1 metallothionein promoter fused to the human estrogen receptor cDND and (2) a receptor plasmid carrying two estrogen-response elements or a reporter plasmid carrying two copies of a progesterone-/androgen-responsive element upstream of the structural gene for β-galactosidase.

Chu et al. (2004) described transrepression of estrogen receptor β signaling by nuclear factor-κB in ovarian granulosa cells.

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Estrogen-Dependent Cell Proliferation

Purpose and Rationale

Specific cell proliferation induced by estrogens is the principle of these assays. Some human breast cancer cell lines, such as MCF-7 and T47-D cells, respond to estrogens with proliferation (Zondek 1935; Eisler 1964). This effect has been used for assays of estrogenic and antiestrogenic activity (Miller and Katzenellenbogen 1983; Scholl et al. 1983; Thompson et al. 1984; Palkowitz et al. 1997; Zacharewski 1997).

Procedure

MCF-7 breast adenocarcinoma cells are maintained in MEM (minimal essential medium), minus phenol red (which is estrogenic at high concentrations) supplemented with 10 % fetal bovine serum (FBS), 2 μM L-glutamine, 1 μM sodium pyruvate,10 μM HEPES, nonessential amino acids, and 1 mg/ml bovine insulin. Ten days prior to assay, MCF-7 cells are switched to maintenance medium supplemented with 10 % dextran-coated FBS in place of 10 % FBS to deplete internal stores of steroids. MCF-7 cells

are removed from maintenance flasks using cell dissociation medium [Ca^{2+}-/Mg^{2+}-free HBSS (phenol red-free) supplemented with 10 µM HEPES and 2 nM EDTA]. Cells are washed twice with assay medium and adjusted to 80,000 cells/ml. Approximately 100 µl (8,000 cells) is added to flat-bottomed microculture wells and incubated at 37 °C in a 5 % CO_2 humidified incubator for 48 h to allow for cell adherence and equilibration transfer. Serial dilutions of test compounds, or dimethyl sulfoxide (DMSO) as a diluent control, are prepared in assay medium and 50 µl transferred to triplicate microcultures, followed by 50 µl assay medium for a final volume of 200 µl. After an additional 48 h incubation, microcultures are pulsed with 1 µCi [^{3}H] thymidine (specific activity 6.7 Ci/mmol) for the last 4–6 h of culture and the assay terminated by freezing at −70 °C. Microcultures are then thawed and harvested using a Skatron semiautomatic cell harvester. Samples are counted by liquid scintillation using a Wallac Betaplate β-counter.

Evaluation

Plotting of dpm versus compound concentration is used to determine the half-maximal effective concentrations EC_{50} or inhibitory concentration IC_{50}.

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In Vivo Methods

Vaginal Cornification Assay

Purpose and Rationale

This is an early bioassay for estrogenic activity based on epithelial proliferation. The Allen–Doisy test for vaginal cornification in rodents (Allen and Doisy 1923) is based on the observations of Stockard and Papanicolaou (1917), who first reported the cyclic cornification of the vaginal epithelium in guinea pigs.

Procedure

Immature female Sprague–Dawley rats weighing about 55 g are ovariectomized. They are kept for about 1 week on standard laboratory diet and water ad libitum. The test compounds are administered orally or subcutaneously in 0.5 % solution of carboxymethylcellulose or in cotton seed oil injected at several doses to groups of 10–20 rats. Doses of 0.02, 0.1, and 0.5 µg estradiol per animal are used as standard. The compounds are dosed twice daily, for example, on two following days at 10:00 a.m. and 5:00 p.m. At 5:00 p.m. of the third day and at 10:00 a.m. of the fourth day, vaginal smears are taken using cotton swabs moistened with saline. The smears are transferred to a glass slide and stained for 10 min with 5 % aqueous methylene blue solution. They are evaluated microscopically according to the following scores:

0 → diestrus smear, mainly leukocytes, few epithelial cells
1 → mixture of leukocytes and epithelial cells
2 → proestrus smear, nucleated or nucleated plus cornified cells
3 → estrus smear, cornified cells only

Only animals showing a score of 2 or 3 of cornification are considered to be positive.

Evaluation

The number of positive animals in each dosage group is recorded. ED_{50} values are calculated, and potency ratios compared with the standard estradiol may be determined.

Modifications of the Method

The sensitivity of the assay is increased by local vaginal application of estrogens in ovariectomized rats or mice (Emmens 1969).

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Uterine Weight Assay

Purpose and Rationale

This is an early bioassay for estrogenic activity based on myometrial/endometrial proliferation. Repeated administration of estrogens induces a dose-dependent increase of uterine weight in ovariectomized rats.

Procedure

Immature female Sprague–Dawley rats weighing about 55 g are ovariectomized. Groups of five to ten animals are injected daily with several doses of the test compound or the standard (estradiol 0.03–0.06 μg per animal s.c.) for 7 days. The test compound is administered orally or subcutaneously in 0.5 % solution of carboxymethylcellulose or in cotton seed oil. Controls receive the vehicle. On the 8th day, the animals are sacrificed and uterine weights determined.

Evaluation

Dose–response curves are established, and potency ratios are calculated, using at least two doses of test compound and estradiol standard.

Similar studies were reported by Zondek (1935), Junkmann (1957), Emmens (1969), Nishino et al. (1991), and Van de Velde et al. (1994).

Modifications of the Method

Rubin et al. (1951) used albino mice 23–25 days of age weighing approximately 8 g. The mice are given subcutaneous injections once daily for 3 days of an oil solution of the hormone. Then, 24 h after the last injection, the animals are sacrificed and uterine and body weights recorded. The uterine ratio is calculated by dividing uterine weight in milligrams by body weight in grams, multiplied by 100 (relative uterine weight). Two groups receive a standard preparation and two groups the unknown.

Bhakoo and Katzenellenbogen (1977) showed that one of the earliest biosynthetic tissue responses after estrogen binding in the rat uterus, the synthesis of a specific uterine protein called "induced protein," is antagonized by progesterone.

In addition to uterus weight, Branham et al. (1993) studied luminal and glandular epithelium height in cross sections of the uterine horns of rats by histomorphology.

Uterine peroxidase activity was proposed as a marker for estrogen action by Lyttle and DeSombre (1977) and Astroff and Safe (1991).

Odum et al. (1997) compared the rodent uterotrophic assay with a yeast estrogenicity assay.

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Chick Oviduct Method

Purpose and Rationale

This is an early bioassay for estrogenic activity based on proliferation of the oviduct. The weight of the oviduct of young chickens is increased dose dependently by natural and synthetic estrogens (Tullner and Hertz 1956; Lerner et al. 1958; Dorfmann 1969).

Procedure

Seven-day-old pullet chicks are injected subcutaneously twice daily with solutions of the test compound in various doses for 6 days. Doses between 0.02 and 0.5 μg estradiol-17β per animal serve as standard. Six to ten chicks are used for each dosage group. On the day after the last injection, the animals are sacrificed and the weights of the body and oviduct determined.

Evaluation

The ratio of oviduct weight/body weight is calculated for each animal (relative weight). Mean values are plotted as dose–response curves in order to calculate potency ratios.

Modification of the Method

The assay was used for evaluation of antiestrogenic activity using simultaneous injection of 0.6 μg estradiol-17β 1 and the inhibitor, e.g., progestogens, and calculation of the percentage of oviduct weight in estrogen-/antiestrogen-treated animals versus the estrogen control group.

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Antiestrogenic Activity

Antagonism of Estrogen Effect on Uterus Weight

Purpose and Rationale

These assays are modifications of estrogen bioassays. The effect of estrogen treatment on uterine weight in ovariectomized rats was antagonized by antiestrogenic compounds.

Procedure

Immature female Sprague–Dawley rats weighing about 55 g are ovariectomized. Groups of five to ten animals are injected daily for 7 days with estradiol 0.03–0.06 μg per animal s.c. and various doses of the test compound or estradiol alone. The test compound is administered in 0.5 % solution of carboxymethylcellulose or in cotton seed oil either orally or injected subcutaneously. On day 8, the weight of the uterus is determined, and the inhibitory effect of antiestrogenic compounds is assessed.

Evaluation

The antiestrogenic effect is expressed as a percentage reduction of estrogen-stimulated uterine weight by test compounds.

Similar studies were reported by Byrnes and Shipley (1955), Dorfmann (1969), Terenius (1971), Wakeling and Bowler (1988), Levesque et al. (1991), Kangas (1992), and Nique et al. (1994).

Modifications of the Method

Three-week-old female NMRI mice can be used for determination of uterus weight (Lerner et al. 1958).

The inhibition by antiestrogens of vaginal cornification induced by estradiol can be used as the assessment parameter.

The chick oviduct weight can be adapted for the assay of antiestrogenic activity (Tullner and Hertz 1956).

Antiestrogenic activity may also be determined in castrated immature male rats. The animals show an increase in seminal vesicle weight when treated with 20 μg estradiol for 5 days, and the effect is counteracted by antiestrogenic steroids (Byrnes et al. 1953).

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Aromatase Inhibition

Purpose and Rationale

Estrogen synthesis by local action of the enzyme aromatase occurs in various tissues and is also found in estrogen-dependent tumors (Brodie 1992). Specific aromatase (estrogen synthetase) inhibitors are potential therapeutic agent for estrogen-dependent tumors, e.g., some breast cancers. 4-Hydroxyandrostenedione (4-OHA) inhibits aromatase completely but also causes destruction of the enzyme (irreversible inhibitor). Test compounds can be evaluated both in vitro (Häusler et al. 1989) and in vivo (Geelen et al. 1991). Tests are based on stimulation of estrogen biosynthesis by luteinizing hormone (LH) and inhibition of this effect by aromatase inhibitors.

Procedure

For in vitro experiments, ovarian tissue from adult golden hamsters (*Mesocricetus auratus*) is used. Estrus cycle is monitored for at least three consecutive 4-day estrus cycles prior to the experiment. The experiments for evaluating inhibitor effects are performed with ovaries obtained from animals sacrificed on day 4 (proestrus), at the time of preferred estrogen synthesis. The ovaries are excised, freed from adhering fat tissue, and quartered. The quarters are transferred to plastic incubation flasks with 2 ml of Krebs–Ringer bicarbonate (KBR) salt solution, pH 7.6, containing 8.4 mM glucose. The flasks are gassed with O_2/CO_2 (95 %/5 %), tightly closed, and placed in a shaker/water bath (37 °C) for incubation of the fragments. The incubation media are replaced with fresh KBR after preincubation for 1 h.

The ovaries are further incubated for 4 h in the presence or absence of ovine LH (100 ng/ml) and inhibitors to be tested. 4-OH-androstenedione is used as standard in concentrations between 0.33 and 330 μM. At the end of the experiment, the incubation media are removed and centrifuged. In the supernatant, estrogen, progesterone, and testosterone are determined by radioimmunoassay.

Evaluation

The results are expressed as percentage inhibition of estradiol synthesis relative to control incubations containing 100 ng/ml LH in the absence of inhibitor. Statistical analyses are performed using Dunnett's *t*-test.

Modifications of the Method

Geelen et al. (1991) determined the in vivo aromatase activity of test compounds in hypophysectomized rats treated with the estrogen precursor dehydroepiandrosterone (DHEA) sulfate, using the inhibition of cornification of vaginal epithelium and estradiol levels in plasma as parameters. Furthermore, aromatase activity was determined in homogenized ovaries. To the supernatant, [1β-^{3}H]androst-4-ene-3, 17-dione was added. Incubation was performed with an NADPH-generating system for 1 h at 37 °C. Incubations were terminated by placing the tubes on ice followed by an extraction with chloroform. After phase separation by centrifugation, the aqueous phase was diluted with an equal volume of dextran-coated charcoal suspension. Following centrifugation, the ^3H_2O content was determined by liquid scintillation counting. The amount of ^3H_2O is a measure of the amount of estrogen produced.

Zaccheo et al. (1989) studied the antitumor activity of aromatase inhibitors in rats with tumors induced by 7,12-dimethylbenzanthracene (DMBA).

Wouters et al. (1993) studied the inhibition of aromatase activity in FSH-stimulated rat granulosa cells by vorozole, a selective, nonsteroidal aromatase inhibitor.

Suzuki et al. (1996) described changes in prostate volume and histopathological findings in androstenedione-treated castrated beagle dogs as a bioassay for an aromatase inhibitor.

Takahashi et al. (1997) tested the inhibition of aromatase activity in the microsomes from fresh human placentae by the amount of tritiated water released from [1β-^{3}H]androstenedione. For

biological proof, the dose-dependent suppression of androstenedione-induced uterine hypertrophy in immature rats was measured.

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Antiestrogenic Effect on MCF-7 Breast Cancer Cells

Purpose and Rationale

The MCF-7 cell line, derived from a pleural effusion of a malignant breast cancer, is a widely studied model for hormone-dependent human breast cancer. These cells contain functional estrogen receptors and show a pleiotropic response to estrogen which can be used to evaluate antiestrogenic effects (Miller and Katzenellenbogen 1983; Scholl et al. 1983; Thompson et al. 1988). The MCF-7 cells proliferate and invade through an artificial basement membrane. Antiestrogens reduce both the proliferation and invasiveness of MCF-7 cells.

Procedure

MCF-7 cells are maintained in T75 flasks in IMEM (Biofluids, Rockville, MD, USA) supplemented with 2 mM glutamine and 10 % fetal bovine serum. To deplete estrogen, the cells are passaged for at least 2 weeks in IMEM supplemented with 5 % calf serum which has been treated sequentially with sulfatase and dextran-coated charcoal (DCC) to remove endogenous estrogen.

Estrogen and Antiestrogen Treatment

Cells are trypsinized, reseeded in tissue culture dishes (1×10^6 cells/10-cm-diameter Falcon dish), and allowed to adhere overnight in a humidified incubator (37 °C, 5 % CO_2, 95 % air). The cells are treated the next day with either 17β-estradiol (10^{-9} M) or the antiestrogen or 0.1 % ethanol alone. Four days later, the cells are harvested with trypsin, washed twice in IMEM containing 0.1 % BSA, counted with a Coulter cell counter, and tested for chemotaxis and chemoinvasion activities.

Chemoinvasion Assay

Boyden chambers are used (Albini et al. 1987). Polycarbonate filters (12-µm pore, polyvinylpyrrolidone-free, Nucleopore) are coated with Matrigel (25 µg/filter), a mixture of basement membrane components (Kleinman et al. 1986), which is dried and then reconstituted at 37 °C into a solid, even layer over the surface of the filter. Fibroblast-conditioned medium, obtained by incubating confluent NIH-3 T3 cells for 24 h with IMEM, is used as the chemoattractant. Cells are harvested with trypsin, washed twice with BSA/IMEM, and added to the top chamber (300,000 cells/chamber).

Chambers are incubated in a humidified incubator at 37 °C in 5 % CO_2 in air for 6, 9, or 12 h. The cells which have traversed the Matrigel and attached to the lower surface of the filter are stained with Diff-Quick (American Scientific Products) and quantitated electronically with the Optimax V image analyzer.

Chemotaxis Assay

Chemotaxis assays are performed as described for the chemoinvasion studies with the single exception that the filter surfaces are coated with 5 μg collagen IV instead of the layer of Matrigel. This coats the interstices of the filters but does not form a barrier over the surface. Chemotaxis assays are performed in parallel to the chemoinvasion assays using the same cells and conditioned medium.

Evaluation

One-way analysis of variance is performed on the data from each experiment.

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Progestational Activity

In Vitro Methods

Gestagen Receptor Binding

Purpose and Rationale

Progesterone receptor preparations may be obtained from numerous sources of tissue: uteri from estrogen-primed rabbits (Ojasoo and Raynaud 1978; Boonkasemsanti et al. 1989; Phillips et al. 1990; Cook et al. 1992), castrated estrogen-treated mice or rats (Philibert and Raynaud 1977; Li et al. 1997), MCF-7 cells derived from human breast tumor (Bergink et al. 1983; Kloosterboer et al. 1988a, 1988b, 1994), breast cancer T47D cells (Meyer et al. 1990), the quail fibroblast cell line QT6 (Schowalter et al. 1991), or human uteri obtained after hysterectomy (Jänne et al. 1976; Pollow et al. 1989a, 1989b, 1992). Tritium-labeled progesterone and R 5020 ([6,7-^{3}H]17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) are used as ligands (Moguilewsky and Raynaud 1979).

Procedure

Relative Binding Affinities

Human uteri obtained after hysterectomy are snap-frozen in liquid nitrogen and stored at −80 °C until use. For cytosol preparations, uterine tissues are minced and homogenized with an ULTRA-TURRAX at 0–4 °C in ice-cold buffer composed of 10 mM KH_2PO_4, 10 mM K_2HPO_4, 1.5 mM EDTA, 3 mM NaN_3, and 10 % glycerol, pH 7.5 (PENG buffer). The homogenates are then centrifuged at 105,000 g at 4 °C for 30 min. The supernatant is taken as cytosol. The cytosol preparations are incubated with ^{3}H-R 5020 as radioligand at a concentration of 8 nmol/l and increasing concentrations (1×10^{-10} to 1×10^{-5} mol/l) of the competitor steroids overnight at 4 °C. Then, unbound steroids are adsorbed by incubating with 0.5 ml of DCC (0.5 % Norit A, 0.05 % dextran T400 in PENG buffer) for 10 min at 4 °C. After centrifugation (10 min at 1,500 g at 4 °C), 0.5 ml of the supernatant is withdrawn and counted for radioactivity.

Association rate and dissociation rate are determined as described for the estrogen receptor.

Evaluation

For the calculation of **relative binding affinity**, the percentage of radioligand bound in the presence of a competitor compared to that bound in its absence is plotted against the concentration of unlabeled steroid. A standard curve for the unlabeled radioligand (progesterone) is constructed with the use of nine to ten concentrations; five or six concentrations of the competitor are tested. The molar concentrations of unlabeled radioligand and steroid competitors that reduce radioligand binding by 50 % are determined. The ratio of unlabeled radioligand and competitor for 50 % competition multiplied by 100 is calculated for relative binding affinity.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}t = (2.3/E_0 - R_0)\log(ER_0/RE_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t = 0$ and time t, respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3\log B/B_0$$

where B_0 and B represent bound steroid at time $t = 0$ and time t, respectively.

Modifications of the Method

For screening procedures, homogenates of rabbit uteri may be used (Philibert et al. 1977).

The binding of the progesterone agonist R 5020 and of the progesterone antagonist RU 486 to the progesterone receptor from calf uterus was characterized by Hurd and Moudgil (1988).

A high-affinity ligand and novel photoaffinity labeling reagent for the progesterone receptor ([^{3}H]DU41165) were described by Pinney et al. (1990).

The different DNA-binding properties of the calf uterine estrogen and progesterone receptors were explained by different dimerization constants (Skafar 1991).

Mutations of the progesterone receptor were found to be responsible for species specificity and have been used for evaluation of agonistic and antagonistic activity (Benhamou et al. 1992; Garcia et al. 1992).

The complete amino acid sequence of the human progesterone receptor has been deduced from cloned cDNA by Misrani et al. (1987).

Structural requirements of the ligand and mapping of the hormone-binding site of the progestin receptor have been discussed by Ojasoo and Raynaud (1990).

Allan et al. (1992) studied conformational changes in the ligand-binding domain induced by various progestins and antiprogestins.

Collins (1994) recommended the ratio between the affinity of a compound to progesterone receptors and the affinity to androgen receptors as a selection criterion for new oral contraceptives.

Comparative pharmacology of newer progestogens has been reviewed by Kuhl (1996).

Oñate et al. (1994) found that the DNA-binding protein HMG-1 enhances progesterone receptor binding to its target DNA sequences.

The concept of two categories of progestin antagonists based on differences in how they interact with and inactivate the progestin receptor has been discussed by Edwards et al. (1995).

Carbajo et al. (1996) studied the binding of [^{3}H]progesterone to the human progesterone receptor existing in two isoforms hPR-A and hPR-B which differ in that hPR-A lacks 164 amino acids at the amino terminus of hPR-B.

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Transactivation Assay for Gestagens

Purpose and Rationale

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone-responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988).

Transactivation assays were used by several authors to test the progestational and antiprogestational activity of steroidal and nonsteroidal compounds (Sobek et al. 1994; Pathirana et al. 1995; Fuhrmann et al. 1996; Jones et al. 1996; Dijkema et al. 1998; Edwards et al. 1998; Schoonen et al. 1998; Zhi et al. 1998).

Procedure

CV-1 cells (African green monkey kidney fibroblasts) are grown in Dulbecco's modified Eagle medium containing 10 % charcoal resin-stripped fetal bovine serum, 2 mM glutamine, and 55 µg/ml gentamicin. Cells are maintained in an environment of 4 % carbon dioxide and routinely passaged from T-255 flasks to 96-well microtiter plates (1.5×10^5 cells/well, 70 % confluent) 1 day before transfection.

Cells are transiently transfected by the standard calcium phosphate coprecipitation procedure with 50 ng/well of plasmids coding for human progesterone receptor-B (hPR-B1) and pRS-β-Gal under constitutive control and a reporter (MTV-LUC) containing a response element for the progesterone receptor. After 6 h, the medium is replaced with a medium containing progesterone at concentrations between 10^{-11} and 10^{-5} M or another progestogen. After a 40-h incubation, wells are washed with phosphate-buffered saline, and the cells are lysed with Triton X-100-based buffer. An aliquot of lysate (20 µl) is then transferred to Dynatech 96-well plates containing 1.6 mM ATP. LUC activity (chemiluminescence upon addition of luciferin substrate) is determined using a Dynatech ML1000 luminometer, according to the equation:

$$\text{LUC units} = \text{Relative LUC unit} \times 10^4.$$

β-Gal activity is determined from the remaining lysate in the original 96-well plates. The substrate, *o*-nitrophenyl-β-galactoside, is added to the plates, followed by incubation at 37 °C. The incubation is terminated by the addition of sodium carbonate when the average absorbance, as determined by visual observation of the yellow product (*o*-nitrophenol), is within a standard range. Absorbance at a wavelength of 415 nm is then quantified spectrophotometrically. β-Gal rates are calculated according to the following equation:

$$\beta - \text{Gal rate} = \frac{\beta - \text{Gal absorbance} \times 10^5}{\beta - \text{Gal incubation time}}.$$

For each set of replicate wells, normalized response is calculated according the following equation:

$$\text{Normalized response} = \text{LUC units}/\beta - \text{Gal rate}.$$

Evaluation

Agonist activity is determined by examining the amount of LUC expression (normalized response). The effective concentration that produces 50 % of the maximal response (EC_{50}) is quantified. The efficacy is a function of the LUC expression relative to the maximal LUC expression produced by the reference agonist, e. g., progesterone. Antagonist activity is determined by testing the amount of LUC expression in the presence of a fixed concentration (equal to its agonist EC_{50}) of reference agonist. The concentration of a test compound that inhibits the gene expression induced by the reference compound by 50 % is quantified (IC_{50}). In addition, the efficacy of antagonists is determined as a function of maximal inhibition (LUC expression = basal activity).

Critical Assessment of the Method

See chapter "▶ Adrenal Steroid Hormones" for the importance of controlling transfection efficacy.

Modifications of the Method

For transactivation studies, hPR-A-MMTV-LUC and hPR-B-MMTV-LUC and stably co-transfected CHO cells were used by Dijekema et al. (1998) and Schoonen et al. (1998).

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Alkaline Phosphatase Assay

Purpose and Rationale

Progestins induce the de novo synthesis of alkaline phosphatase (Di Lorenzo et al. 1991, 1993). The progestin induction of the nonspecific tissue alkaline phosphatase is not altered by other steroid hormones or synthetic analogs. This finding has been used by Sobek et al. (1994), Pathirana et al. (1995), and Li et al. (1997) to measure the progestogenic and antiprogestogenic actions in a microplate assay.

Procedure

T47D cells are grown in 96-well plates to near confluence and then treated with 0.1–0.2 % alcohol (control) or steroid agonists, otherwise R 5020 plus potential antagonist for 2 days. The cells are then fixed with 3.7 % formaldehyde in phosphate-buffered saline for 15 min at 15 °C, washed with 200 µl phosphate-buffered saline, and stored at −90 °C. After thawing, each well is incubated with 100 µl *p*-nitrophenyl phosphate (1 mg pNPP/ml) in DEAM (1 M diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$ and 20 µM $ZnSO_4$). The enzyme reaction is allowed to proceed in the dark at 37 °C. Formation of *p*-nitrophenol is monitored periodically at 405 nm in a microplate reader. In each microtiter plate, blanks, *p*-nitrophenol, and alkaline phosphatase standards are measured together with the samples. One unit of alkaline phosphatase is defined as the amount of enzyme capable of transforming 1 µmol of substrate in 1 min at 37 °C. Enzyme assays are performed under conditions of linearity relative to the substrate and to the concentration of proteins.

Evaluation

Progestin concentrations corresponding to half the maximal increase in alkaline phosphatase

activity (ED_{50}) are calculated graphically and by computer-assisted analysis using the ImmunoFit EIA/RIA analysis software (Beckman Instruments).

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In Vivo Methods

Clauberg (McPhail) Test in Rabbits

Purpose and Rationale

In this historical bioassay, Clauberg (1930a, b, c, d) first described the histological changes of the endometrium in estrogen-pretreated rabbits after administration of progestational compounds. The test was further studied by Butenandt et al. (1934) and systematically examined by McPhail (1934) who introduced scoring the changes of the endometrium.

Procedure

Immature female rabbits (Yellow Silver or New Zealand strain), weighing 550–650 g, receive daily injections of 5.0 μg estradiol benzoate per animal in sesame oil solution for a period of 6 days (priming). From days 7–12 (treatment for 5 days), the test compound or standard is administered at several doses, three to four animals per group. Standard doses are 0.02, 0.08, and 2.0 mg progesterone per day and animal s.c. in sesame oil solution or 0.01, 0.02, and 0.04 mg medroxyprogesterone acetate orally. Controls receive either the vehicles or estradiol benzoate only. At autopsy on day 15, both horns of the uterus are removed and fixed in 10 % formalin. Sections from the middle part of each uterine horn are low examined by histology.

Evaluation

Pretreatment with estradiol stimulates uterine weight development (priming); progestogens induce further proliferation and secretory transformation. The following scores are established:

0 → ramification of the uterus mucosa, but no proliferation (estrogen treatment only)
1 → slight proliferation of the uterus mucosa
2 → medium proliferation of the uterus mucosa, slight additional ramification
3 → pronounced proliferation of the uterus mucosa
4 → very pronounced proliferation of the uterus mucosa, pronounced ramification

The scores from each dosage group are averaged. Dose–response curves are constructed in order to calculate potency ratios versus the standard (progesterone). The morphological evaluation needs to be done by an experienced investigator. Preliminary experiments with standard drugs and photographical documentation of the histological findings are recommended.

Similar studies were reported by Zondek (1935), Junkmann (1957), Elton and Edgren (1958), Miyake (1962b), and Wiechert and Neumann (1965).

Modifications of the Method

In order to study the prolonged activity of progestogen esters, the animals are primed with estradiol for 6 days, followed by a single subcutaneous injection of the test compound or hydroxyprogesterone caproate standard. Daily treatment with estradiol is continued and the rabbits sacrificed at increasing intervals up to 4 weeks.

McGinty et al. (1939) established a local **progestational test** which involves the direct injection of progesterone into a uterine segment. The test is performed in immature rabbits primed for 6 days with estrogen. On day 7, the uterus is exposed by laparotomy. The upper middle segment of each horn is ligated without disturbance of blood circulation. A solution of the gestagen in oil is injected into the lumen of one segment through the lower ligature, which is closed tightly after the injection. In the opposite horn, only the vehicle is injected. The animals are sacrificed 3 days later, and sections of the horn are evaluated histologically according to the McPhail scores (Tayama et al. 1979).

Pincus et al. (1957) improved the quantitative aspects of the McPhail test with the planimetric measurement of the endometrial proliferation.

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Endometrial Carbonic Anhydrase Assay

Purpose and Rationale

Carbonic anhydrase activity in the endometrium is increased after administration of progesterone. Carbonic anhydrase activity in the endometrium of rabbits was used for a quantitative progestin assay (Lutwak-Mann 1955; Pincus et al. 1957;

Elton and Edgren 1958; Miyake and Pincus 1958; Eisler 1964).

Procedure

Immature female rabbits are estrogen primed and progestin treated as described in the Clauberg test. After sacrifice of the animals, the uteri are opened longitudinally. Endometrium is dissected, weighed, and homogenized with a tenfold volume in a glass homogenizer. After centrifugation, carbonic anhydrase is determined in the supernatant with the colorimetric method of Philpot and Philpot (1936) using bromothymol as indicator.

Evaluation

Mean values of carbonic anhydrase activity/g wet tissue are calculated and potency ratios for test compound and standard established.

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Deciduoma Formation

Purpose and Rationale

This is a classical bioassay for progestogens. The deciduoma tests are based on the fact that the endometrium of the estrogen-primed, progesterone-treated rodent is sensitive to local stimuli such as scratching (Astwood 1939), chemical irritation, and electrical stimulation. The effect of local irritation produces a deciduoma, similar to the formation of a maternal placenta (Astwood 1939; Elton and Edgren 1958; Zarrow et al. 1958; Miyake 1962; Eisler 1964; Sreenivasulu et al. 1993).

Procedure

Adult female Sprague–Dawley rats weighing 200–250 g are ovariectomized and 1 week later treated with 0.5 μg estradiol/animal once daily subcutaneously for 4 days, followed by 9 days of progesterone or the test compound in various doses. The uterus is exposed on day 5 of progesterone treatment, and 1.0 mg histamine dihydrochloride is injected into the lumen of one horn. The animals are sacrificed after the last treatment. Both uterine horns are removed and weighed. The degree of deciduoma formation is evaluated by the percentage increase in weight of the histamine-injected uterine horn as compared with the untreated control horn.

Evaluation

Dose–response curves are established plotting percentage increase in the weight of the treated uterus horn versus logarithm of the dose of the test compound and standard in order to calculate potency ratios.

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Pregnancy Maintenance Assay

Purpose and Rationale

This is a historical bioassay for progestational activity. In the rat, ovariectomy performed during the first half of pregnancy terminates gestation, but ovariectomy during the second half of pregnancy does not result in abortion, because the placenta now provides estrogens and progesterone for maintenance of pregnancy. In early pregnancy, maintenance of pregnancy after ovariectomy can be achieved by sufficient exogenous progestin with and without estrogen (Stucki 1958; Miyake 1962; Eisler 1964; Hebborn 1971; Phillips et al. 1984; Kuhnz und Beier 1994).

Procedure

Mature Sprague–Dawley female rats are inseminated by being placed with males overnight. The presence of sperm is assessed by vaginal lavage and, if positive, is considered to be day 1 of pregnancy. On day 8, the females are ovariectomized if found pregnant upon examination of the uterus. Test compounds are administered once daily, subcutaneously, for 13 days beginning immediately after ovariectomy. Estradiol (0.1 μg/day) is administered concomitantly with the test compound. At autopsy on day 21, the presence or absence of implantation sites and the numbers of live embryos are recorded.

Evaluation

Normal pregnant rats have an average of 11 implantation sites and about ten live embryos. Maintenance of pregnancy is assessed with reference to these normal values.

Modifications of the Method

Similar tests have been performed in rabbits (Elton and Edgren 1958), in mice (McGinty 1959), and in hamsters (Shipley 1965). In rabbits, abortion can be induced by intravenous injection of oxytocin from day 30 of pregnancy onward. The abortifacient effect can be suppressed by progestogens.

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Antiprogestational Activity

Progesterone Antagonism (Antiprogestins)

Purpose and Rationale

The antiprogestational activity of a test compound may be determined by antagonism against the effect of progesterone in the Clauberg/McPhail assay in rabbits (see section "Clauberg (McPhail) Test in Rabbits") or the McGinty test (see section "Clauberg (McPhail) Test in Rabbits") and in the deciduoma formation assay in rats (see section "Deciduoma Formation") (Miyake 1965; Tamaya et al. 1979; Oettel and Kurischko 1980; Philibert et al. 1985; Cook et al. 1994; Teutsch and Philibert 1994).

Modifications of the Method

Progesterone control of cervical ripening in the guinea pig and the tree shrew *Tupaia belangeri* has been used for the evaluation of progesterone antagonists (Chwalisz et al. 1991; Chwalisz 1994). The effects of progesterone antagonists on surgically induced endometriosis in rats have been studied by Stöckemann and Chwalisz (1993).

Michna et al. (1991) developed a bioassay which allows quantification of the antiproliferative potency of progesterone antagonists on the mammary gland in rats. Female Wistar rats with a body weight of 100 g were ovariectomized. One week after ovariectomy, the rats were substituted with 10 μg estrone and 3 mg progesterone for 3 days. The animals in the experimental groups simultaneously received the progesterone antagonist. The animals were then sacrificed and the inguinal mammary glands dissected: the right gland for biochemical analysis (DNA) and the left for morphometrical analysis. The entire inguinal mammary gland was prepared for conventional paraffin sections and stained with ferric ammonium sulfate. Microphotographs were taken in transmitted light. The number of tubuloalveolar buds was counted in the whole mount preparations using a 40-fold magnification. In the neighborhood of the inguinal node, a square of 2.5 mm^2 was quantified and calculated for a tissue volume of 100 mm^3 in more than ten animals. The antiproliferative action of progesterone antagonists on the amount of tubuloalveolar buds was estimated for an 80 % confidence interval of mean inhibition.

Critical Assessment of the Method

This method is very detailed and time consuming; it appears to be suitable for the advanced stages of drug evaluation for antiprogestins. In contrast to the only biochemical assessment, the method provides detailed information which is also applicable to toxicological evaluations.

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Luteolytic Activity of Prostaglandins

Purpose and Rationale

Synthetic prostaglandins are used for synchronization of estrus and for treatment of anestrus caused by a persistent corpus luteum in cattle. Furthermore, synthetic prostaglandins were studied for termination of early pregnancy in humans (Karim et al. 1977; Takagi et al. 1977, 1978; Toppozada et al. 1979; Dwivedy 1979). The most useful animal model for luteolytic activity is the hamster (Gutknecht et al. 1971; Labhsetwar 1971, 1972a; Dukes et al. 1974; Bartmann et al. 1979; Galliani et al. 1984; Roy et al. 1987). The effects of prostaglandins on reproductive function in other species such as rat (Fuchs et al. 1974), mouse (Labhsetwar 1972b), and guinea pig (Blatchley and Donovan 1969) have also been studied.

Procedure

Adult female golden hamsters (*Mesocricetus auratus*), weighing approximately 100 g, with regular, 4-day estrus cycles are housed under controlled light and temperature conditions and given a standard diet. They are caged with fertile males on the day before expected vaginal discharge, and the next morning, vaginal smears are taken. If clumps of spermatozoa are found in the smear, this day is designated as day 1 of pregnancy (see similar procedure in rats). Groups of ten animals are treated on days 4, 5, and 6 with four different doses of standard (prostaglandin $F_{2\alpha}$) or test compound. On day 13 of pregnancy, the hamsters are sacrificed, and the number of implantation scars in the uterus is counted as the parameter of luteolytic activity causing early termination of pregnancy.

Evaluation

The luteolytic activity is expressed as the median effective dose for termination of pregnancy in 50 % of the treated animals (ED_{50}).

Modifications of the Method

The mode of action of luteolysis by prostaglandins has been studied by various authors both in vitro (Speroff and Ramwell 1970; O'Grady et al. 1972; Henderson and McNatty 1975; Kenny and Robinson 1986; Brambaifa 1988) and in vivo (Pharriss and Wyngarden 1969; Johnston and Hunter 1970; McCracken et al. 1970; Chatterjee 1973; Buhr et al. 1983; Torjesen and Aakvaag 1984, 1986).

Cao and Chan (1993) investigated the effects of oxytocin and luteal prostaglandins on the functional regression of the corpus luteum in pseudopregnant rats.

Motta et al. (1996) studied the effect of an oxytocin receptor antagonist on ovarian and uterine synthesis and release of prostaglandin $F_{2\alpha}$ in pseudopregnant rats.

Stocco and Deis (1998) examined the participation of intraluteal progesterone and prostaglandin $F_{2\alpha}$ in LH-induced luteolysis in pregnant rats.

Luteolysis by prostaglandins in the rhesus monkey has been studied by Auletta and Kelm (1994) and Auletta et al. (1995).

The ewe as a model for regulation of luteal regression has been recommended by Hoyer (1998).

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Testicular Steroid Hormones

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Contents

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Testicular Steroid Hormones

Castration of Male Rats (Orchiectomy)

Procedure

Castration of young male rats is performed with minimal bleeding in animals weighing less than 60 g. The animal is anesthetized. A small transversal incision is made in the skin on the ventral site over the symphysis. The testis lying in the scrotum is gently pushed into the abdominal cavity. With a pair of fine forceps, the abdominal wall is opened. The epididymal fat pad, easily seen, is grasped with the forceps, and the testis with the epididymis is pulled out from the wound. The ductus deferens with the testicular vessels is crushed with artery forceps and the testis together with the epididymal fad pad cut off with a pair of fine scissors. There is almost no bleeding in young animals. In older animals, ligation of the testicular vessels together with the ductus deferens may be necessary. The same procedure is performed on the other side. The skin wound is closed with one or two wound clips. The animal recovers immediately. With some skill, the operation can be performed very rapidly (Bomskov 1939).

Modifications of the Methods

Dorfman (1969) recommended removing the testes through an incision in the tip of the scrotum. In our hands, the procedure described above was preferable.

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Caponizing of Cockerels (Orchiectomy)

Procedure

This is a classical bioassay for androgens. White Leghorn cockerels are used for surgery at approximately 6 weeks of age. The animals, fasted 24 h prior to surgery, were anesthetized with ether and placed on their sides. An incision is made between the last two ribs, the muscle layer is divided, and the incision is pulled apart with small retractors. The testis is found close to the midline of the posterior abdominal wall, alongside the vena cava. The capsule enclosing the testis is cut and the gonad is removed. It is imperative to remove the testis intact, as fragments left behind are usually vascularized and persist, giving rise to incompletely caponized animals. The incision is closed by a suture. The contralateral testis is removed in a similar fashion (Bomskov 1939).

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Androgenic and Anabolic Activity

In Vitro Methods

Androgen Receptor Binding

Purpose and Rationale

Rat ventral prostate (Bonne and Raynaud 1974; Liao et al. 1974; Grover and Odell 1975; Ojasoo and Raynaud 1978; Raynaud et al. 1979; Winneker et al. 1989; Duc et al. 1995) and mouse kidney (Isomaa et al. 1982) serve as sources for androgen receptors. Human androgen receptors have been prepared from transfected COS-1 cells (Teutsch et al. 1994). Labeled androstanolone, 5α-dihydrotestosterone, testosterone, and, more recently, methyltrienolone (R 1881) have been used as radioligands.

Procedure

Androgen Receptor Assay

Cytosol is prepared from ventral prostate glands of adult male rats castrated approximately 24 h before use. The tissue is homogenized in TMDG buffer (10 mM Tris, 20 mM sodium molybdate, 2 mM dithiothreitol, 10 % glycerol, pH = 7.4) at room temperature using a motor-driven glass homogenizer and centrifuged at 135,000 g for 1 h. Aliquots of the supernatant (cytosol) are diluted to contain 40 mg tissue/ml and incubated for 1 h or overnight with [17α-methyl-^{3}H]R 1881 (methyltrienolone, 5 nM final concentration, 87 Ci/mmol, New England Nuclear) in either the absence or presence of increasing concentrations (1 nM to 10 μM) of R 1881 or test compounds. Because R 1881 binds weakly to progesterone and glucocorticoid receptors, cytosols are pretreated with 1 μM triamcinolone acetonide to block these interactions. After a 1- or 18-h incubation period, a suspension of dextran-coated charcoal (1 % charcoal, 0.05 % dextran T-70, 0.05 % BSA) is added to the ligand–cytosol mixture and incubated for 5 min. The charcoal is removed by centrifugation at 1500 g for 10 min and the

supernatant (protein-bound [^{3}H]R 1881) counted using 10 ml of scintillation fluid (New England Nuclear) in a liquid scintillation spectrometer.

Nuclear Androgen Receptor Exchange Assay

Ventral prostates are homogenized at 100 mg/ml in hexylene glycol buffer (1 M hexylene glycol, 1 mM $MgCl_2$, 2.0 mM dithiothreitol, 5.0 mM EGTA, 1.0 mM PIPES, pH = 7.4) using a motor-driven ground glass homogenizer. Homogenates are centrifuged at 1500 g for 10 min. The nuclear pellet is washed three times in homogenization buffer by gently resuspending the pellet in a Dounce homogenizer and centrifugation at 1500 g for 10 min. The washed nuclear pellet is resuspended in pyridoxal-5′-phosphate extraction buffer (20 mM sodium barbital, 5 mM pyridoxal-5′-phosphate, 5.0 mM dithiothreitol,1.5 mM EDTA, 150 mM KCl, 20 % glycerol, pH = 7.4) for 60 min at a final concentration of 60 mg tissue/ml. The extracted nuclei are centrifuged at 25,000 g for 30 min with the resulting supernatant being used in the same single saturating dose assay as described for prostate cytosol.

Evaluation

Binding of test substances to the androgen receptor (receptor affinity) is quantified by calculating the relative binding affinity (ratio of the molar concentration of unlabeled R 1881 to test substance required to inhibit the binding of [^{3}H]R 1881 by 50 % after correction for nonspecific binding) and equilibrium inhibitory binding constant ($K_i = IC_{50}/(1 + C)/K_d$, where C = the concentration of [^{3}H]R 1881 and the K_d for R 1881 is 1.3 nM).

In the nuclear androgen receptor exchange assay, treatment group means are compared to control means, using ANOVA and Dunnett's multiple comparison tests.

Modifications of the Method

Cell assays and animal assays are described for evaluation of androgens and antiandrogens (Raynaud et al. 1975; Liang et al. 1977; Sivelle et al. 1982; Liao et al. 1984; Traish et al. 1986; Stobaugh and Blickenstaff 1990; Christiansen et al. 1990; Humm and Schneider 1990; Neubauer et al. 1993).

Brown et al. (1981) studied antiandrogen effects on androgen receptor binding in cultured human newborn foreskin fibroblasts.

Tezón et al. (1982) studied the intracellular distribution of the androgen receptor in the rat epididymis under the influence of androgens and antiandrogens.

The use of tritiated 7α,17α-dimethyl-19-nortestosterone for the assay of androgen receptors was recommended by Schilling and Liao (1984).

Characterization and expression of a cDNA encoding the human androgen receptor was described by Tilley et al. (1989).

Hoyte et al. (1993) recommended 7α-methyl-17α-(E-2′-[^{125}I]iodovinyl)-19-nortestosterone as radioligand for the detection of the androgen receptor.

Structure–affinity relationships of various steroids structurally related to nomegestrol and progesterone for [^{3}H]testosterone binding to rat ventral prostate cytosol were reported by Botella et al. (1987).

Molecular cloning of human androgen receptor complementary cDNA has been reported by Chang et al. (1988) and Lubahn et al. (1988).

DNA binding of androgen receptor overexpressed in COS-1 cells has been reported by Von Krempelhuber et al. (1994).

Thoth et al. (1995) studied in vitro binding of 16-methylated C-18 and C-19 steroid derivates to the androgen receptor using cytosol of castrated rat prostate and [^{3}H]R 1881 as radioligand.

Chang et al. (1995) reviewed the structure and function of the androgen receptor and its role in the function of the mammalian system.

The interaction of androgen receptors with the androgen-response element in intact cells was investigated by Karvonen et al. (1997).

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Transactivation Assay for Androgens

Purpose and Rationale

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone-responsive elements of hormone-regulated genes, inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

Procedure

Vector Construct

CV-1 cells are stably transfected with the rat androgen receptor and pMMTV-CAT7. The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

The culture medium of CV-1 cells stably transfected with the rat androgen receptor and pMMTV-CAT7 is supplemented with 400 µg/ml G418 (Gibco BRL) and 5 µg/ml puromycin.

Stable and transient transfections are performed using Lipofectin Reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80 % confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg hAR expression plasmid and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish,

gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10 % fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled, and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3 % charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. Cells are cultured with 1 % ethanol as a negative control for the reporter gene induction. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

Evaluation

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to demonstrate the potency of the test hormone. The synthetic androgen R 1881 (10^{-10} to 10^{-6} mol/l) is used as the standard.

For antiandrogenic activity, CAT activity in the presence of 0.5 nmol/l R 1881 is set as 100 %, and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the antihormone.

Critical Assessment of the Method

See chapter "► Anterior Pituitary Hormones"

Modifications of the Method

Warriar et al. (1993) examined the ability of dihydrotestosterone (DHT) and various antiandrogens to stimulate or to inhibit the transcription activation of mouse mammary tumor virus-bacterial chloramphenicol acetyltransferase (MMTV-CAT) in CV-1 cells.

White et al. (1994) described a simple and sensitive high-throughput assay which can be adapted for several classes of steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein–Barr virus episomal vector which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses a high level of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone (DHT). These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase (CAT) assay.

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In Vivo Methods

Chicken Comb Method for Androgen Activity

Purpose and Rationale

This is a historical bioassay based on the response of the comb of castrate cockerels (capons). The method has been used by many authors to assay androgenic activity and has been found to be extremely useful for the isolation and structural elucidation of natural androgens. Many modifications have been published (reviewed by Dorfman 1969).

Procedure

Prior to assay, the surface area (sum of the length plus height of each individual comb) is determined by a millimeter rule placed directly on the comb. The capons are injected q.d. intramuscularly for 5 days consecutively with a solution or suspension of the test compound, and reference animals are treated with the androgen standard in 1 ml olive oil. Then 24 h after the last injection, the combs are measured again, and the growth of the comb is expressed as the sum of length and height in millimeters. Groups of eight animals are used for at least two doses of the test compound and the standard. In this method, animals can be used repeatedly.

Evaluation

The experimental parameter is the surface area of the comb. The mean values of each group are calculated, dose–response curve for the test compound and the standard is plotted, and potency ratios are calculated where possible.

Similar studies were reported by Gallagher and Koch (1935), Greenwood et al. (1935), and Oesting and Webster (1938).

Modifications of the Method

The hormones, dissolved in oil, have been applied locally to the capon's comb instead of by injection. A greater sensitivity has been achieved with this modification (Fussgänger 1934; McCullagh and Cuyler 1939).

Newly hatched chicks of either sex have been used to study the growth of combs after systemic or local administration (Frank et al. 1942; Dorfman 1948). White Leghorn chicks are used at an age of 2–3 days. They are kept in a brooder with thermostatic control. An oily solution (0.05 ml) of the test compound or the standard is applied to the comb daily for a period of 7 days. Then 24 h after the last application, the animals are autopsied. Body weights are determined. The combs are removed by two longitudinal incisions along the base of the comb at its juncture with the scalp. The comb is freed from the scalp, touched lightly on a towel to remove blood, and weighed. Dose–response curves are established.

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Weight of Androgen-Dependent Organs in Rats

Purpose and Rationale

Androgens affect the development of secondary sex organs in the male rat (ventral prostate, seminal vesicles). The growth of the ventral prostate, the seminal vesicles, and the musculus levator ani is dependent on the presence of male sexual hormones. Weight development of the musculus levator ani was considered to indicate anabolic activity, and weight development of the ventral prostate and seminal vesicles was considered to indicate androgenic activity (Dorfman 1969).

Procedure

Immature male Sprague–Dawley rats weighing about 55 g are orchiectomized. Animal body weight is recorded at the beginning and at the end of the experiment.

The animals are treated with test compounds by gavage (orally) in 0.5 ml 0.5 % carboxymethylcellulose or by subcutaneous injection in 0.2 ml sesame oil suspension once per day over a period of 10 days. Testosterone is given in doses of 0.02, 0.1, and 0.5 mg per animal subcutaneously and methyltestosterone orally in doses of 0.25, 1.5, and 5 mg per animal. Controls receive the respective vehicle. Ten animals are used for each group. On day 10 at autopsy, the seminal vesicles, ventral prostate, and levator ani muscle are dissected out and weighed. The seminal vesicles are squeezed and dried to remove the fluid.

Dissection of the levator ani muscle is performed after removal of the skin in the scrotal area between the base of the penis and the anus. The posterior aspect of the perineal complex is cleared of fat and connective tissue with forceps, particular care being taken to expose the constrictions at either end of the levator ani where it joins the bulbocavernosus muscle. The rectum is transected just caudad to the point where the musculus levator ani loops around it dorsally. The body of the levator ani is then freed of the rectum and is removed by incisions at the points of attachment to the bulbocavernosus muscle. The levator ani is cleared of any connective tissue and weighed to the nearest 0.1 mg.

Evaluation

The ratio of organ weight to body weight is calculated for every animal and each organ (relative organ weights). Dose–response curves are constructed for each organ comparing the test compound with the standard in order to calculate potency ratios. An increase in the weight of seminal vesicles and ventral prostate indicates androgenic activity, whereas an increase in the weight of musculus levator ani is considered to indicate anabolic activity.

In evaluating steroids for possible use as anabolic agents, Hershberger et al. (1953) suggested the use of the levator ani to ventral prostate ratio, which is defined as the ratio of the increase in levator ani weight divided by the increase in ventral prostate weight.

The method has been used by several authors (Korenchevsky and Dennison 1935; Eisenberg and Gordon 1950; Junkmann 1957; Kincl 1965; Eisler 1964; Dorfman 1969; Kuhnz and Beier 1994).

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Nitrogen Retention

Purpose and Rationale

Anabolic agents induce positive nitrogen balance in the living organism (Polish 1964; Dorfman 1969). Many modifications of this assay principle have evolved. Stafford et al. (1954) suggested a method involving the measurement of nitrogen excretion in the castrated rat fed a liquid diet and in nitrogen balance.

Procedure

Twenty-five-day-old rats are castrated and kept untreated for 67 days, reaching about 300 g in body weight on normal laboratory diet. After 67 days, they are changed to a liquid diet, forced-feeding regime. The diet contains carbohydrates and fat, as well as casein and brewer's yeast as a nitrogen source. At the start, the rats receive 10 ml of feed per day, and this is increased to 26 ml per day. Feeding is continued for 30 days with simultaneous administration of the test drug once a day. Twenty-four-hour urine specimens are collected three times weekly and analyzed for total nitrogen.

Evaluation

Indices are calculated, such as greatest daily retention, which is defined as the difference between the lowest daily nitrogen value after the beginning of treatment and the preinjection mean; the total nitrogen retention, which is the sum of the differences between the preinjection excretion and the daily values during the retention period; and the number of days in the retention period.

Modifications of the Method

A method for the assay of anabolic steroids in the monkey (*Macaca mulatta*) has been suggested by Stucki et al. (1960). Nitrogen retention expressed as total nitrogen retained per day during the treatment period is chosen as the end point.

Critical Assessment of the Method

The assay is time consuming and labor intensive, and its use is no longer recommended.

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Antiandrogenic Activity

General Considerations

Antiandrogens may exhibit their activity both peripherally on androgen-dependent tissues and by feedback action at a central site (Neumann et al. 1970, 1977; Mainwaring 1977; Neri 1977; Raynaud et al. 1977; Neumann 1985; Moguilewski and Bouton 1988). They compete with the peripheral androgen receptors and thus inhibit the effect of endogenous or exogenous androgens. Centrally, they inhibit gonadotropin secretion and thereby diminish testosterone production by the gonads. In addition to their effects on reproduction and accessory sexual organs, antiandrogens inhibit sebum production (anti-acne drugs) and delay androgen-dependent hair loss (alopecia). The methods to detect and quantify gonadotropin inhibition are described in chapter "► Vitiligo Models".

Inhibition of 5α-reductase, an enzyme located in tissues such as the prostate, is one pharmacological approach to inhibit benign prostate hyperplasia in men. Such inhibitors reduced the conversion from testosterone to 5α-dihydrotestosterone (DHT).

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In Vitro Methods

Inhibition of 5α-Reductase

Purpose and Rationale

Testosterone is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase which is specifically localized in some androgen-responsive target tissues (e.g., prostate, seminal vesicle, epididymis, skin, and sebaceous glands), whereas in other androgen-sensitive tissues, such as the skeletal muscles and the central structures, the androgenic hormone is testosterone. Inhibition of 5α-reductase provides a selective approach to androgen deprivation in DHT-target tissues, such as the prostate. The 5α-reductase inhibitors are applied in the therapy of benign prostate hyperplasia.

Procedure

5α-Reductase preparations are obtainable from prostates of various species, such as human, dog, and rat.

Frozen human prostates from benign prostatic hyperplasia patients are thawed and minced with a pair of scissors. The minced tissue is homogenized in three tissue volumes of medium A (20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose, 1 mM dithiothreitol, and 50 µM

NADPH) with a Brinkmann Polytron and a glass–glass homogenizer. The homogenate is centrifuged at 140,000 g for 60 min, and the pellets are washed with approximately three tissue volumes of medium A. The washed pellets are suspended at a concentration of 5–10 mg protein/ml in 20 mM potassium phosphate, pH 6.5, containing 20 % glycerol and 1 mM dithiothreitol.

Dog prostatic particulates are prepared from either fresh or frozen specimens of male mature dogs as described for human prostate. The washed pellets are suspended in medium A at a concentration of 30–60 mg protein/ml.

Ventral prostates from male Sprague–Dawley rats weighing 400 g are processed as described for human prostate, except that medium A without NADPH is used throughout the procedures. NADPH prevents inactivation of human and dog 5α-reductases during the preparation; the rat enzyme however is stable without the coenzyme.

For the 5α-reductase assay, reaction solutions are prepared in duplicate tubes containing 1 μM [^{14}C]testosterone, 1 mM dithiothreitol, 40 mM buffer (potassium phosphate, pH 6.5, for the rat and for the dog enzymes; Tris–citrate, pH 5.0, for the human enzyme), prostatic particulate (1 mg protein), and NADPH (50 μM for reaction with rat enzyme, 500 μM for reaction with human and dog enzyme) in a final volume of 0.5 ml. Test compounds or standard as inhibitors is added in 5 μl ethanol at concentrations between 10^{-9} and 10^{-5} M. The control tubes receive the same volume of ethanol. The reactions for the rat and dog enzymes are started by the addition of the prostatic particulates. The human prostatic particulate is premixed with NADPH before starting the reaction. The reactions are linear for at least 1 h at 37 °C. The reactions are carried out for 10–30 min and are stopped with 2 ml ethyl acetate containing testosterone, 5α-dihydrotestosterone, and androstenedione (10 μg each). After centrifugation at 1000 g for 5 min, the ethyl acetate phase is transferred to a tube and evaporated under nitrogen to dryness. The steroids are taken up in 50 μl ethyl acetate. The solutions are applied to Whatman LK5DF silica plates, and the plates are developed in either ethyl acetate to cyclohexane (1:1) at 25 °C or chloroform to methanol (96:4) at 4 °C. The plates are air-dried and the chromatography is repeated. Nonradioactive steroid standards are located by UV and by spraying with 1 % $CeSO_4$/10 % H_2SO_4 solution followed by heating. The radioactivity profiles are determined by scanning the plates or by scraping the silica in sections and counting in a scintillation counter. 5α-Dihydrotestosterone is the only radioactive product for the rat and human enzymes. With the dog enzyme 5α-dihydrotestosterone, 3α,17β-androstanediol, androstan-3,17-dione, and androstenedione are formed. The radioactivities of the first three products are combined for the calculation of the 5α-reductase activity.

Evaluation

IC_{50} values are calculated based on at least five dilutions of test preparations or standard.

Modifications of the Method

The method has been used by several authors (Bruchovsky and Wilson 1968; Corvol et al. 1975; Brooks et al. 1981; Liang et al. 1985; Rhodes et al. 1993; di Salle et al. 1993, 1994; Sudduth and Koronkowski 1993).

Using human genital skin fibroblasts and simian COS cells, specific inhibition of 5α-reductase type 1 has been observed (Hirsch et al. 1993).

Wennbo et al. (1997) reported that **transgenic mice** overexpressing the prolactin gene develop dramatic enlargement of the prostate gland.

Sigimura et al. (1994) described age-related changes of the prostate gland in the senescence-accelerated mouse and recommended this strain as a model of age-related changes in the prostate gland.

Neubauer et al. (1993) measured prostatic 5α-reductase in rats both in vitro and *ex vivo* and determined in vivo uptake of [^{3}H]testosterone by the prostate.

At least two isoforms of 5α-reductase have been isolated (Andersson and Russell 1990; Jenkins et al. 1992). Recombinant human prostatic 5α-reductase types I and II were expressed using the baculovirus-directed insect cell expression system (Iehlè et al. 1993).

Iehlè et al. (1995) and di Salle et al. (1998) tested synthetic 5α-reductase inhibitors against both isoforms.

Tolman et al. (1995) identified a 4-azasteroid as a scalp isoenzyme selective inhibitor.

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In Vivo Methods

Chick Comb Method

Purpose and Rationale

This method is now obsolete for the evaluation of antiandrogens. Several modifications of the chick comb method have been described for antiandrogens applied either systemically or locally to the comb of intact cockerels (Dorfman and Dorfman 1960; Dorfman 1969).

Procedure

Male or female, 1–3-day-old White Leghorn chicks are housed at constant temperature in a heated incubator. Testosterone is incorporated into the finely ground chick starting mash at a concentration of 80 mg/kg of food to stimulate comb growth. The chicks are placed on this diet on day 1. The antiandrogen to be tested is dissolved in sesame oil and injected for several days. At 24 h after the last injection, the animals are sacrificed, the combs removed and blotted to remove blood, and weighed rapidly to the nearest 0.5 mg. Body weights are also determined.

Evaluation

The results may be expressed as absolute comb weights or as milligram of comb per gram of body weight. Dose–response curves for groups treated with increasing doses of antiandrogen are plotted.

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Antagonism of Androgen Action in Castrated Rats

Purpose and Rationale

In this modification, antiandrogens are administered to reduce or inhibit testosterone actions on androgen-dependent organs.

Procedure

Male Sprague–Dawley rats weighing 50–70 g are orchiectomized. Starting 1 day after castration, the rats are injected once daily for 7 days with 0.15 mg testosterone propionate in 0.1 ml sesame oil (agonist action). The test compounds (antiandrogens) are dissolved in sesame oil or suspended and injected subcutaneously daily at a separate site for the same test period of 7 days. Six to ten animals are used per group. At autopsy on day 8, weights of ventral prostate, seminal vesicles, and musculus levator ani as well as body weight are recorded.

Evaluation

The organ weight to body weight ratio is calculated for each order, preferably based on relative organ weight to 100 g of body weight. The inhibition by the antiandrogen is compared with agonist action in the groups of animals receiving testosterone propionate alone. Dose–response curves may be plotted for each organ and expressed as percentage inhibition of the agonist effect of testosterone by the antiandrogen.

Modifications of the Method

Dorfman (1962) described an antiandrogen assay using the castrated mouse. Weights of prostate and seminal vesicles were determined after injection of the antiandrogen test compounds and simultaneous injections of 2 mg testosterone over a period of 7 days.

Applications of the Method

The intrinsic antiandrogenic activity is an important parameter in the evaluation of the pharmacological activity of H_2-receptor antagonists (Winters et al. 1979; Broulik 1980; Baba et al. 1981; Sivelle et al. 1982;

Foldesy et al. 1985; Takeda et al. 1982; Neubauer et al. 1990). In general pharmacology studies, there is rarely any need for specific anti-hormonal tests. However, in toxicology studies, tests for antiandrogen activity may be warranted when the effects on the testes and androgen-dependent organs are found in repeated-dose studies.

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Antiandrogenic Activity in Female Rats

Purpose and Rationale

This is another historical bioassay of interest due to the design of exploring antiandrogenic activity. Neumann and Elger (1966) described a method for testing the antiandrogenic activity of compounds in immature female rats. The inhibition by the antiandrogen cyproterone of the trophic effect of testosterone on uterine and preputial growth was studied in intact as well as in castrated female rats (Neri et al. 1972; Snyder et al. 1989).

Procedure

Female Sprague–Dawley rats weighing 40–45 g are ovariectomized. One week later, the treatment is started for 12 days with daily subcutaneous injections of 0.3 mg testosterone propionate and several doses of the antagonist. Controls receive testosterone propionate only. At autopsy on day 13, the uteri and preputial glands are distracted out and weighed. The weight increase of female accessory sexual organs caused by the testosterone's action is dose-dependently reduced by the antiandrogen (in this case, cyproterone). Similar results are found using intact immature female rats.

Evaluation

Dose–response curves were established for increasing doses of the antiandrogen at a given dose of testosterone propionate or for increasing doses of testosterone propionate at a given dose of the antiandrogen, cyproterone.

Critical Assessment of the Method

The method is time consuming and was directed to the specific investigation of cyproterone and cyproterone acetate in precocious puberty and androgen-dependent disorders.

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Intrauterine Feminizing/Virilizing Effect

Purpose and Rationale

From clinical observations as well as from experimental data (Neumann and Junkmann 1963; Neumann and Kramer 1964), it is well known that the external genitalia of female fetuses can be masculinized by tumors secreting endogenous androgens or by steroids with androgenic activity. This effect can be antagonized by an antiandrogen.

The method is of interest for assessment of reproductive toxicology.

Procedure

Adult female Sprague–Dawley rats are mated, and the beginning of pregnancy is determined by vaginal smears. From day 16 to day 19 of pregnancy, the antiandrogens are administered in various doses subcutaneously in sesame oil. Testosterone propionate is used in doses between 1.0 and 10.0 mg as the androgenic stimulus. The dams are sacrificed on the 20th day of pregnancy and the external genitalia of the female embryos examined. The sex of the embryos is recognized by the presence of ovaries and uterus. A dose of 10 mg testosterone propionate leads to total masculinization of female embryos with the loss of

female and the appearance of male sex characteristics. The anogenital distance in female rat fetuses measured macroscopically and microscopically is dose-dependently increased by testosterone propionate. This characteristic androgen effect is diminished by an antiandrogen.

Evaluation

The androgen-dependent decrease of the anogenital distance in female fetuses by various doses of the antiandrogen is expressed as percentage inhibition of the testosterone-induced virilization.

Modifications of the Method

Feminization of male rats was induced by treatment of pregnant rats during the second half of gestation and of the newborn fetuses during weeks 1–3 postpartum with an antiandrogen, e.g., cyproterone acetate (Neumann and Elger 1966; Nishino et al. 1988; review by Neumann 1994). A decrease of the anogenital distance in the male fetuses of antiandrogen-treated rats is expressed as percentage inhibition relative to fetuses from untreated mothers.

In feminized male rats, nipples and associated glandular tissues develop after birth as in normal female rats (Neumann and Elger 1967).

Feminization of male rats treated in utero was also observed with nonsteroidal antiandrogens and a 5α-reductase inhibitor (Imperato-McGinley et al. 1992).

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Antiandrogenic Activity on Sebaceous Glands

Purpose and Rationale

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. This effect is used for the morphometric evaluation of topical antiandrogens.

The method is described in detail in chapter "► Vitiligo Models".

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Antiandrogenic Activity in the Hamster Flank Organ

Purpose and Rationale

This is another bioassay preferably for topical antiandrogens. The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemic or topical antiandrogens.

The method is described in detail in chapter "► Vitiligo Models".

Effect of 5α-Reductase Inhibitors on Plasma and Tissue Steroid Levels

Purpose and Rationale

5α-Reductase inhibitors change the ratio of plasma testosterone to dihydrotestosterone (DHT) as well as the tissue concentrations particularly in the prostate tissue.

Procedure

Treatment of Animals

Male Sprague–Dawley rats are treated subcutaneously with the 5α-reductase inhibitor or vehicle beginning on postnatal day 3 until the age of 4 or 7 weeks. After sacrifice, blood is withdrawn for testosterone and DHT determinations (George et al. 1989). Moreover, intraprostatic concentrations of testosterone and DHT are determined as an index of antiproliferative activity (di Salle et al. 1993).

Radioimmunoassay for Testosterone and Dihydrotestosterone

Serum testosterone and DHT are measured by radioimmunoassay (RIA) in serum or serum extracts using specific antisera without prior chromatography (Falvo and Nalbandov 1974). Serum samples of 0.5 ml may be extracted with 2 ml of freshly purified, peroxide-free diethyl ether by shaking for 60 s on a vortex mixer. The aqueous phase is frozen at −70 °C, then the ether phase containing steroids is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is redissolved in BSA/phosphate buffer (1 % BSA = bovine serum albumin) for RIA. [1,2,6,7-^{3}H]-Testosterone or [1,2,6,7-^{3}H]-dihydrotestosterone and specific antisera are added and tubes incubated over a period of 24 h at +4 °C under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal. The activity of each sample is determined by beta-spectrometry.

Commercially available RIA kits can be used with suitable validation.

Evaluation

The hormone concentrations in the sample are calculated from a standard curve by a computer program (e.g., RIA-Calc, LKB), using appropriate control sera. The ratios of testosterone to DHT in rats treated with different doses of 5α-reductase inhibitors are compared with those of vehicle-treated intact control rats.

Modifications of the Method

di Salle et al. (1998) measured prostatic concentrations of testosterone and 5α-dihydrotestosterone in rats by specific RIAs after treatment with a dual type I and II 5α-reductase inhibitor. Similar measurements of tissue testosterone to DHT ratios have been performed in dogs, in the

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Thyroid Hormones

Jürgen Sandow

Contents

General Considerations

The purpose of this section is twofold: to describe the methods for the determination of hormones secreted by the thyroid gland and to describe the investigation of thyroid function in experimental models. For information on the hypothalamic–pituitary–thyroid system and its investigation, see the sections on anterior pituitary hormones (N.7) and hypothalamic hormones (N.9).

The thyroid gland secretes two types of hormones: the **thyroid hormones**, i.e., l-**thyroxine** (T_4) and **triiodothyronine** (T_3) which have metabolic functions and are involved in neuronal development, and the **calciotropic hormone**, **calcitonin**. The functional system for metabolic regulation subserved by the thyroid hormones is entirely different from the complex system for regulation of calcium and phosphate balance fulfilled by (thyro)calcitonin, parathormone from the parathyroid glands, and the calciferol hormones (formerly vitamin D) produced by the liver and kidneys and activated in the skin.

The main biological effects of **T3** and **T4** are on growth and development (e.g., maturation of tadpoles), their calorigenic effect (increase of basic metabolic rate), cardiovascular function (increased sensitivity of the heart to catecholamines), and metabolic functions (lipid, carbohydrate, and collagen metabolism). The primary feedback effect is inhibition of thyroid-stimulating hormone (TSH) secretion. These effects can be used to test thyroid hormone

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analogs and metabolites. The thyroid hormones regulate iodine uptake and utilization in the thyroid, and their action can be inhibited by antithyroid drugs.

Historical bioassays rely on morphogenesis and neuronal development in **amphibia** (Biedl 1916; Pitt-Rivers and Tata 1959; Copp et al. 1962; Turner and Premachandra 1962). Thyroid hormones induce premature metamorphosis in amphibian tadpoles. Since the first observation by Gudernatsch (1913a, b), this phenomenon has been studied by numerous workers with the purpose of adapting this response for the assay of thyroidal substances (Bomskov 1937). Within a short period of time, the treatment with thyroid hormones induces the transformation of the **tadpole** into a small frog with growth of limbs, lungs, and other terrestrial accouterments and stimulates the synthesis of enzymes mediating morphogenesis and transformation.

The **axolotl** (*Ambystoma mexicanum* or *tigrinum*) has been used as a test object to study metamorphosis induced by thyroid hormones. This animal loses the gills and forms lungs, changing the shape of its tail at the same time (Huxley and Hogben 1922; Zavadovsky and Zavadovsky 1926; Haffner 1927).

Another principle action of T_3 and T_4 is metabolic activation and increased energy expenditure. Kreitmair (1928) standardized thyroid preparations using the **weight loss of guinea pigs** after 1 week of treatment as a parameter. A guinea pig unit was defined as the dose which reduces the body weight of guinea pigs with an initial weight of 250–300 g within 7 days by at least 10 %.

A different functional role is subserved by calcitonin. The **hypocalcemic hormone calcitonin** was discovered by Copp (Copp et al. 1962; Copp 1964). Calcitonin originates from parafollicular C cells of the thyroid. Its functional antagonist is parathyroid hormone. The bioassay of calcitonin preparations is performed by assessing their ability to **lower the plasma calcium** in the rat. Assay of serum (thyro)calcitonin has a significant diagnostic role for thyroid carcinoma.

As with other hormones, research methods have progressed from bioassays of thyroid hormone action to direct measurement of the thyroid hormones (thyroxine and triiodothyronine) and their metabolites, to studies on enzymatic steps in thyroid hormone synthesis and inactivation, to the identification of thyroid hormone receptors as members of the superfamily of nuclear receptors, and to signaling induced by binding of thyroid hormones to their receptors.

Thyroid Hormone Receptors

Nuclear triiodothyronine-binding proteins were purified and characterized by Torresanai and Anselmet (1978). Ichikawa and DeGroot (1987a, b) described the purification and characterization of rat liver nuclear thyroid hormone receptors and thyroid hormone receptors in a human hepatoma cell line. Apriletti et al. (1988) reported large-scale purification of the nuclear thyroid hormone receptor from rat liver and sequence-specific binding of the receptor to DNA. Ichikawa et al. (1988) and Ichikawa and Hashizume (1991) published methods of an aqueous two-phase (dextran and polyethylene glycol) partitioning study of nuclear thyroid hormone receptors. Glucocorticoids, other steroid hormones, thyroid hormones, and vitamin-derived hormones (including retinoids) all exert their effects by the regulation of hormone-responsive target genes within the cell nucleus. William and Franklyn (1994) reviewed the physiology of the steroid–thyroid hormone nuclear receptor superfamily. A nuclear hormone receptor-associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors was described by Burris et al. (1995). Two different genes encode two different thyroid hormone receptors, thyroid hormone receptor-α and thyroid hormone receptor-β, and these two thyroid hormone receptors are often co-expressed at different levels in different tissues. Chiellini et al. (1998) designed a high-affinity subtype-selective agonist ligand for the thyroid hormone receptor-β. The expression of thyroid hormone receptor isoforms in rat growth plate cartilage in vivo was described by Ballock et al. (1999). Yuan et al. (1998)

described a component of a thyroid hormone receptor-associated protein (TRAP) co-activator complex which interacts directly with nuclear receptors in a ligand-dependent fashion. The sequence of the thyroid hormone response element and the recruitment of retinoid X receptors for thyroid hormone responsiveness were investigated by Wu et al. (2001).

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Thyroidectomy

Purpose and Rationale

Experiments for pharmacological evaluation of thyroid hormones and analogs were performed in thyroidectomized rats. Bomskov (1937) described the method of thyroidectomy in various animal species, such as tadpoles, frogs, birds, goats, dogs, cats, rabbits, guinea pigs, rats, and mice, based on the clinical experience with thyroid resection in humans.

Procedure

The thyroid in rats consists of three lobes (left, median, and right). The rat is anesthetized, e.g., with pentobarbital, and placed on a surgical table. The fur of the neck is removed with electric clippers and the area disinfected. A median skin incision of 2.0 cm length is made. On both sides, large salivary glands and maxillary lymph nodes are found. They are pushed aside, making visible the musculus hyoideus covering the trachea. This muscle is split in the midline. The isthmus of the thyroid connecting both lobes is located below the thyroid cartilage. The lobes and the isthmus are separated with blunt forceps from the trachea and the blood vessels ligated. Alternatively, the thyroid can be removed by electrocauterization. In most cases, the parathyroid glands are severed by the operation, and postoperative substitution with calcium lactate 1 % in drinking water is advised.

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In Vivo Tests for Thyroid Hormones

Oxygen Consumption

Purpose and Rationale

Basal metabolic rate, oxygen consumption, and CO_2 production are increased by thyroid hormones. This has been used for diagnostic procedures in humans as well as for evaluation of thyroid hormones and their derivatives in animals (indirect calorimetry). The historical method based on survival time of mice placed individually into tightly closed glass jars (Smith et al. 1947; Basil et al. 1950; Gemmill 1953) was modified, measuring time until occurrence of convulsions. The method was based on the increase in oxygen consumption associated with the markedly increased metabolic rate at high doses of thyroid hormones.

Procedure

This is a description of the now obsolete assay: mice are placed individually into 200-ml wide-necked bottles. The bottom of the bottles is covered with filter paper to soak up the urine. The bottles are tilted to an angle of 60° and rotated five times per minute in a special apparatus. The time until asphyctic seizures occur is noted. Immediately after observation of seizures, the mouse is released for recovery. Due to the defined muscle work, the time to seizures is shortened in controls to 20–30 min.

Evaluation

Average time to seizures was calculated, and dose–response curves were established.

Modifications of the Method

Similar studies were reported by Bomskov (1937), Lilienthal et al. (1949), MacLagan and Sheahan (1950), Reineke and Turner (1950), Anderson (1954), Heming (1964), and Turner (1969).

Several apparatuses have been designed to measure oxygen consumption in animals, e.g., by Holtkamp et al. (1955).

Stock (1975) described an automatic, **closed-circuit oxygen consumption apparatus** for small animals. A Perspex animal chamber is surrounded by a water jacket except for one end, which has a removable cover plate. This cover, as well as allowing access to the chamber interior, also holds the connections for the oxygen delivery line and the pressure line. For experiments involving injections, infusions, and blood sampling, catheters are passed through and sealed into rubber bungs which are then forced into holes in the cover plate. A rubber gasket forms an airtight seal between the cover and the chamber. Within the chamber, the animal is supported on a wire grid over a layer of self-indicating soda lime and silica gel. A major determinant of sensitivity in this system is the dead space of the chamber. Chambers with internal dimensions of 20 × 10 × 10 cm are suitable for animals such as mice and rats up to about 250 g body weight. Fixed volumes of oxygen are introduced into the chamber by an automatic syringe dispenser (Fisons Scientific) which draws pure oxygen from a spirometer through a drying tube filled with silica gel. When chamber pressure exceeds atmospheric by about 3 mmH_2O, the microdifferential pressure switch (KDG Instruments) inactivates the dispenser. The dispenser is reactivated when the pressure differential drops below this threshold value. The volume of oxygen dispensed is adjusted to the smallest volume that, with a single action of the syringe, will return chamber pressure to above the threshold value. The particular dispenser used in this system has the advantages of being (1) gastight and (2) when activated will complete its pump cycle even if the chamber pressure exceeds the threshold value in mid-cycle. A discrete fixed volume of oxygen is delivered each time it is activated. To obtain the rate of oxygen consumption, it is merely necessary to record the pump rate.

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Inhibition of Iodine Release

Purpose and Rationale

The thyroid gland has a high avidity for iodine, uptake of which may be measured by isotope-labeled iodine (^{131}I), in a dose-related and time-dependent manner. The release of ^{131}I from the thyroid in rats is inhibited by treatment with thyroxine (Wolff 1951), and the degree of inhibition is related to the dose administered (Perry 1951). This phenomenon was used to compare activity of thyroid hormone derivatives with the standard thyroxine.

For analytical and diagnostic purposes, direct quantitation of thyroid hormones is now achieved by methods such as radioimmunoassay and HPLC chromatography and by measuring feedback inhibition of thyroid hormones directly via the decrease in serum TSH.

Procedure

Male Sprague–Dawley rats weighing 180–240 g are fed a commercial laboratory chow without or with addition of 0.03 % propylthiouracil (reference compound for thyroid peroxidase inhibition). Food is withheld 8 h before the injection of 25 μC ^{131}I or ^{125}I intraperitoneally. Radioactivity over the thyroid region of the neck is determined 40 h later (if necessary under sedation). This reading is taken as time zero, and all further counts made at 24-h intervals may be expressed as a percentage of time zero counts after correction for physical decay of the ^{131}I isotope. After the reading at time zero, the diet is changed to a feed containing 0.03 % propylthiouracil, and several doses of the test preparation or the standard are injected subcutaneously at 24-h intervals up to a total of four doses. The daily loss of ^{131}I is inversely proportional to the dose of thyroid hormone.

Evaluation

Percentage of time zero counts after 96 h of ^{131}I remaining in the thyroid after the last of four doses is plotted against logarithm of dose. From these dose–response curves, potency ratios are calculated.

The method has been used by several authors: Reineke and Turner (1950), Anderson (1954), and Turner and Premachandra (1962).

Critical Assessment of the Method

The assay described here was used for quantitative estimates and has now been replaced by

analytical determination of thyroid hormone contents. For human drug formulations, bioequivalence studies are required when generic formulations are assessed. This approach of measuring the uptake and release of labeled iodine may be modified for short-term uptake of ^{131}I or ^{125}I as a parameter of thyroid peroxidase inhibition by antithyroid drugs and other drugs affecting thyroid function.

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Anti-Goitrogenic Activity

Purpose and Rationale

Thyroid weight and size are controlled by the action of thyroid-stimulating hormone (TSH) on thyroid tissue. In rats, increased secretion of TSH induces thyroid enlargement and weight increase within a few days (addressed as goiter formation). In normal animals, the secretion of TSH by the pituitary is regulated by feedback of thyroid hormones. The administration of goitrogenic compounds which block thyroid hormone synthesis and/or secretion reduces the concentrations of circulating thyroid hormones (T_4/T_3) and their pituitary effect (negative feedback inhibition of TSH secretion), releasing TSH from its feedback inhibition. The TSH rise induces hyperplasia of the thyroid follicles as indicated by the dose-related increase of thyroid weight. Hyperplasia is prevented by injection of thyroxine, triiodothyronine, or thyroid hormone analogs.

Procedure

Male Sprague–Dawley rats weighing 150–180 g are used in groups of eight to ten animals. During the treatment period, 0.1 % propylthiouracil (PTU) is added to the food or to the drinking water, in order to achieve a stable baseline of thyroid weight. Over a period of 2 weeks, the rats are treated (preferably by gavage) with various doses of the test compound or the thyroxine standard (10–40 μg/kg). PTU controls are treated with the suspension medium or saline injections only. At autopsy on day 14, the thyroid glands are dissected out and weighed rapidly to avoid evaporation loss. Thyroids may also be lyophilized first to weigh dry matter. The two- to threefold increase of thyroid weight by PTU is reversed dose dependently to normal values by thyroid active substances.

Evaluation

Dose–response curves are plotted and potency ratios with confidence limits may be calculated.

Modifications of the Method

Similar studies were reported by Reineke et al. (1945), Pitt-Rivers and Tata (1959), Turner and Premachandra (1962), Wiberg et al. (1964), Ortiz-Caro et al. (1983), and Pisarev et al. (1994).

The effect of PTU-induced baseline suppression is monitored and ascertained by measuring serum TSH, T_4 and T_3. The dose-related inhibition of the TSH rise by thyroid substances is used as the parameter to assess goiter prevention.

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Tensile Strength of Connective Tissue in Rats, Modified for Thyroid Hormones

These studies are an example of evaluating the biological effect of high doses of thyroid hormones on tissues other than those involved in the increase of metabolic rate. Thyroid hormone secretion affects almost all tissues in the body, and high doses may exert unwanted effects on connective tissue.

Purpose and Rationale

The decrease of tensile strength after a single high-dose injection of thyroid hormones is dose dependent and can be used for evaluation of thyroid hormone derivatives. Short-term treatment with corticosteroids increases the strength of connective tissue (Vogel 1969). This effect is antagonized by thyroid hormones (Ther et al. 1963; Vogel and Ther 1964). Thyroid hormones per se have a biphasic effect: short-term treatment decreases the dose-dependent tensile strength of epiphyseal cartilage, tail tendons, and skin strips, whereas treatment over 10 days increases tensile strength, probably mediated by the activation of endogenous adrenal secretion (similar to the effect of corticosteroid treatment).

Procedure

Male Sprague–Dawley rats weighing 110 ± 10 g are injected subcutaneously with thyroid hormones (dose range of the standard L-triiodothyronine 0.1–1.0 mg/kg). After 24 h, the animals are sacrificed, and the tensile strength of distal femoral epiphyseal plates, tail tendons, or skin strips is tested as described in chapters "▶ Adrenal Steroid Hormones" and "▶ Acne Models".

Evaluation

Doses–response curves of test compounds and standard are established and potency ratios may be calculated. T_3 (L-triiodothyronine) is about 3 times more active than T_4 (levothyroxine), in accordance with its metabolic activity. The mechanism of action of this short-term effect of thyroid hormones on connective tissue has not been explored.

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Antithyroid Drugs

General Considerations

Antithyroid drugs interfere with synthesis, release, and/or the peripheral action of the thyroid hormone, lowering the basal metabolic rate. They are used in the treatment of thyroid disorders (hyperthyroidism). The suppression of T_4/T_3 secretion reduces thyroidal inhibition of the pituitary gland, increasing TSH secretion, and then induces the goitrogenic response. This response was used to detect antithyroid drugs and has been widely used for screening procedures. It is, however, nonspecific and may be caused by several

different mechanisms, including enzyme induction of glucuronyltransferases. The goitrogenic response is of considerable interest in toxicology, because it may be produced by several compounds during early drug evaluation which modify the biosynthesis and/or inactivation of thyroid hormones in an unexpected manner.

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Inhibition of Iodine Uptake in Rats

Purpose and Rationale

Propylthiouracil (PTU) and a wide spectrum of drugs may inhibit thyroid hormone synthesis. Some of these drugs are used to treat thyrotoxicosis. As a consequence of thyroid peroxidase inhibition, the iodine uptake by and content in the thyroid are decreased. This phenomenon is dose dependent and may occur at lower doses than those increasing thyroid weight in rats (McGinty and Bywater 1945). The historical parameter of iodine content has been replaced by measuring the uptake and release of ^{131}I.

Procedure

Groups of male Wistar rats age 26–28 days, weighing 40–45 g, are placed into metabolism cages. They are fed normal diet, and potassium iodide is added to the drinking water. In one modification of the method (for toxicology studies), the test compounds or the reference standard (several concentrations) may be added to the diet over a period of 10 days, and the amount of compound ingested by each rat is then calculated from the total food consumption over 10 days and expressed in milligram daily per kilogram of body weight. After 10 days of treatment, the rats are sacrificed and the thyroids dissected free from adjacent tissue and capsule. The thyroid is weighed and iodine content determined. In daily doses of between 0.1 and 10.0 mg/kg, thiouracil decreases the iodine content of the thyroid in a dose-dependent manner. Definitely higher doses are necessary to increase thyroid weight.

Evaluation

Dose–response curves of test compounds and reference standard are plotted, and potency ratios with confidence limits may be calculated.

Modifications of the Method

Walker and Levy (1989) used implantable pellets of propylthiouracil to induce thyroid dysfunction in rats. Uptake of labeled iodine is measured instead of iodine content. Release of labeled iodine may be stimulated by protirelin (TRH) injection in order to assess thyroid function or as a quantitative bioassay for the effect of the hypothalamic hormone TRH.

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Antithyroidal Effects in Animal Assays

The oxygen consumption in iodine-treated mice has been used as a bioassay, modified for antithyroid activity.

Purpose and Rationale

The historical bioassay is based on oxygen consumption, which is increased in acutely potassium iodide-treated mice, resulting in a decrease of asphyxiation time (thyroid activation). This effect is dose dependently antagonized by antithyroidal compounds, and the time to convulsions is prolonged due to the reduced metabolic rate. The methods based on increased oxygen consumption after thyroid hormones (section "Oxygen Consumption") are applied.

Modifications of the Method

Thyroid weight was an early parameter for the detection of antithyroid activity. Rabbits treated with goitrogenic compounds or fed exclusively with cabbage (Chesney et al. 1928; Marine et al. 1929) showed an up to tenfold increase of thyroid weight, histologically manifested as hyperplasia without colloid formation. These phenomena were reversed by iodine treatment (Bomskov 1937).

Goiter formation as a side effect of nonsteroidal anti-inflammatory drugs was studied by Müller et al. (1985).

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Calcitonin

General Considerations

The calciotropic hormone (thyro)calcitonin was discovered in C cells of the thyroid gland by Copp (Copp et al. 1962; Copp 1964; 1994). This hypocalcemic hypophosphatemic principle of the thyroid gland (Austin and Heath 1981) was designated thyrocalcitonin by Hirsch et al. (1964), Munson and Hirsch (1966), Raisz et al. (1967), and MacIntyre (1992). Its calciotropic effects on bone and kidney function are opposite to those of the parathyroid hormone. Calcitonin originates from parafollicular C cells of the thyroid. Calcitonin secretion can be evaluated in vitro using the isolated perfused porcine thyroid (Pento 1985). Radioimmunoassays for calcitonin are available (Tashjian and Voelkel 1979), and species-specific methods for calcitonin determination need to be considered. Assays for calcitonin receptors have been described (Nissenson et al. 1985). Surveys on the effects of exogenous calcitonin were given by Deftos (1989), Braga (1994), and Wallach et al. (1999). The biology and clinical relevance of calcitonin gene peptides have been reviewed (Reginster 1993; Silverman 2003; Zaidi et al. 1990).

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Decrease of Serum Calcium in Rats

Purpose and Rationale

The bioassay of calcitonin preparations is performed using their ability to lower the plasma calcium in the rat. This procedure has also been adopted by pharmacopeias, using the International Reference Preparation for Calcitonin (porcine) consisting of freeze-dried purified pork calcitonin and the International Reference Preparation for Calcitonin (salmon) consisting of freeze-dried purified synthetic salmon calcitonin. These assays for calcitonin quantitation, however, have now been replaced by a physicochemical method for pharmaceutical quality control. Either intravenous or subcutaneous administration can be chosen. International standards for salmon calcitonin, eel calcitonin, and the Asu^{1-7} analog of eel calcitonin have been elaborated (Zanelli et al. 1990). A second international standard for porcine and human calcitonins has been established by an international collaborative study group based on the in vivo rat hypocalcemia bioassay (Zanelli et al. 1993).

Procedure

Groups of at least five female Wistar rats, weighing 100–120 g, are used. Three doses of standard preparation (1, 3, and 9 mU per rat) and three doses of test preparation are injected intravenously. Then 1 h after injection, blood is withdrawn under light anesthesia. Plasma calcium is determined by flame photometry or by atomic absorption photometry.

Evaluation

Dose–response curves of decreases in plasma calcium are established, and potency ratios with confidence limits are calculated.

Modifications of the Method

Similar studies were reported by Kumar et al. (1965), Munson et al. (1968), Rittel et al. (1976), Schwartz et al. (1981), Michelangeli et al. (1983), Findlay et al. (1985), Buck and Maxl (1990), and Deming et al. (1994).

Yates et al. (1990) assessed the acute hypocalcemic responses to single subcutaneous injections of calcitonin preparations in intact young male ICR Swiss mice weighing 12–20 g.

Calcitonin of the stingray and of the goldfish was characterized by Sasayama et al. (1992, 1993).

Kapurniotu and Taylor (1995) performed in vitro hypocalcemic assays in mice by analysis of serum calcium 1 h after subcutaneous injection of lactam-bridged analogs of human calcitonin.

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Effect of Calcitonin on Osteoclasts In Vitro

Purpose and Rationale

Calcitonin acts primarily by inhibition of osteoclastic bone resorption (Friedman and Raisz 1965;

Aliapoulios et al. 1966). Zaidi et al. (1990, 1994) reported the development and validation of three microbioassays for calcitonin based on calcitonin-induced inhibition of the activity of isolated osteoclasts.

Procedure

Femora and tibiae are removed from newborn Wistar rats. The bones are freed from adherent soft tissues and cut across their epiphyses in HEPES-buffered medium 199 supplemented with heat-inactivated fetal calf serum, benzyl penicillin (100 μU/ml), and streptomycin (100 μg/ml). The osteoclasts are mechanically disaggregated by curetting the bones of each rat with a scalpel blade into 1 ml medium and agitating the suspension with a pipette. Larger fragments are allowed to settle for 10 s, before the supernatant is dropped onto appropriate substrate (bone slices, plastic Petri dishes, or glass coverslips).

Motility-Based System

The morphological appearance of stained osteoclasts is used as an index to assess the state of cytoplasmic activity. Osteoclasts are settled on coverslips in microtiter wells and are incubated for 20 min at 37 °C. The coverslips are removed, washed with medium 199, and placed in separate wells, each containing 100 μl medium.

Following a further incubation for 30 min (37 °C), serial dilutions (tenfold) of salmon or human calcitonin or test preparations or appropriate dilutions of plasma samples are added. The cells are finally incubated for 2 h, fixed in 10 % glutaraldehyde, and stained with toluidine blue. The state of motility of each osteoclast on each coverslip is scored by observing the characteristic shape change these cells undergo when motility is inhibited; a motile cell is characterized by a smooth outline with increased staining intensity over all or part of its periphery, whereas an immotile cell typically shows an irregular pale outline without ruffled edges. The number of immotile cells is counted on each coverslip and expressed as a percentage of the total number of cells counted.

Cytoplasmatic Spreading System

Osteoclasts are settled in tissue culture dishes (35 mm) and are incubated at 37 °C for 20 min to allow sedimentation and attachment. The cells are then washed with medium 199, and 2 ml of the same medium is placed in each well. The dishes are placed in the incubation chamber of an inverted phase-contrast microscope. Images of the osteoclasts are recorded on a time-lapse video recorder. A tracing of their outlines is transferred through a digitizing system into a computer, programmed to measure the area within each tracing.

The outlines of each osteoclast are recorded before or after the addition of calcitonin or vehicle to the cultures. For each variable, the outline of six osteoclasts is traced after a 60-min incubation in the chamber and again 40 min following the addition of the hormone. The mean surface area covered by six osteoclasts after incubation is expressed as a percentage of the mean surface area of the osteoclasts before the addition of hormone or vehicle.

Bone Resorption System

Specimens of human femoral cortical bone are obtained from donors (patients who died without evidence of bone disease). The adherent soft tissue is removed and the bone cortex cut longitudinally into slices (0.1 mm thick). The slices are then cut into pieces (approximately 3 mm^2). They are cleaned by ultrasonication (15 min, in sterile distilled water), dehydrated by immersion in 80 % aqueous ethanol for 2 h, and stored to dry at room temperature. Osteoclasts isolated in medium 199 are dropped onto 12–16 bone slices placed in a well of an 18-mm multiwell dish. Following incubation (37 °C, 15 min), slices are removed and washed gently in minimal essential medium supplemented with 10 % FCS and antibiotics as described above. They are placed in separate wells, each well containing 5–6 slices in 900 μl medium. After further incubation (37 °C, 10 % humidified CO_2, 10 min), 100 μl of medium containing the test concentration of the hormone or the test solution is added. Human $PTH_{(1–34)}$ (0.1 U/ml) is used to assess functional effects of contaminating osteoblasts.

The calcitonin analogs are tested at various concentrations (tenfold dilutions). Finally, bone slices are incubated overnight (37 °C, 10 % humidified CO_2, 18 h). The cells are fixed in glutaraldehyde, stained with toluidine blue, and examined by transmitted light microscopy. Osteoclasts and mononuclear cells are counted. The slices are then bleached by immersion in sodium hypochlorite solution for 30 min and dehydrated in 80 % aqueous ethanol. Finally, they are sputter coated with gold, randomized, and examined in a scanning electronic microscope. The numbers of osteoclastic excavations, each defined by a continuous border, are counted. The area of bone surface resorbed is calculated by tracing the outline of the concavities into a digitizing tablet, linked to a microcomputer. Resorption surface areas may be expressed as a percentage of the mean of the control response.

Evaluation

Data of each assay are analyzed using classical methods for analysis of parallel line assays. Estimates of relative potencies are calculated from parallel log dose–response lines of test preparations and a reference preparation.

Osteoclasts are mechanically disaggregated from neonatal rat long bones and dispersed at low densities on slices of devitalized bovine cartilage bone. The resulting areas of bone excavation are quantified with micrometric precision by scanning electron microscopy together with computer-assisted image analysis. These findings are used to develop a formal bioassay for calcitonin.

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Receptor Binding and cAMP Accumulation in Isolated Cells

Purpose and Rationale

The human breast cancer cell line T47D responds to calcitonin and its analogs by receptor binding and accumulation of cAMP. This can be used as a biological assay (Findlay et al. 1980; 1983, 1985; Grauer et al. 1992; Sexton and Hilton 1992; Blind et al. 1993).

Procedure

The human breast cancer cell line T47D was originally established from a pleural effusion from an infiltrating ductal breast cancer (Horwitz et al. 1978). For binding experiments, cell monolayers are washed with 0.02 % EDTA before treatment with 0.125 % trypsin in 0.02 % EDTA for 2 min at 37 °C, addition of complete medium before centrifugation at 200 *g*, and resuspension in complete medium.

Iodination of calcitonin is performed with ^{125}I using the chloramine-T method.

For binding experiments, T47D cells suspended in isotonic buffer are added to ^{125}I-labeled salmon calcitonin mixed with varying concentrations of unlabeled calcitonin or analogs and incubated at 20 °C for 1 h. Nonspecific binding is assessed as the binding of ^{125}I-labeled salmon calcitonin in the presence of excess (2 μg/ml) unlabeled salmon calcitonin.

Stimulation of adenylate cyclase in intact T47D cells by calcitonin analogs is assessed by measuring [^3H]cAMP production in cells prelabeled with [^3H]adenine. Cellular ATP pools are labeled by incubation with 2,8-[^3H]adenine (0.5–2 μCi/ml) for 2 h at 37 °C in 12-well culture dishes in

RPMI 1640 medium containing 0.1 % BSA. Cells are then washed twice with serum-free medium and incubated for a further 20 min in medium containing 0.1 % BSA and 1 mM isobutylmethylxanthine (IBMX) before treatment with calcitonin and its analogs for 10 min at 37 °C in the same buffer. Incubations are terminated by removing medium and adding 100 µl 20 % trichloroacetic acid at 4 °C. This is followed by 800 µl of a 5 mM solution of ATP, ADP, AMP, cAMP, and adenine. The [^{3}H]cAMP is isolated by chromatography on Dowex and alumina. Radioactivity is counted in a scintillation counter.

Evaluation

For both parameters, full dose–response curves are generated, and the concentrations required for half-maximal responses are calculated.

Modifications of the Method

Yates et al. (1990) measured stimulation of adenylate cyclase activity by calcitonin analogs in primary cultures of mouse renal cortex which were prepared according to the methods of Fukase et al. (1982) from 4-week-old ICR Swiss mice and used at confluence after 4 days of culture.

A radioreceptor assay for potency determinations of formulations of salmon calcitonin was described by Sjödin et al. (1990).

Albrandt et al. (1993) cloned two receptors with high affinity for salmon calcitonin from the nucleus accumbens region of rat brain.

Likewise, Sexton et al. (1993) identified in rats two isoforms of the calcitonin receptor, designated C1a and C1b.

Functional aspects of the isoforms C1a and C1b were discussed by Martin et al. (1995).

The calcitonin receptor isoforms C1a and C1b were localized in rat brain using in vitro autoradiography by Hilton et al. (1995).

Keustner et al. (1994) cloned and characterized a second form of the human calcitonin receptor from T47D cells.

Sexton et al. (1994) assayed the cloned renal porcine calcitonin receptor cDNA expressed by transient transfection in COS-1 cells or stable transfection in HEK-293 cells for interaction with calcitonin, amylin, and calcitonin gene-related peptide. The results suggested that amylin may act as a natural ligand for the renal porcine calcitonin receptor.

The various pathways in signal transduction by calcitonin were discussed by Horne et al. (1994).

Houssami et al. (1994) found that different structural requirements exist for calcitonin receptor binding specificity and adenylate cyclase activation.

Suva et al. (1997) synthesized benzophenone-containing calcitonin analogs and tested them for receptor binding and stimulation of cAMP accumulation.

Povzek et al. (1997) investigated the structure–function relationship of salmon calcitonin analogs as agonists, antagonists, or inverse agonists in heterologous calcitonin receptor expression systems using two calcitonin receptor cell clones, B8-H10 and G12-E12, which express about 5 million and 25,000 C1b receptors/cell, respectively.

The location of the phosphorylation of the human calcitonin receptor by multiple kinases was studied by Nygaard et al. (1997).

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Parathyroid Hormones

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Contents

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General Considerations

The primary function of parathyroid hormone (PTH) is to maintain a constant concentration of Ca^{2+} in the extracellular fluid. Processes that are regulated include the absorption of Ca^{2+} from the gastrointestinal tract, the deposition and mobilization of bone Ca^{2+}, and the excretion of Ca^{2+} in urine, feces, sweat, and milk. PTH is the functional antagonist of calcitonin. The most prominent effect is to promote the mobilization of Ca^{2+} from the bone. In the kidney, the tubular reabsorption of Ca^{2+} is increased and tubular reabsorption of phosphate is inhibited. Clinically, idiopathic or postoperative hypoparathyroidism results in hypocalcemia followed by tetany.

PTH is used for the treatment of hypoparathyroidism, and the symptoms of the disease can also be effectively antagonized by oral administration of dihydrotachysterol. A survey on the structure and function of the parathyroid gland in animals was published by Capen and Rosol (1989). The biologically active synthetic human parathyroid hormone 1–34 fragment (active sequence of PTH) is used for diagnostic testing (Mallette 1988).

An experimental model for secondary hyperparathyroidism with elevated levels of parathyroid hormone in rats has been described by Sancho et al. (1989).

Radioligand assays for parathyroid hormone receptors have been described (Habener and Potts 1976; Nissenson et al. 1985; Schneider et al. 1993).

Immunoassays for PTH are those measuring intact hormone (N-terminal, intact) and those measuring inactive fragments and partial sequences of the intact hormone (mid-region, C-terminal, polyvalent) (Endres et al. 1989). An immunochemiluminometric assay has been described by Klee et al. (1992).

Parathyroid hormone-related protein (PTHrP) was first identified and cloned from malignant tumor cells and tissues from patients with a syndrome called humoral hypercalcemia of malignancy (Moseley et al. 1987; Strewler et al. 1987; Suva et al. 1987). PTHrP-(1–34) and PTH-(1–34) act via a single species of cloned receptor (Abou-Samra et al. 1992; Schipani et al. 1993), although other studies have shown that specific receptors for each of these peptides exist (Usdin et al. 1995; Behar et al. 1996; Bergwitz et al. 1997; Yamamoto et al. 1997; Fukayama et al. 1998). PTHrP is an example of the widening array of sequences identified by molecular endocrinology, with functional characterization of their endocrine role often following after a considerable delay.

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Receptor Binding Assay for PTH

Purpose and Rationale

In many instances, the presence of receptors is explored as the counterpart of the presence of hormonally active substances in tissues and organs. However, the presence of binding sites does not immediately indicate functional activation of the respective tissue, and there are indeed circulating "free receptors" which have been identified as subunits of fragments of "whole receptors" located in cell membranes. Cloned receptors are frequently used for advanced research, to identify compounds which might interact with functional receptors in tissues, and acquire relevance as pharmacological tools, for example, for high-throughput screening. Furthermore, using radioligand assays to determine the receptor affinity of PTH analogs may provide helpful structure–activity information. Three receptors that are activated by PTH have been cloned. PTH and PTHrP bind to the PTH_1 receptor, for which many studies are published (Abou-Samra et al. 1992; Uneno et al. 1992; Schipani et al. 1993; Ureña et al. 1993; Kaufmann et al. 1994; Schermer et al. 1994; Orloff et al. 1995; Usdin et al. 1995; Bergwitz et al. 1996, 1997; Gardella et al. 1996; Yasuka

et al. 1996; Bisello et al. 1997; Guo et al. 1997; Yamamoto et al. 1997; Fukayama et al. 1998; Yaghoobian and Drueke 1998).

Behar et al. (1996b) reported ligand binding in stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor and HEK/BP16 cells that express the hPTH2 receptor.

The PTH_2 receptor has about 50 % amino acid sequence identity with the PTH_1 receptor and is not activated by PTHrP, but by a peptide isolated from bovine hypothalamus, TIP39, which is only distantly related to PTH and PTHrP (Usdin 2000).

The PTH_3 receptor has been isolated from zebra fish (Rubin and Juppner 1999).

It is part of the problem of advanced research that cloning of receptors frequently precedes the validation of physiological relevance of receptor subclasses.

Procedure

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994) and HEK/BP16 cells that express the hPTH2 receptor (Behar et al. 1996a), are maintained in DMEM supplemented with 10 % FBS.

The cells are incubated with [^{131}I]PTH-(1–34) (100,000 cpm/well) with or without competing unlabeled PTH-(1–34) or other ligands in binding buffer for 2 h at room temperature. Cells are washed twice with PBS and then solubilized in 0.5 ml of 0.1 M NaOH. Aliquots are taken for the determination of bound radioactivity by γ-counting.

Evaluation

Specific binding is expressed as counts per minute bound per well (raw data) and converted to the percentage specific binding of radioligand. Affinity constants and binding capacity of these receptors in tissue may be calculated, and dose–response curves of ligands are compared as a first step toward proof of signaling and biological activity.

Modifications of the Method

McCuiag et al. (1994) reported the molecular cloning of the gene encoding the receptor for the parathyroid hormone/parathyroid hormone-related peptide in the mouse.

Inomata et al. (1995) characterized a PTH receptor with specificity for the carboxy-terminal region of PTH-(1–84).

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PTH Assay by Serum Calcium Increase

Purpose and Rationale

The classical method by Collip and Clark (1925) involves measurement of the rise in serum calcium after administration of parathyroid extracts to dogs. Hamilton and Schwartz (1932) use rabbits treated with oral loads of calcium chloride. The intact rat is very insensitive to injected parathyroid hormone; however, parathyroidectomy produces an increase in sensitivity (Holtz 1937; Davies and Gordon 1953; Davies et al. 1954; Thorp 1969; Zull and Malbon 1976).

Procedure

Male Wistar rats weighing 200–250 g are anesthetized with pentobarbital sodium intraperitoneally. Parathyroidectomy is performed by cauterization. After a recovery period of 1 week, blood is withdrawn by retro-orbital puncture (baseline). Various doses of the test preparation or standard are injected subcutaneously to groups of six to ten animals. Blood samples are obtained again 21 h later. Serum calcium is determined by flame photometry. The increase of calcium 21 h after PTH injection is calculated for each animal.

Evaluation

Mean values of the increase in serum calcium are plotted versus logarithm of dose, and potency ratios versus PTH standard may be calculated for the test compounds.

Modifications of the Method

An increase of whole-body calcium and skeletal mass after repeated-dose treatment with parathyroid hormone in normal rats and in rats with osteoporosis induced by pregnancy and lactation under a low-calcium diet was found by Hefti et al. (1981).

Parathyroid hormone prevented bone loss and augmented bone formation in ovariectomized rats (Kalu et al. 1990; Liu and Kalu 1990).

The active sequence of PTH has been used instead of the full secreted sequence.

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Serum Phosphate Decrease After PTH

Purpose and Rationale

Tepperman et al. (1947) developed a method using the fall in serum inorganic phosphorus in the rat after injection of parathyroid hormone (Thorp 1962).

Procedure

Male Wistar rats weighing 150–200 g are fed Purina dog chow for at least 2 weeks prior to the experiment. During the experiment, only water is allowed. Blood samples (baseline and PTH stimulated, 3 h after PTH) are taken from the tail and 0.6 ml is collected from each rat into tubes and centrifuged for 10 min and 0.2-ml samples of the serum are pipetted into 6 ml of 10 % trichloroacetic acid; solutions are centrifuged and 5-ml aliquots of the protein-free solution are used for the estimation of inorganic phosphorus, by the method of Fiske and Subbarow (1925), for example. Serum phosphorus is measured before and 3 h after subcutaneous administration of various doses of test preparation or standard.

Evaluation

Dose–response curves showing a linear relationship between log dose and response are suitable for the calculation of potency ratios.

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cAMP Release in Isolated Perfused Rat Femur

Purpose and Rationale

This is an in vitro assay. The effect of parathyroid hormone and analogs on release of cAMP from adult bone can be measured in a perfusion system of isolated rat femora (Sugimoto et al. 1985; Lopez-Hilker et al. 1992).

Procedure

Five-week-old Wistar rats anesthetized with pentobarbital (45 mg/kg) are heparinized and used as donors; the femora are removed. Adhering muscles are stripped from the bone. A hole with about half of the depth of the cortex is made with a fine drill at the nutrient foramen below the femoral neck.

Then a 21-gauge needle is inserted into this hole and fixed by dental cement to avoid leakage of the perfusate. The bone is then placed in an apparatus for liver perfusion and perfused at a flow rate of 1 ml/5 min by a pump with Krebs-Ringer bicarbonate, continuously gassed with 95 % O_2 and 5 % CO_2 and containing 1 mg/ml glucose. Once the perfused bone is assembled, the bone is allowed to equilibrate for 45 min. Samples are collected into a chilled tube for the last 5 min for determination of basal cAMP levels. Then various doses of the test preparations or the standard are infused for 5 min and serial samples are collected every 5 min. In the perfusate, cAMP is measured by radioimmunoassay.

Evaluation

Time–response curves and dose–response curves are established allowing the calculation of potency ratios with confidence limits.

Modifications of the Method

Nissenson et al. (1981) measured the activation of canine renal cortical plasma membrane adenylate cyclase activity produced by parathyroid hormone standard and test sera from parathyroid venous effluent of patients with primary hyperparathyroidism, in the presence of the hydrolysis-resistant GTP analog 5′-guanylimidodiphosphate.

Gundberg et al. (1995) compared the effects of parathyroid hormone and parathyroid-hormone-related protein on osteocalcin release in the isolated rat hindlimb and in intact and thyroparathyroidectomized rats.

Saito et al. (1987) established a new biological assay system for simultaneous measurement of bone resorption and bone mineralization in **organ cultures of chick embryonic femur**. Eleven-day-old chick embryonic femur was labeled with ^{45}Ca in vitro. $T_{1/2}$ of calcium efflux was calculated from the sequential release of the label into the medium. Parathyroid hormone increased calcium mobilization, indicating enhanced bone resorption, whereas hydrocortisone and sodium fluoride inhibited bone resorption. Calcitonin was ineffective.

Barling et al. (1989) measured the adenylate cyclase response to parathyroid hormone in cultured rabbit marrow fibroblast cells.

Docherty and Heath (1989) used osteosarcoma cells for an in vitro bioassay determining cAMP formation.

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Renal and Metatarsal Cytochemical Bioassay

Purpose and Rationale

Cytochemical bioassays using renal and metatarsal tissue are sensitive enough to detect plasma levels of parathyroid hormone and useful for determining the agonist and antagonist activities of fragments and analogs (Chambers et al. 1978; Goltzman et al. 1980; Bradbeer et al. 1988; Loveridge et al. 1991; Zaman et al. 1991; Bourdeau et al. 1990; Wood 1992).

Procedure

For the **renal cytochemical assay**, kidney segments from vitamin-D-depleted guinea pigs are maintained in nonproliferative organ culture for 5 h using Trowell's T8 medium (GIBCO). The medium is then changed for 8 min before exposure to various doses of the parathyroid hormone standard, the parathyroid hormone fragment, or the analog for an additional 8 min. In the experiments for antagonistic activities, each segment is exposed to a single concentration of hPTH-(1–84) (106 fmol/l) or to hPTH-(1–34) (255 fmol/l) in the presence or absence of the antagonist to be tested. The segments are then shock-frozen to − 70 °C in *N*-hexane before being sectioned at 16 μm on a cryostat. The sections are examined for glucose-6-phosphate dehydrogenase activity using specific staining. The precipitated formazan is quantified in the cells of the distal convoluted tubules by means of a microdensitometer (wavelength 585 nm). Ten readings with each of two duplicate sections are made and the results presented as the mean integrated absorbance × 100 (±SEM).

For the **metatarsal cytochemical assay**, the metatarsals of young female Wistar rats weighing 50–100 g are removed and the growth plates are isolated. Only the four longest metatarsals have growth plates large enough to be of use. The metatarsals are maintained individually in nonproliferative organ culture in 5–10 ml Trowell's T8 medium buffered to pH 7.6 in an atmosphere of 95 % O_2 and 5 % CO_2 at 37 °C for 5 h. After the culture period, the medium is removed and each metatarsal exposed to fresh medium (buffered to pH 7.6 by bubbling with a 95 % O_2 and 5 % CO_2 mixture) containing a low priming dose of PTH (0.5 fg/ml) for 8 min, followed by exposure to known concentrations of a standard PTH preparation or various concentrations of the analog or to dilutions of plasma for 8 min. The metatarsals are then briefly dipped in a 5 % solution of polyvinyl alcohol and chilled immediately in *N*-hexane to −70 °C.

Each bone is sectioned at 10 μm in a cryostat. The sections are reacted for glucose-6-phosphate dehydrogenase activity in a similar way as described for the renal cytochemical assay. The activity in hypertrophic chondrocytes is linear with respect to time from 10 min, at which time there is enough formazan to be measured, up to 40 min when the density of the formazan formed is too great for a reliable linear response. The enzyme activity in each section is measured in ten individual hypertrophic chondrocytes or osteoblasts lining the metaphyseal trabeculae by scanning and integrating microdensitometry at a wavelength of 585 nm. The results are presented as the mean integrated extinction × 100 (±SE) of ten measurements from each of two sections of each metatarsal.

Evaluation

Dose–response curves are tested for linearity and parallelism and potency ratios are calculated.

Critical Assessment of the Method

The cytochemical assays are sensitive but very time-consuming when compared with other analytical methods for the detection of PTH and PTH fragments.

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cAMP Accumulation in Cultured Cells

Purpose and Rationale

Parathyroid hormone and parathyroid hormone-related protein (PTHrP) stimulate intracellular cAMP accumulation in various cell types in a dose-dependent manner, for example, in PTHrP-overexpressing ROS cells, an osteoblast-like cell line Motomura et al. (1996); in UMR106 cells, a rat osteosarcoma cell line (Oldenburg et al. 1996); in the human osteosarcoma SaOS cell line (Rodan et al. 1987; Fukayama and Tashjian 1994); in COS-7 cells transfected with the human PTH-1 (PTH/PTHrP) receptor and the human PTH-2 receptor (Bergwitz et al. 1997); or in stably transfected human embryonic kidney cells (HEK-293/C-21 cells that express the hPTH/PTRrP receptor and HEK/BP16 cells that express the hPTH2 receptor) (Behar et al. 1996b).

Procedure

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994) and HEK/BP16 cells that express the hPTH2 receptor (Behar et al. 1996a), are maintained in DMEM supplemented with 10%FBS. For cAMP determination, C-21 or BP-16 cells are incubated in 24-well tissue plates with various peptides for 10 min in DMEM in the presence of 1 mM 3-isobutyl-1-methylxanthine. The incubation is terminated by the removal of the cell culture medium and addition of perchloric acid (final concentration 30 %, vol/vol). Samples are neutralized with potassium bicarbonate and acetylated with acetic anhydrate. Total cAMP values (medium plus cells) are determined by RIA. Data are presented as the percentage stimulation, from the ratio of maximal accumulated cAMP levels obtained in the presence of the highest concentration of the agonist PTH-(1–34) compared to the basal cAMP level. The accumulated cAMP levels (picomoles) are calculated per 100,000 cells. Cell number is determined in a Coulter counter.

Evaluation

Data are presented as the triplicate mean of $([cAMP]_c/[cAMP]_{PTHmax}) \times 100$ (±SEM) or sample stimulation vs. maximal PTH induced stimulation. $[cAMP]_c$ is the concentration of cAMP accumulated in response to a given concentration of a ligand, and $[cAMP]_{PTHmax}$ is the concentration of cAMP accumulated at the maximal dose (10^{-6} M) of PTH-(1–34).

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Bone Anabolic Activity in Ovariectomized, Osteopenic Rats

Purpose and Rationale

This is an in vivo bioassay which may be used for the beneficial effect of PTH on bone. Vickery et al. (1996) tested an analog of human parathyroid hormone-related protein (1–43) (hPTHrP) in ovariectomized, osteopenic rats.

Procedure

Groups of 3-month-old virgin female rats are subjected to bilateral ovariectomy or sham surgery. The ovariectomized animals are treated by daily subcutaneous injections of 0, 9, 29, or 80 μg/kg of test compound or human parathyroid hormone-related protein (1–43), starting on day 17 after surgery and continuing for 21 days. On days 12 and 19 following the first day of treatment, animals are dosed intraperitoneally with 6 mg/kg of a 3.75 mg/ml solution of calcein (Sigma) in 2 % sodium bicarbonate saline. Body weights are recorded, following an 18-h fast, on the last day of treatment. The animals are sacrificed immediately following the last injection, and the femurs, tibiae, and L2 vertebrae are excised.

The distal part of the right femur is cut in half longitudinally after removal of the epiphysis. The bone marrow is flushed out using a stream of water. The trabecular and cortical bones are separated using a dental drill. Calcium is extracted by immersion of the trabecular bone for 3 days and the cortical bone for 5 days in 5 % trichloroacetic acid. The calcium content of the extracts is determined. Measurements are expressed as

mean ± SEM in units of milligrams of Ca^{2+}/distal half femur per 100 g of body weight.

For histomorphometry, the left tibiae from sham, ovariectomized, and high-dose-treated rats are removed and fixed in formalin. The tibiae are cut with a diamond disk into three pieces: the proximal 1 cm, 2 cm of the diaphysis, and the distal end. The proximal and diaphyseal portions are transferred to Villanueva stain for 72 h (Villanueva and Lundin 1989). The proximal specimen is then dehydrated through increasing concentrations of ethanol (70 %–100 %), defatted in acetone, and embedded in modified methyl methacrylate. Pairs of 5-μm-thick frontal sections are prepared from the anterior aspect with a microtome. The first section is stained with a modified Masson trichrome technique (Goldner 1938) and the second left unstained.

The diaphyseal portion is also dehydrated, defatted, and embedded in methyl methacrylate. Five 150-μm-thick sections are prepared, ground to 100 μm thickness, and mounted unstained.

Growth cartilage thickness is measured by finding the distance between the proximal border of the epiphyseal growth cartilage and the metaphyseal junction at 12 places spaced 0.2 mm apart.

On each slide, a rectangular window is defined as the field for evaluation. The top line is drawn under the primary spongiosa, and the second line is drawn parallel to it and 3.5 mm distally. The side lines are drawn near to, but not touching, the endocortical surface.

Histomorphometric variables are calculated for mean trabecular volume and structure (trabecular thickness, number, spacing, mineralizing surface, individual osteoblast activity, and osteoclast surface). Surface-based, bone-volume-based, and total tissue-volume-based bone formation rates are also calculated.

In the cortical bone of the tibial diaphysis, the periosteal and endocortical surfaces are measured separately. Using transmitted light at 25 × magnification, the periosteal and endo-osteal perimeters are measured. The periosteal single-labeled and double-labeled surface and endo-osteal single-labeled and double-labeled surface are measured using 160 × magnification.

Vertebral processing and electron microscopy are performed in sham, ovariectomized, and compound-treated groups. The second lumbar vertebra is removed, dissected from soft tissue, and split sagittally with a diamond saw. The two halves are fixed in a mixture of glutaraldehyde and formaldehyde for 24–48 h at 4 °C. The tissue is decalcified in 10 % EDTA in 0.1 M cacodylate, pH 7.2, postfixed for 1.5 h in OsO_4, stained in block with uranyl acetate, dehydrated, and fixed in Epon. Then 50-nm sections are cut and examined in an electron microscope. Microphotographs are printed at a final magnification of 1,300 ×. Each microphotograph is overlaid with a grid with a total test line length of 2.22×10^3 μm. Each intercept of the line with the trabecular surface is scored into one of four categories, depending on the cell type adjacent to the intercept: osteoblasts, osteoclasts, lining cells, or no cells. The proportion of the trabecular bone surface area covered by each cell type is estimated by pooling the pictures from each rat and summing the grid points that intersect each cell type and then dividing by the total number of intersection, excluding the "no cell" counts.

Evaluation

The treatment groups are compared by one-way analysis of variance (ANOVA) followed by Fisher's least-significance difference test to compare each treatment group with the ovariectomized vehicle control group. For the histomorphometric determinations, the Kruskal-Wallis test is applied to assess *r* group differences. For relative surface area cell coverage estimation, the treatments are compared overall as to the proportions of osteoblasts, osteoclasts, lining cells, and no cells using one-way ANOVA.

Modifications of the Method

Anderson et al. (1990) proposed the ovariectomized, lactating Sprague-Dawley rat as an experimental model for the rapid development of osteopenia which may be used to test the

effectiveness of bone-retentive drugs, potentially useful for treating osteoporotic women. Rats were ovariectomized on day 2 postpartum and were kept on a low-calcium diet. Measurements of serum total calcium, ionic calcium, albumin, and parathyroid hormone were conducted between days 4 and 21 of lactation.

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Anterior Pituitary Hormones

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Contents

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Much of the early research on anterior pituitary hormones was performed with extracts obtained from animal pituitary glands and with animal models for the specific analytical determination of the concentrations of anterior pituitary hormones in serum, in pituitary tissue, and in some cases by measurement of urinary excretion, for hormones which are excreted during pregnancy, for example. Biosynthetic hormone preparations are now available for human therapy, and species-specific hormone standards are also available for numerous animal species. For an understanding of the development of endocrine research, and the approach to understanding physiology that was necessary, helpful, and effective, reference is made extensively to historical methods that are no longer required. As with other fields, the receptors for hypothalamic hormones and anterior pituitary hormones have been characterized and studied, for both a better understanding of regulation and in several cases structure–activity studies. This approach has also been very helpful in understanding how the sensitivity of the pituitary gland is regulated by both negative feedback of gonadal steroids and the impact of hypothalamic hormones.

Hypophysectomy in Rats

Purpose and Rationale

This is a classical technique for assessment of the biological effects of pituitary hormones (Collip et al. 1933b; Burn et al. 1952; Vogel 1965, 1969b). The pituitary gland is removed entirely (anterior and posterior pituitary), the functional deficiencies are identified, and hormone preparations are injected for substitution. Due to the presence of multiple deficiencies after the operation, results by this technique are limited. The approach is of considerable historical interest but has now been replaced by more specific investigation of single hormonal systems. Various techniques of hypophysectomy have been described by Biedl (1916), Thompson (1932), Collip et al. (1933b), Anselmino and Pecharz (1935), and Bomskov (1939) in several animal species, including dog, cat, rabbit, ferret, guinea pig, rat, mouse, chicken, and frog. For some time, hypophysectomy in the rat has been applied to biological standardization of anterior pituitary hormones.

Nowadays, hypophysectomized rats may be obtained from certified suppliers who are licensed to perform the required operation and who are experienced in postoperative handling and maintenance.

Procedure

In the rat, the parapharyngeal approach is less destructive than the transauricular approach and is preferred for bioassay. Male Wistar or Sprague–Dawley rats weighing 110–150 g are anesthetized with modern anesthetics and placed on a surgical table in a recumbent position. The head is tilted backward to expose the operating field, the fur is removed with electric clippers, and the field of surgery is cleaned with alcohol. A median incision is made 2.5 cm in front of the sternum. The large salivary glands and the maxillary lymph nodes are retracted, the muscles over the trachea are divided at the midline, slightly below the thyroid a hole is made in the trachea ("tracheotomy"), and tracheal mucus is removed by vacuum aspiration. The insertion of a tracheal cannula is not necessary.

A deep blunt dissection is made directly medial to the tendon of the left or right digastric muscle. The ipsilateral glossopharyngeal nerve and the blood vessels are drawn to one side, and the pharynx and trachea are drawn to the other side, for an approach to the midline of the base of the skull. For the subsequent procedure, the use of an operating microscope is recommended (stereo microscope).

The base of the skull is cleaned using small pellets of cotton wool. The spheno-occipital suture running between both mastoid processes is exposed. From behind, the crista occipitalis runs longitudinally up to the middle of this suture. Just at this point, a small hole is drilled at the base of the skull with a dental drill, taking care to leave the surrounding sinus blood vessels intact. The pituitary is now visible as a small, lens-shaped

organ. With the tip of a fine glass tube, attached to a suction device, the pituitary gland is removed by aspiration. Occasional bleeding is stopped with a cotton-wool pellet. The trachea is now cleaned by suction pump and the wound closed. Animals are placed carefully in padded recovery cages, at an ambient temperature of 20–22 °C for recovery. The removal of the posterior pituitary gland causes the animals to have experimentally induced diabetes insipidus, and therefore they need access to sufficient quantities of drinking water.

Modifications of the Method

Instead of a parapharyngeal approach, the **transauricular approach** may be used (S. Jung and H. G. Vogel unpublished data) to facilitate surgery. Male rats weighing 120–150 g are anesthetized and placed on their right side. The head is fixed between the thumb and the index finger of the left hand. In the perpendicular direction, a dental drill with a diameter of 2.2 mm is introduced into the meatus acusticus externus osseus. First, the fine lamellae of the middle ear are passed. Then, the drill is directed from the external ear to the contralateral orbita. In this way, the thin inner wall of the bulla tympani is perforated. The junction of the bulla tympanica, os occipitale, and os sphenoidale is located here. A small, straight forceps is introduced to remove bone fragments, and a blunt cannula diameter 1.6 mm is introduced to remove the pituitary gland by suction. The procedure needs much more technical skill than the current pharyngeal approach and is more destructive.

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Gonadotropins

General Considerations

Selection of assay methods depends on the study's objectives, i. e., whether it is the biological activity of gonadotropins or the quantitative response of elements in the signaling chain being assessed.

The biological assays have been widely replaced by methods for receptor binding and receptor-induced response (signaling).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins which exist in multiple molecular isoforms (microheterogeneity) with different molecular weights and with different degrees of glycosylation (Ulloa-Aguirre et al. 1988; Dahl and Stone 1991). The fine differences identified by analytical methods are not relevant for the bioassay, which measures the sum of biological activities. The immunological methods provide limited information on biological activity and need to be validated for detection of the intact hormone or cross-reactivity with inactive subunits. In vivo animal bioassays are available (e.g., the Steelman–Pohley assay), and in vitro cell bioassays with a high degree of sensitivity and specific radioreceptor assays have been developed (Simoni and Nieschlag 1991). Striking differences in assay results were found when comparing estimates obtained by in vivo bioassays, in vitro cell assays, receptor-binding assays, and immunoassays with the International Standard for Pituitary FSH, when assayed in terms of the Second International Reference Preparation of Human Pituitary FSH and LH (Storring and Gaines Das 1989).

For binding assays, gonadotropin receptors of various animal species have been cloned (Poyner and Hanley 1992). Time-resolved fluoroimmunoassay for gonadotropins has been proposed by Iwasawa et al. (1994). Application of methods for detection and quantification depends on the research objective, which may be targeted to physiological functions or to the biochemical identification of isoforms and subunits (Imse et al. 1992).

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Follicle-Stimulating Hormone (FSH)

Some of the methods described here are used for quantal assay of biological FSH activity (in animals and in vitro); others are used for FSH activity in samples from nonclinical and clinical studies.

Ovarian Weight in hCG-Primed Rats

Purpose and Rationale

Follicle-stimulating hormone (FSH) increases the weight of ovaries in immature rats by inducing follicular maturation in a dose-related manner. This effect is greatly enhanced by simultaneous administration of a constant dose of human chorionic gonadotropin (hCG) for luteinization of the follicles, allowing the detection of low amounts of FSH (Steelman and Pohley 1953).

Procedure

Immature female Sprague–Dawley rats weighing 40–45 g are treated for 3 days by subcutaneous injections of FSH (twice per day), using three doses of the standard (e.g., ovine NIH-FSH-S-9, National Institute of Health, Bethesda, Md., USA) or the test preparation (at the same dose interval). A constant amount of 25 IU hCG (Primogonyl, Schering, Berlin) is added. Hormone preparations are injected in physiological saline containing 2 % gelatin. Six to eight animals are used per group. Then, 18 h after the last injection, the animals are sacrificed, and then the ovaries are dissected out and weighed to the nearest 0.1 mg.

Evaluation

Dose–response curves for standard and test compounds are plotted, and potency ratios with confidence limits are calculated.

Critical Assessment of the Method

The Steelman–Pohley test is rather specific for FSH. The addition of luteinizing hormone (LH), thyroid-stimulating hormone (TSH), adrenocorticotropin (ACTH), human growth hormone (hGH), or prolactin did not influence the dose–response curves of FSH (Christiansen 1972b). The method has been adopted by pharmacopoeias (e.g., British Pharmacopoeia 1988).

Modifications of the Method

Similar studies were reported by Evans et al. 1939; Brown 1955; Segaloff 1962; Parlow and Reichert 1963; Christiansen 1972a; and Storring and Gaines 1989

Igarashi and McCann (1964) reported a bioassay for FSH in mice using an added constant dose of hCG for augmentation and uterine weight as the endpoint.

Brown and Wells (1966) described an assay of human urinary FSH with hCG augmentation, using ovarian weight in mice as the endpoint. The method has been used by Wide and Hobson (1986) to identify the effect of the assay method used to select the most active forms of FSH from human pituitary extracts.

Lamond and Bindon (1966) recommended the use of immature hypophysectomized mice for the assay of FSH with hCG augmentation, using uterine weight as endpoint.

Gans and van Rees (1966) studied a testicular augmentation assay method for FSH. Immature male rats were hypophysectomized, and on the same day the right testis was removed and weighed. Treatment with various doses of FSH together with a constant dose of 20 IU hCG was started on the next day and continued for 6 days. On the 7th day, the animals were sacrificed, and the remaining left testis was removed and weighed. The difference between weights of the right and left testis in each animal was used as the endpoint.

Uberoi and Meyer (1967) used the uterine weight of the immature rat as a pituitary gonadotropin assay, with augmentation by hCG.

Results obtained with the in vivo bioassay (Steelman and Pohley 1953) have been compared with an in vitro bioassay based on estradiol production by cultured Sertoli cells and with an FSH immunoassay by Storring et al. (1981).

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[^{3}H]Thymidine Uptake in Cultured Mouse Ovaries

Purpose and Rationale

Follicle-stimulating hormone stimulates [^{3}H]thymidine uptake by cultured mouse ovaries in a dose-dependent manner, using the tissue-specific proliferation response of the follicles (Ryle 1971; Boggins and Ryle 1972).

Procedure

Intact ovaries are obtained from 15-day-old mice. They are dissected carefully with the aid of a stereomicroscope and transferred to culture dishes. Each ovary is placed on a strip of lens tissue supported on a stainless steel mesh grid 4 mm above the floor of a plastic Petri dish and incubated in Eagle's medium supplemented with glucose and glutamine. The dishes are gassed with 5 % CO_2 in air at 37 °C. Three replicate dishes are used for each concentration of the standard (0.1 and 0.4 IU/ml) and of the test preparation. [^{3}H] Thymidine (0.02 μC) is added to each dish the day after the cultures are set up. Three days later, the tissue is prepared for counting. Each grid is irrigated with about 5 ml saline solution. The ovary is then transferred to a counting vial and dissolved for counting the incorporated radioactivity in a liquid scintillation counter.

Evaluation

Dose–response curves of [^{3}H]thymidine uptake are established for the standard and the test preparation, and potency ratios with confidence limits are calculated. Advantages over animal bioassays include increased specificity and a reduced requirement for a purified test preparation, which may be available only in small quantities.

Critical Assessment of the Method

The [^{3}H]thymidine uptake by cultured mouse ovaries has found less acceptance than other in vitro methods described below.

Modifications of the Method

Follicle cultures from mouse ovaries for the study of follicular metabolism have been described by Boland et al. (1993).

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Granulosa Cell Aromatase Assay in Vitro

Purpose and Rationale

The granulosa cell aromatase assay in vitro is based on the principle of the in vivo bioassay of Steelman and Pohley (1953), in which the ovarian weight response is the result of FSH-induced follicular maturation and induced estradiol production, which stimulates ovarian growth. The coadministration of human chorionic gonadotropin (hCG) at a standard amount enhances androgen production by theca cells, which in turn provides the androgen substrate for FSH-dependent aromatase activity in granulosa cells. Hsueh et al. (1983) and Jia and Hsueh (1985) developed a sensitive in vitro bioassay for FSH based on the stimulation of estrogen production by cultured granulosa cells in serum-free medium containing androstenedione.

Procedure

Intact Sprague–Dawley rats (21–22 days old) are implanted with Silastic capsules containing diethylstilbestrol to stimulate granulosa cell proliferation. Four days after implantation, the animals are sacrificed, and ovaries are dissected for granulosa cell collection. The ovaries are decapsulated, follicles are punctured with 27-gauge hypodermic needles, and granulosa cells are carefully expressed into McCoy's 5a medium. An aliquot is diluted with trypan blue stain, and viable cells are counted with a hemocytometer. Cells are cultured in 16-mm, 24-well culture plates for 2–3 days at 37 °C in a humidified, 95 % air–5 % CO_2 incubator. Each well contains 5×10^4 viable cells in 0.5 ml McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10^{-7} M diethylstilbestrol (DES), $10 - {}^{6}$ M androstenedione (as aromatase substrate), 0.125 mM 1-methy-3-isobutylxanthine (MIX), 1 μg/ml insulin, and 30 ng/ml hCG.

For the measurement of FSH bioactivity in serum samples, each test is performed in triplicate at three dose levels (5, 10, and 20 μl). To ensure a constant volume of 20 μl serum in the total incubation volume of 500 μl, gonadotropin-free serum is added as required. For the FSH standard curve, 4 % gonadotropin-free serum is added to the culture medium. The sensitivity is increased when the serum is pretreated with 12 % polyethyleneglycol (PEG, mol. wt. 8,000). After a culture period of 3 days, estradiol content in the medium is measured by radioimmunoassay (RIA).

Evaluation

RIA data are analyzed using weighted logit-log regression analysis. Calculation of FSH bioactivity in serum samples or test preparations is performed using a standard curve fitted with a second-order polynomial.

Modifications of the Method

Dorrington et al. 1975; Hsueh et al. 1984; Jia and Hsueh 1986; Dahl et al. 1989; Fauser et al. 1989; Storring and Gaines 1989; Matzkin et al. 1990; Simoni and Nieschlag 1991; YoungLai et al. 1992

Sensitivity may be improved by using a specific response of the follicles. Beers and Strickland (1978), Wang and Leung (1983), Combarnous et al. (1984), and Thakur et al. (1990) found that FSH led to a dose-dependent increase of plasminogen activator production in cultures of rat granulosa cells. This assay has been found to be extremely sensitive: as little as 10^{-15} mol of FSH could be detected.

Ax and Ryan (1979) found that FSH dose-dependently stimulates ^{3}H-glucosamine incorporation into proteoglycans by porcine granulosa cells in vitro.

Bhargava et al. (1989) used normal human ovarian cells for long-term cultures. These cells produced progesterone from cholesterol or

pregnenolone and estrone from androstenedione when stimulated by FSH.

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Sertoli Cell Aromatase Assay in Vitro

Purpose and Rationale

The Sertoli cell aromatase assay was first described by Van Damme et al. (1979). This in vitro bioassay was developed following the observation by Dorrington et al. (1975) that FSH, but not LH, stimulates estradiol production by cultured Sertoli cells from immature rats.

The assay has been further improved by Ritzén et al. (1982), Shah and Ritzén (1984), and Padmanabhan et al. (1987). These assays are used to determine bioactive FSH in serum for diagnostic use, and they are also applicable for measuring the activity of FSH preparations intended for therapy.

Procedure

Sertoli cells are obtained from 7- to 10-day-old male Sprague–Dawley rats. Testes are decapsulated, the testicular tissue is chopped twice, at right angles, with a mechanical tissue slicer and incubated in culture medium containing 0.03 % collagenase and 0.003 % trypsin inhibitor for 5–10 min at 34 °C. After the initial dispersal, seminiferous tubules settle to the bottom of the incubation flask, and the medium is decanted to remove interstitial cells. The tubules are washed several times; resuspended in medium that contains collagenase, trypsin inhibitor, and 0.03 % DNase (to prevent clumping of cells), and then incubated for 30 min at 34 °C. During incubation, dispersion of Sertoli cells is hastened by repeated aspiration with a Pasteur pipette. The resulting cell suspension is washed three times; cell number and viability are determined by counting in trypan-blue-dye-containing medium. Cells are suspended in a density of 5×10^5 viable cells/ml. One milliliter of cell suspension is transferred to each well of Falcon multiwell culture dishes (16 mm diameter, Falcon Plastics, Oxnard, Calif., USA).

Sertoli cells are cultured in medium comprised of the following constituents: 1:1 (vol/vol) mixture of Ham's F-10 nutrient mixture and Dulbecco's Modified Eagle's Medium that contains 1.2 g/l sodium bicarbonate, 20 mg/l gentamicin, and 1 mg/l amphotericin. Also included in the medium are 1 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml epidermal growth factor, 20 pg/ml T_4, 10^{-8} M hydrocortisone, and 10^{-6} M retinoic acid. The cell cultures are incubated in this medium in a water-saturated atmosphere that consists of 95 % air and 5 % CO_2 at 37 °C.

After an initial incubation of the cell monolayers for 72 h, the medium is removed; the cells are washed once with the medium and reincubated in the medium described above, which contains 2.5×10^{-6} M 19-hydroxyandrostenedione, FSH standard or unknown samples at various concentrations, and 0.1 mM methylisobutylxanthine (MIX). After a 24-h incubation, the medium is aspirated and centrifuged. The resultant supernatants are stored frozen until estradiol measurement by radioimmunoassay (England et al. 1974).

Evaluation

The results are expressed as picograms of estradiol formed per milliliter of culture medium. To evaluate changes in estradiol secretion with changes in FSH concentration, regression analyses are performed. From these data, activity ratios with confidence limits are calculated.

Modifications of the Method

Similar studies were reported by Dorrington and Armstrong 1975; Marana et al. 1974; Storring et al. 1981; Zaidi et al. 1981; Foulds and Robertson 1983; Wide and Hobsen 1983, 1986; Khna et al. 1984; Harlin et.al. 1988; Storring and Gaines 1989; Simoni and Nieschlag 1991; Rao and Ramachandran (1975) used isolated rat seminiferous tubule cell preparations free of Leydig cells for determination of cyclic AMP production as an in vitro assay for FSH.

Sairam and Manjunath (1982) used rat seminiferous tubular suspensions containing Sertoli cells, incubated them with FSH preparations, and measured cyclic AMP formation as the endpoint.

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Receptor Binding Assay for FSH

Purpose and Rationale

Significant differences between the receptor binding activity of FSH preparations and biological activity have been found by Marana et al. (1979), Zaidi et al. (1981), Foulds and Robertson (1983), and Burgon et al. (1993). This is attributed to measuring binding activity, which may differ

from subsequent intracellular signaling. Several receptor binding assays have been described [Cheng (1975); Andersen et al. (1983) using bovine testes; Reichert (1976) using rat testes tubule tissue].

Procedure

Membrane preparations from bovine testes are used according to Cheng (1975) and Andersen et al. (1983). Fresh bovine testes or testes from rats weighing 220–280 g are decapsulated and rinsed with cold 0.025 M Tris–HCl buffer at pH 7.2, containing 0.3 M sucrose, and then minced and homogenized with a Polytron homogenizer at maximum speed for 30 s at a concentration of 5 ml buffer per gram of tissue. The homogenate is first filtered through four layers, and the filtrate is again filtered through eight layers of cheesecloth. The filtrate is then centrifuged at 12,000 *g* for 30 min at 4 °C. The pellet is discarded and the supernatant further centrifuged at 100,000 *g* for 1 h at 4 °C. The supernatant is discarded and the pellet resuspended in cold 0.025 M Tris–HCl buffer at pH 7.2, containing 10 mM $MgCl_2$, at a concentration of 1 ml buffer per gram of the original weight of the testis. The isolated membranes are stored at −70 °C in aliquots of 10 ml per vial until use.

For assays, 12/75 mm glass disposable tubes are used. To each tube, 0.2 ml of 0.025 M Tris–HCl buffer at pH 7.2, containing 10 mM $MgCl_2$ and 0.1 % BSA, 0.1 ml of standard FSH or unknown samples in the same buffer, 0.1 ml of ^{125}I-hFSH tracer labeled by the lactoperoxidase method (50,000 cpm, approximately 2 ng), and finally 0.1 ml of plasma membrane receptors of appropriate dilution (approximately 1–2 mg/ml) are added to reach a final volume of 500 µl per tube. All the above solutions are kept at 4 °C before use. The tubes are then shaken vigorously and incubated at room temperature for 20 h. Following incubation, the reaction is stopped by adding 3.0 ml of cold 0.025 M Tris–HCl buffer containing 0.1 % BSA. After centrifugation at 4,000 rpm for 30 min, the supernatant is drained and the tip of each tube dried. The pellet remaining at the bottom of the tube is counted in an automatic gamma counter.

Evaluation

Specific binding (%) is defined as, $(C_B - C_N) \times 100/C_{T,}$ where C_B is the cpm bound to the testicular receptor (pellet), C_N is the nonspecific bound cpm (not displaced by 1,000-fold excess of unlabeled hFSH), and C_T is the total cpm added per tube. For standard curves, the specific binding of tracer ^{125}I-hFSH in the absence of cold hormone (6 %–8 % by 150–200 µg of receptor protein) is taken as 100 % bound ^{125}I-hFSH and the nonspecific bound ^{125}I-hFSH (1 %–2 % of the total cpm added) as the baseline. The specific binding of ^{125}I-hFSH over a range of hFSH concentrations is used for calculating a standard curve and sample concentrations.

Modifications of the Method

The method has been used by several authors: Lee and Ryan 1973; Schwartz et al. 1973; Ketelslegers and Catt 1974; Reichert and Bhalla 1974; Calvo et al. 1986; Storring and Gaines 1989

Simoni et al. (1993a) analyzed the biological and immunological properties of the international standard for FSH 83/575 by isoelectrofocusing and made a comparison with other FSH preparations. The results suggested that the international standard 83/575 is not fully representative of pituitary and serum FSH, and its use for calibration of immunometric methods based on monoclonal antibodies is unlikely to resolve problems of inaccuracy in the measurement of serum FSH.

Grasso et al. (1993) studied the effects of a testicular toxicant on FSH binding to membranes of cultured rat Sertoli cells as a method in toxicology.

Wakabayashi et al. (1997) reported the cDNA cloning and transient expression of a chicken gene encoding an FSH receptor.

Simoni et al. (1993b) analyzed the molecular heterogeneity of two batches of commercially available urofollitropin by immunofluorometric assay, radioligand receptor assay, and in vitro bioassay.

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Luteinizing Hormone (LH) = Interstitial Cell Stimulating Hormone (ICSH)

Some of the methods described here are classical animal bioassays for LH/ICSH preparations; others are in vitro bioassays used in research and for clinical samples.

Prostate Weight in Hypophysectomized Rats

Purpose and Rationale

The assay is based on a biological response. Injection of luteinizing hormone (ICSH) in hypophysectomized male rats causes enlargement of

the testes and the secondary sexual organs. The interstitial cells of the testes (Leydig cells) are stimulated by LH/ICSH and secrete androgens which in turn stimulate the accessory organs, such as the ventral prostate of the rat.

Procedure

The method has been used by several authors: Greep et al. 1942; Segaloff et al. 1956; Segaloff 1962; British Pharmacopoeia 1988. Immature male Sprague–Dawley rats aged 21 days are hypophysectomized. From the 2nd to the 5th day, they receive daily subcutaneous injections of two or more doses of the test preparation and the LH/ICSH standard. They are sacrificed on the 6th day. Both testes and the ventral prostate are prepared and weighed. LH/ICSH – but not FSH – induces a specific dose-dependent increase of prostate weight, whereas FSH also induces an increase of testis weight. A predominant LH activity in the test material mainly stimulates prostate weight but not testis weight.

Evaluation

Dose–response curves are plotted for the ventral prostate of each group of test preparation or standard allowing the calculation of activities and potency ratios with confidence limits.

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Superovulation in Immature Rats

Purpose and Rationale

An assay for evaluation of luteinizing hormone (LH) and the luteinizing component of human chorionic gonadotropin (hCG) was described by Zarrow et al. (1958). The sensitivity of the response is enhanced by priming with pregnant mares' serum gonadotropin (PMSG – mainly follicle-stimulating hormone activity) and by intravenous injection of the LH test preparation.

Procedure

Groups of five immature female rats (Charles River strain, age 21 days) are treated with a priming dose of 30 IU of PMS to induce follicular development. Increasing doses of LH reference or test preparations are injected intravenously 56 h later, to induce ovulation. Then 24 h after the final injection, the animals are sacrificed, the fallopian tubes are removed and examined under a dissecting microscope (magnification of 40×) φ for the presence of oocytes (ova). Ovulation is easily noted by an enlarged translucent segment of the tube, through which the ova can be seen. The segment is punctured with a dissecting needle and the entire cumulus clot containing the ova expelled. The clump containing the oocytes is treated with hyaluronidase, and the number of ova is counted.

Evaluation

Dose–response curves are obtained using three doses of standard/reference and test preparation for the calculation of potency ratios with confidence limits.

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Ascorbic Acid Depletion of Ovaries in PMSG/hCG-Primed Rats

Purpose and Rationale

Luteinizing hormone (LH, also known as interstitial cell-stimulating hormone or ICSH) induces

dose-dependent depletion of ascorbic acid in the ovaries of rats primed with pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (Parlow 1961; Parlow and Reichert 1963; Sandow et al. 1972.

Procedure

Female Sprague–Dawley rats weighing 40 ± 5 g are injected with 50 IU PMSG (e.g., Anteron, Schering, Berlin) in 0.2 ml saline subcutaneously on day 1 at 3:00 p.m. On day 3, the rats receive 25 IU hCG (e.g., Primogonyl, Schering, Berlin) subcutaneously at 9:00 a.m. On day 7, three different doses of the standard (e.g., NIH LH-S-1, National Institute of Health, Bethesda, Md., USA) and the test substance are injected subcutaneously. Eight animals are used per group. Three hours later, the animals are sacrificed, and both ovaries are removed, weighed, and homogenized for determination of ascorbic acid content.

Evaluation

Dose–response curves for standard and test compound are plotted and activity ratios with confidence limits calculated.

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Testosterone Production by Leydig Cells in Vitro Induced by LH

Purpose and Rationale

Luteinizing hormone (LH) increases the biosynthesis and secretion of androgens in the Leydig cells of the testes. This can be used for an in vitro assay using isolated Leydig cells.

Procedure

For Leydig cell isolation, male Wistar or Sprague–Dawley rats weighing 250–300 g are sacrificed, the testes removed, and immediately decapsulated. Two testes are then digested with 7 ml of collagenase solution (1 mg collagenase/1 ml Gibco medium 199) at 37 °C for 18 min. Plastic tubes of 40 ml capacity with tight-fitting caps are used and are placed longitudinally in the water bath with constant shaking (75 cycles/min). After incubation, 15 ml of cold saline is added to each tube. The tubes are inverted a few times and then left at 4 °C for 10 min. The supernatants are then carefully siphoned off from the top. The clear supernatant is mixed with an equal volume of 26 % Ficoll/0.4 % BSA/medium 199 at pH 6.5 and then centrifuged at 1,500 *g* for 10 min at 4 °C. The cell pellet obtained from the centrifugation is suspended in a known volume of incubation medium (Medium 199 containing 1 % BSA and 0.01 % lima bean trypsin inhibitor). An aliquot of the cell suspension is taken for counting in a Coulter counter, and the balance is diluted with the incubation medium giving a density of 4×10^6 cells/ml.

For each assay, 0.25 ml of the cell suspension is used. The assay is carried out in 12 × 75 mm plastic tubes incubated with graded doses of test compound at 37 °C. Constant shaking (100 cycles/min) under a 95 % O_2/5 % CO_2 atm for 3 h is required. At the end of the incubation, each tube is centrifuged, and the supernatant is assayed for testosterone by a radioimmunoassay (Dufau et al. 1972). The assay is carried out in triplicate and needs to be repeated at least once with different batches of donor animals.

Evaluation

Dose–response curves are obtained with various doses of standard and test preparation to allow calculation of activities and potency ratios with confidence limits.

Modifications of the Method

Similar studies were reported by: Van Damme et al. 1974; Dufau et al. 1976; Janszen

et al. 1976; Dufau et al. 1977; Khan et al. 1984; Liu et al. 1984; Harlin et al. 1988; Stadler et al. 1989; Haavisto et al. 1990; and Rodgers et al. 1992

The effect of chemically deglycosylated human chorionic gonadotropin on cAMP and testosterone production in rat Leydig cells has been used by Chen et al. (1982) for characterization.

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Receptor Binding Assay forLH

Purpose and Rationale

LH receptors are membrane receptors that mediate the initial hormone-binding step; they are suitable for structure–activity studies and ex vivo analysis of receptor concentration changes. Luteinizing hormone is a glycoprotein composed of an α- and a β-subunit. Carbohydrate moieties are attached to both. Removal of the carbohydrate chains reduces bioactivity but enhances receptor binding.

Procedure

For the preparation of Leydig cell receptors, the partially purified Leydig cells obtained from Ficoll gradient centrifugation as described above for the testosterone formation test are used. The cell pellet obtained from the Ficoll gradient centrifugation is first washed twice with unlabeled radioligand buffer (0.1 M Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$, 0.1 M sucrose, and 0.1 % BSA). The pellet is resuspended in a known volume of the same buffer. An aliquot of this suspension is taken for cell counting in a Coulter counter, and the balance is homogenized in a Polytron homogenizer for 10 s to break up the cells. This prevents internalization of the hormone molecule during the binding assay. The iodination of LH is carried out according to the chloramine-T method (Liu et al. 1977). After the addition of 2.5 ng of ^{125}I-labeled LH tracer, the binding assay is carried out at 37 °C for 2 h with constant shaking. The hormone–receptor complex is then separated by centrifugation, and bound radioactivity is counted in a Packard gamma counter.

Evaluation

Competitive–inhibition curves for specific binding are established and binding capacity calculated.

Modifications of the Method

Similar studies were reported by Liu et al. 1974; Catt et al. 1976; and Liu et al. 1984

Lee and Ryan (1972) described specific binding of human luteinizing hormone (LH) to homogenates of luteinized rat ovaries.

Storring and Gaines-Das (1993) described the second International Standard for Human Pituitary LH. Two new batches (coded as 80/552 and 81/535) were compared with the International Reference Preparation of Human Pituitary LH for Immunoassay (IRP 68/40) by 19 laboratories in 11 countries, using in vivo and in vitro bioassays, a receptor assay, and immunoassays. An activity of 35 International Units of Human Pituitary LH was assigned to the contents of each ampoule coded 80/552.

Selvaraj and Moudgal (1993) used sheep luteal membranes for an LH receptor assay capable of measuring serum LH/chorionic gonadotropin (CG) in a wide variety of species.

Chen and Bahl (1993) reported a high expression of the hormone-binding active extracellular domain (1–294) of rat lutropin receptor in *Escherichia coli*.

Jia et al. (1993) developed an LH/CG bioassay using 293 cells permanently transfected with the human LH receptor cDNA and a luciferase reporter gene driven by a cAMP-dependent promoter.

Selvaraj et al. (1996) established a radioreceptor assay for LH/CG in human sera using immortalized granulosa cells transfected with LH/CG receptor.

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Other Gonadotropins

General Considerations

The pituitary gonadotropins are species specific and exist in various isoforms containing different carbohydrate moieties. In the treatment of infertility, they were substituted by gonadotropin preparations from human urine purified by extraction (human menopausal gonadotropin, hMG, and human chorionic gonadotropin, hCG, from urine in pregnancy), containing variable proportions of follicle-stimulating and luteinizing activity. Pituitary gonadotropins for the treatment of infertility are now obtained by biosynthesis (recombinant DNA methods). Their structure closely resembles human FSH and LH.

Human chorionic gonadotropin (*hCG*) is excreted in the urine of pregnant women, and may also be excreted by tumors, such as hydatidiform mole and chorionepithelioma. hCG is measured as a tumor marker and for the diagnosis of early pregnancy. hCG has mainly LH-like activity. Purified preparations of hCG and hMG are tested by bioassays.

Human menopausal gonadotropin (*hMG*) is excreted in the urine of postmenopausal women. hMG has predominantly FSH-like activity and was used to induce follicular maturation.

A gonadotropin preparation previously used in therapy is *pregnant mares' serum gonadotropin* (*PMSG*). Pregnant horses secrete large amounts of pituitary gonadotropin, which is not excreted in the urine and accumulates in serum. PMSG has predominantly FSH-like activity.

Corpus Luteum Formation in Immature Mice (Aschheim–Zondek Test)

Purpose and Rationale

Aschheim and Zondek (1927, 1935) injected the urine of pregnant women (containing FSH and LH activity) into immature female mice and observed the ovulation and formation of corpora lutea. This procedure was widely used as a biological pregnancy test and later replaced the hCG assays. Further studies were performed by Hamburger and Pedersen-Bjergaard (1937).

Procedure

Groups of 10–20 mice, 21 days of age, are treated with 5 equal subcutaneous injections of urinary extracts over the course of 48 h. The animals are sacrificed 96 h after the first injection. The ovaries are dissected and the formation of corpora lutea observed by examination with a lens or a stereomicroscope. Rupture of follicles is indicated by blood spots.

Evaluation

The number of ovaries showing the formation of corpora lutea was expressed as dose–response curves.

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Biological Assay of hCG in Immature Male Rats

Purpose and Rationale

The LH-like activity of hCG can be determined by measuring the weight increase of the prostate and seminal vesicles in immature male rats.

Procedure

Immature male Sprague–Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e.g., 4, 8, and 16 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 4 days. On the 5th day, the animals are sacrificed and the seminal vesicles and prostate glands prepared and weighed.

Evaluation

Dose–response curves of the weights of seminal vesicles and ventral prostate glands are established for standard and test preparations allowing calculation of potency ratios with confidence limits.

Modifications of the Method

The US Pharmacopeia USP 23 (1995) requests standardization of hCG in immature female rats at an age of 20–23 days. The animals are injected subcutaneously with three different doses of test preparation or standard daily on 3 days consecutively. They are sacrificed on the 5th day and the uteri prepared and weighed.

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Receptor Binding Assay for hCG

Purpose and Rationale

The initial binding step to the luteinizing hormone (LH) receptor is used for quantification; the receptors may be obtained from superovulated rat ovaries or from other tissues. Selvaraj et al. (1996) described an in vitro bioassay and radioreceptor assay for LH/chorionic gonadotropin (CG) in human sera using immortalized granulosa cells transfected with the LH/CG receptor.

Procedure

Ovaries from pseudopregnant rats primed with pregnant mares'serum gonadotropin (PMSG) and hCG are homogenized. The homogenates are centrifuged at 2,000 *g* for 15 min and the pellets washed three times with 40 mM Tris buffer and resuspended in the same buffer. The suspension is filtered through four layers of cheesecloth prior to use. Fresh 2,000-*g* fraction is prepared for each experiment. Purified hCG is labeled by the chloramine-T method resulting in a specific activity of approximately 60–70 μCi/μg.

For assessment of binding to receptors, a mixture consisting of the 2,000-*g* fraction (equivalent to 2.5 or 5 mg of wet ovary or 45–90 μg of protein) and $(0.1–30) \times 10^{-10}$ M labeled gonadotropin or other test substances is incubated in a final volume of 1 ml of 40 mM Tris buffer (pH 7.4) containing 0.1 % BSA at 25 °C for 16 h. Then, 1 ml ice-cold Tris buffer is added to the medium. This is

immediately filtered, with suction, through Millipore EHWP filters (pore size 0.5 μm) previously wetted with 4 % BSA to reduce nonspecific binding. The adsorbed material is washed with another 10 ml of ice-cold Tris buffer. The radioactivity on the filter is measured. Binding in the presence of a large excess of unlabeled hCG (200 IU/ml) is used to assess nonspecific binding.

Evaluation

Specific binding is calculated and dose–response curves obtained.

Modifications of the Method

Similar studies were reported by Catt et al. 1972, 1976; Lee and Ryan 1973; Saxena 1976; and Keutmann et al. 1983.

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Human Menopausal Gonadotropin (hMG)

Biological Assay of hMG in Immature Rats

Purpose and Rationale

The FSH-like activity of hMG can be measured by the ovarian weight response of immature rats. The LH-like activity of hMG can be determined in immature male rats by the increase of prostate and seminal vesicle weights.

Procedure

Female Sprague–Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e.g., a total dose of 1.5, 3, and 6 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 3 days. On the 4th day, the animals are sacrificed and the ovaries removed and weighed.

Evaluation

Dose–response curves for FSH-like activity are established, and potency ratios with confidence limits may be calculated using hMG standard preparations.

References and Further Reading

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Pregnant Mares' Serum Gonadotropin (PMSG)

Biological Assay of PMSG in Immature Female Rats

Purpose and Rationale

The predominant FSH-like activity of equine gonadotropin preparations is determined by the ovarian weight response of immature rats as described for hMG (Hamburger 1950).

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Immunoassays of Gonadotropins

Immunoassays of gonadotropins are important in clinical diagnostics (RIA, ELISA, and related methods). For pharmacological and biological experiments, they are measured in blood, urine, and pituitary tissue, e.g., for evaluation of gonadotropin-releasing hormones (as described below). Many comparisons for results of in vivo and in vitro bioassays versus immunoassays showed significant discrepancies when expressed as the ratios of bioactive to immunoreactive LH (B/I ratio). However, there are numerous validated species-specific methods which are used for experimental endocrinology and clinical diagnosis.

Similar studies were reported by Faiman and Ryan 1967; Wide and Hobson 1983; Ulloa-Aguire et al. 1988; Seth et al. 1989; Terouanne et al. 1989; Armbruster and Haws 1990; Wheeler 1991; Rosenfield an Helke 1992; Weiss et al. 1992; YoungLai et al. 1992; and Haavisto et al. 1993.

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Gonadotropin Inhibition

General Considerations

Gonadotropin inhibition is suspected when in early testing of compounds or in toxicology studies, weight reduction of gonads and the endocrine-dependent organs is found. Early tests used the physiological feedback effects of endogenous steroids in rats, e.g., the parabiosis experiment. This technique in rats was applied to the relationship between central and peripheral

endocrine organs, the pituitary–gonadal axis. Basically, the deficit in gonadal steroid hormones after castration of one parabiotic partner induces increased secretion of gonadotropin which in turn stimulates hypertrophy of the gonads of the noncastrated partner. The procedure is described in detail by Byrnes and Meyer (1951) and Shipley (1962). The parabiosis technique is now obsolete but was applied again a few years ago with remarkable success in the identification of the adipose tissue hormone leptin (see section on Leptin elsewhere).

References and Further Reading

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Inhibition of Gonadotropin Secretion in Intact Animals

Purpose and Rationale

The suppression of gonadotropin secretion by steroid compounds (as an example) can be detected in the endocrine survey test, described earlier in this chapter. Young rats, e.g., 80–100 g initial body weight, are treated with gonadal steroids or their derivatives to induce gonadotropin suppression by negative feedback of steroids (McGinty and Djerassi 1953; Saunders and Drill 1958; Shipley 1962). In intact rats, a reduction of gonadal weight indicates gonadotropin inhibition, whereas in castrated control groups (females or males), the weight increase of the sexual-hormone-dependent organs indicates the intrinsic estrogenic or androgenic property of the test compound.

Procedure

Immature male and female Sprague–Dawley rats weighing 55–65 g or young rats 80–100 g are used. Groups of ten animals per sex and dosage group are treated daily over a period of 21 days with doses of the test compound usually by the oral or subcutaneous route. Controls receive the vehicle only. Testosterone may be used as reference compound (standard) in males and estradiol as the reference compound in females. Twenty-four hours after the last treatment, the animals are sacrificed and body weights recorded: in males, testes, seminal vesicles, ventral prostate, and musculus levator ani and in females, ovaries and uterus are dissected out and weighed. In addition, the gonadotropin content of the pituitary glands at autopsy may be determined to detect inhibition of gonadotropin synthesis.

Evaluation

Organ weight averages are calculated for each treatment group and compared with controls. The reference steroids testosterone and estradiol suppress gonadal weight by pituitary gonadotropin inhibition (negative feedback) in intact animals and increase gonadal weight in castrated animals by direct action. Dose–response curves can be established for the test compounds and the standard, and the relative potency of gonadotropin inhibition is calculated.

Modifications of the Method

As an example of a classical bioassay, gonadotropin inhibition can be tested in semicastrated male rats (H. G. Vogel unpublished data 1964). Male Sprague–Dawley rats weighing 50–60 g are orchiectomized on the left side. The testis is weighed (without epididymis and epididymal fat). Test compounds in various doses or the standard are administered subcutaneously once daily for a period of 10 days. Controls receive the vehicle only. Standard compounds may be medroxyprogesterone acetate 0.4, 2.0, and 10.0 mg per animal per day or 17-ethinyl-19-nortestosterone acetate 0.1, 0.5, and 2.0 mg per animal per day subcutaneously.

On day 11, the animals are sacrificed and the remaining testis, the adrenals, the seminal vesicles, the ventral prostate and the musculus levator ani dissected out and weighed. Gonadotropin suppression results in atrophy of the remaining (contralateral) testis as compared with testis weight of

the untreated control. A decrease in adrenal weight indicates feedback inhibition of the pituitary–adrenal axis. The weight of seminal vesicles, the ventral prostate, and the musculus levator ani indicates androgenic activity of the test compound. Dose–response curves for the test compound and the standard as well as potency ratios are calculated.

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Inhibition of Ovulation and Luteinization

Purpose and Rationale

These methods were used frequently in research on contraception and selection of contraceptive steroids. Described here is an assay for progestational steroids. Progestagens inhibit pituitary gonadotropin secretion and synthesis. Progestional compounds, e.g., cyproterone acetate, inhibit ovulation and corpus lutea formation in young female rats by reducing luteinizing hormone secretion.

Procedure

Female rats (35 days old) are treated once daily over a period of 7 days with increasing doses of gestagens, e.g., 0.125–2.0 mg progesterone or 0.025–0.40 mg 6α-methyl-17α-acetoxy-progesterone, subcutaneously. Controls receive the vehicle only. On the 8th day, the animals are sacrificed and body weight, ovarian weight, and number of corpora lutea recorded. Progestogens inhibit follicular maturation, ovulation, and corpus lutea formation in this assay.

Evaluation

Dose–response curves are established for average number of corpora lutea per animal and ovarian weight.

Similar studies were reported by Junkmann 1957; Shipley 1962; Hebborn 1971; Phillips et al. 1987; and Uilenbroek 1991

Modification of the Method

Ovum count can be performed in the oviduct of immature mice or rats after treatment with low doses of estradiol (Austin and Bruce 1956; May 1971). Immature rats or mice are injected with estradiol or stilbestrol at several dose levels to initiate estrus and ovulation. Then 72 h later, the animals are sacrificed, and the oviducts are removed and examined under a stereomicroscope for the presence of ova. They can be seen through the swollen translucent walls of the oviduct. The swollen part of the oviduct is punctured with dissecting needles to release the ova and count the number of ova per animal. The rate of ovulation (number of ova) is related to the dose of estrogenic compound. In a modification of the method, inhibition of the rate of ovulation induced using a standard dose of estradiol by antiovulatory test compounds may be determined.

Hahn et al. (1977) treated rats in diestrus with test compounds administered orally in 0.5 ml sesame oil and again on the following day when the animals were in proestrus. The animals were sacrificed on the next day, at which time they would normally have ova in the proximal segment of the fallopian tubes. Oviducts were separately flushed with saline onto a glass microscope slide, and the condition and number of ova were noted.

Inhibition of ovulation by gestagens can be studied in rabbits (Shipley 1965). Progesterone has a time-dependent effect on ovulation (stimulating or inhibiting secretion of pituitary gonadotropins). The timing of progesterone injection relative to the anticipated time of ovulation is important. Progesterone injected less than 4 h before ovulation in rabbits facilitates ovulation. In contrast, when injected more than 4 h before the anticipated time of ovulation (Sawyer 1952), progesterone inhibits ovulation. The rabbit

ovulates within a few hours after mating, after mechanical stimulation of the vagina, or after an intravenous injection of copper acetate (0.3 mg/kg). Progesterone injected 24 h before induction of ovulation will prevent ovulation.

Sexually mature female rabbits weighing 3–4 kg are treated with various doses of a standard progestogen or the test compound, and 24 h later an ovulation-inducing stimulus is given. The rabbits are sacrificed and the ovaries are examined 18–24 h later. The total number of ovulation points on both of the ovaries is recorded for each animal. Dose–response curves for standard and test preparations are calculated.

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Ovary–Spleen Transplantation

Purpose and Rationale

This is a historical assay for gonadal steroids. Venous outflow from the spleen is exclusively to the liver. The steroids secreted by an ovary grafted to the spleen do not reach the peripheral circulation; they are degraded to inactive metabolites in the liver and cannot exert their systemic feedback inhibition. The increasing gonadotropin secretion stimulates the growth of the transplanted ovary. This effect can be diminished or prevented by systemic application of gonadal steroids. Several studies were reported by Mardones et al. 1956; Shipley 1962; Biskind and Biskind 1990; and D'Albora et al. 1992. The method by Mardones et al. (1956) for the guinea pig and by Desclin (1959) for the rat was modified by H. G. Vogel and S. Jung (unpublished data, 1962).

Procedure

Female Sprague–Dawley rats weighing 100–120 g are anesthetized. The abdominal wall is opened by an incision lateral to the linea alba. Both ovaries are removed. One ovary is freed from the capsule and connective tissue and weighed to the nearest 0.1 mg. An incision is made at the cranial end of the spleen forming a deep pocket. The decapsulated ovary is pushed into this pocket. The incision is closed, and animals are treated with daily subcutaneous injection of an estrogen (e.g., 0.01, 0.1, 1.0, and 10 μg estradiol in 0.1 ml sesame oil) or a gestagen (e.g., 0.001, 0.01, 0.1, and 1.0 mg 6α-methyl-17α-acetoxyprogesterone) for a period of 5 weeks. Controls receive the vehicle only. In controls, the weight of the ovary may increase about eightfold, from an average weight at implantation of about 10.0 mg to values around 80.0 mg.

Estradiol at doses starting from 0.1 μg and 6α-methyl-17α-acetoxyprogesterone at a dose of 1.0 mg suppress ovarian growth completely by inhibition of LH secretion. The weight of the uterus is increased by direct action of estradiol too.

Evaluation

Dose–response curves of test compounds and reference standards are calculated.

Critical Assessment of the Method

The ovary–spleen transplantation method is similar to the parabiosis experiment, with much less stress to the animals.

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Inhibition of Fertility

Purpose and Rationale

Fertility in rats can be inhibited by treatment of females with steroids that inhibit pituitary gonadotropin secretion or compete at the progesterone receptor. The test was used for contraceptive steroids (Shipley 1962; Philibert et al. 1985; Dhar et al. 1994).

Procedure

A colony of about 100 adult female Sprague–Dawley rats weighing between 200 and 250 g is established. Daily vaginal smears are taken at noon for 5 days. Then,15 regularly cycling females in proestrus are selected and caged separately. The first drug dose is administered at 3:00 p.m. (day 1). At 5:00 p.m., two males are placed with each female. On day 2, vaginal smears are taken for sperm count at 8:00 a.m. The second drug dose is administered at 4:30 p.m. On day 3, vaginal smears are taken for sperm count at 8:00 a.m. again. The third drug dose is administered and sperm counts are taken at 4:30 p.m. The males are then removed. From the 4th to the 7th day, the test drugs are administered in the morning. Controls receive the vehicle only. On day 9, the animals are sacrificed and the uterus examined for implantation sites.

Evaluation

Compounds which prevent conception, as evidenced by the absence of implantation sites at autopsy, were given further consideration. Minimal effective doses preventing ovulation in all animals of a test group were determined.

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Prolactin

There are considerable sequence homologies in the structures of growth hormone, prolactin, and placental lactogen; however, their biological activities are rather different, and each hormone exerts its specific pattern of activities on tissues, with growth hormone being an anabolic hormone and prolactin predominantly a reproductive hormone.

General Considerations

The classical procedure for the bioassay of prolactin is based on the work of Riddle and Bates (1939), namely, the increase in the weight of the crop sacs of doves and pigeons. Other methods are based on the induction of secretory changes in the mammary glands of rodents. Most of these methods can now be replaced by cell-based assays using suitable cell lines responding to prolactin.

The clinical measurements rely on immunoassays (Shiu and Friesen 1976; Jacobs 1979; Jeffcoate et al. 1986). Due to the species specificity of prolactin, a special radioimmunoassay for rats was developed.

Leroy-Martin et al. (1995) reported an immunocytochemical study of human prolactin receptors using anti-idiotypic antibodies in human breast cancer.

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Radioimmunoassay of Rat Prolactin

Purpose and Rationale

Prolactin is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin-releasing hormone activity or prolactin-inhibiting factor activity, a homologous assay is necessary. The reagents for rat prolactin and guidance for the method are provided by National Pituitary Agency, USA (Jeffcoate et al. 1986).

Procedure

Reagents

Standard:	NIAMDD-rat-prolactin-RP-1
Antiserum:	Rabbit-anti-rat-prolactin (NIAMDD-S-6)
Tracer:	^{125}I-rat-prolactin (NIAMDD-I-3)
Second antibody:	Goat-anti-rabbit-gammaglobulin (Cat. No. OTP 14/15)
Buffer:	0.01 M-phosphate-saline/0.1 % bovine serum albumin, pH 7.4

Assay Procedure

Standards:	0.03–1 ng/tube, 200 µl/tube
Antiserum:	1:4000 100 µl/tube
Tracer:	Specific activity 250 µCi/µg, 10,000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum for 24 h at +4 °C; the tracer is added and incubated for another 48 h. Then, the second antibody (1:50), 200 µl/tube, is added and incubated for 48 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline,

pH 7.4, the vials spun at 1300 *g* for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma counter.

Counting equipment required: gamma spectrometer.

Evaluation

Data processing: standard curves and sample data are calculated by any suitable computer program, e.g., using a spline function.

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Jeffcoate SL, Bacon RRA, Beastall GH, Divers MJ, Franks S, Seth J (1986) Assays for prolactin: guidelines for the provision of a clinical biochemistry service. Ann Clin Biochem 23:638–651

Pigeon Crop Method

Purpose and Rationale

This is again a historical bioassay no longer applied. Riddle and Bates (1939) showed that the secretion of "crop milk" by pigeons and doves is initiated and maintained by a factor from the anterior pituitary of these birds that is identical to the lactogenic hormone from mammals. Suitable assay methods have been developed on this basis (Meites and Turner 1950; Segaloff 1962; Cowie and Forsyth 1995). Pigeons are exquisitely sensitive to lactogenic hormone, and the assay can be done with considerably less material than the mammalian assays. The most sensitive assay is the so-called micromethod of local intracutaneous injections of the crop sac.

Procedure

Pigeons of either sex (2–3 months old) but of uniform strain, e.g., White Carneaux, are injected intramuscularly (intrapectorally) with various doses of the test preparation or the standard once daily for 4 days. On the 5th day, the birds are sacrificed. A midventral incision is made through the skin and crop wall from keel to head. The contents of the crop and the adhering crop milk are removed. The two lateral pouches are removed, the fat cleaned from the back of the glands, and the wet weight of the glands determined. Thus, the unstimulated tissue in the dorsal midline and that around the proximal and distal opening of the crop are not weighed.

Evaluation

Mean values of at least two doses of test preparation and standard are plotted versus logarithms of doses and the potency ratios with confidence limits calculated.

Modifications of the Method

The micromethod is based on the observation that only a small area directly over the site of injection is stimulated when lactogenic hormone is injected intradermally over the crop sac forming a "bleb" at the site of injection. A direct comparison of the potency of two different preparations can be made by a similar injection over the other crop sac. The birds are sacrificed on the 5th day and the entire crop sacs removed and examined by transmitted light (Lyons and Page 1935).

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Lactation in Rabbits

Purpose and Rationale

Only the rabbit and guinea pig have proven satisfactory for mammalian assays of lactogenic

hormones. The assays are based on the prolactin-induced mammary growth and milk secretion of pseudopregnant animals.

Procedure

Pseudopregnancy is induced in mature estrus rabbits by intravenous injection of 50 IU of human chorionic gonadotropin. On the 14th day, the rabbits are examined for the presence of well-developed mammary glands characteristic of pregnancy. Various doses of the test preparation or the standard of lactogenic hormone are injected subcutaneously once daily for 6 days. On the 7th day, the animals are sacrificed and the abdominal skin is incised in the midline and separated from the mammary gland underneath. The degree of enlargement of the glands with secretion is rated as follows:

–	Absence of response
+	All ducts are filled with milk
++	All ducts and most lobules are filled with milk though not greatly thickened
+++	Entire gland is filled with milk
+++ +	Mammary glands are greatly extended with milk throughout

Evaluation

The mean values for groups of six rabbits are compared with the values of the standard groups.

Modifications of the Method

A more sensitive rabbit assay method is based on the ability of lactogenic hormone to act directly on mammary tissue (Lyons 1942). Small amounts of test compound are injected directly into one or more of the six milk ducts of a castrated rabbit pretreated with estrone-progesterone. A localized lactation appears in the gland sector stimulated. A dose-dependent reaction was found. The assay is suitable for prolactin, placental lactogen, and growth hormone.

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Growth Hormone (GH)

General Considerations

Growth hormone (GH) has been isolated from the pituitary glands of several species including humans and is species specific; for therapy, it has been replaced by rDNA preparations. Several methods have been described for the detection and assay of GH activity (Russell 1955; Hughes 1985; Isaksson et al. 1985; Rudd 1991).

Human GH consists of 190 amino acids with two disulfide bridges between cysteine residues. Human GH synthesis is controlled by two genes: hGH-N and hGH-V.

GH secretion is stimulated by growth-hormone-releasing hormone (see chapter "▶ Hypothalamic Hormones"). GH is released in a pulsatile fashion and has a half-life of about 10 min.

Receptors for GH are found in several organs, mainly in liver and muscle.

Receptor binding assays (Ilondo et al. 1991) and radioimmunoassays for GH in several animal species (Greenwood et al. 1963; Peake et al. 1978) and for somatomedins (Chochinov and Daughaday 1978) have been developed.

Amit et al. (1992) measured serum GH by radioimmunoassay and GH-binding protein by a binding assay with dextran-coated charcoal separation.

An immunoradiometric assay for GH was described by Hofland et al. (1989).

Mertani et al. (1995) studied the cellular localization of the GH receptor/binding protein in the human anterior pituitary gland.

A high-performance receptor binding chromatography assay for GH was described by Roswell et al. (1996).

Strasburger et al. (1996) developed an immunofunctional assay for human GH (hGH). An anti-hGH monoclonal antibody recognizing binding site 2 of hGH is immobilized and used to capture hGH from the serum sample. Biotin-labeled recombinant GH-binding protein in a second incubation step forms a complex with those hGH molecular isoforms that have both binding sites for the receptor. The signal is detected after a short third incubation step with labeled streptavidin.

Functional characterization of monoclonal antibodies specific to GH receptor is described by Wang et al. (1996).

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Weight Gain in Female Rats ("Growth Plateau Rats")

Purpose and Rationale

Female rats 6 months of age, having reached maturity, gain weight at a very slow pace; the

slowing down of the growth rate is described as "plateauing." Such rats can readily be induced by growth hormone to accelerate growth and weight gain (Greenspan et al. 1950; Papkoff and Li 1962).

Procedure

Groups of ten adult female rats (Long-Evans or Wistar strain), 6 months old and weighing between 220 and 280 g, are used. Only animals which fail to gain more than 10 g in a 20-day period are used. At least two doses of the hormone preparation and the standard dissolved in saline are injected subcutaneously daily over a period of up to 20 days. During this time, weight gains between 10 g and 40 g can be achieved. A straight line relationship exists between the logarithm of the daily dose and the growth response by body weight increase (Marx et al. 1942).

Evaluation

The weight gains after administration of two doses of the test preparation and of the standard are used for 2 + 2-point assays, and potency ratios are calculated.

Critical Assessment of the Method

The test needs a relatively large amount of test material. In spite of the species specificity of growth hormone, rats respond to growth hormone from many other species over a limited time period, before developing neutralizing antibodies.

Modifications of the Test

Immature female rats hypophysectomized at 26–28 days of age can be used (Li et al. 1945; Groesbeck and Parlow 1987). Smaller amounts of test substance are necessary for weight gain than in adult rats.

A cell proliferation assay using a stable clone of the myeloid cell line FDC-P1, transfected with the full-length growth hormone receptor (FDC-P1-hGRH), was described by Roswell et al. (1996) as an alternative to the classical hypophysectomized rat weight gain bioassay.

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Tibia Test in Hypophysectomized Rats

Purpose and Rationale

Hypophysectomy is followed by cessation of epiphyseal growth due to growth hormone deficiency. The width of the epiphyseal cartilage is markedly reduced after hypophysectomy. Administration of growth hormone to hypophysectomized rats induces a remarkable increase in the width of the epiphyseal cartilage plate.

Procedure

Female rats of the Long-Evans or Sprague–Dawley strains are hypophysectomized at the age of 26–28 days. They are used for the bioassay 12–14 days after the operation. The

increase of body weight during this period of time has to be less than 0.5 g per day to indicate complete hypophysectomy. Six to ten animals are used for each group of two doses of test preparation and standard. The solutions are administered intraperitoneally twice daily for 4 days.

On the 5th day, the animals are sacrificed, both tibiae dissected free of soft tissue, and the bones split in half with a sharp razor at the proximal end in the mid-sagittal plane. The halves are washed in water for 10 min, immersed in acetone for 6 min, and washed again in water for 3 min. They are then placed in 2 % silver nitrate solution for 2 min and rinsed with water. During the water rinse, they are exposed to a strong light which turns the calcified portions of the bone dark brown. The stained tibiae are then transferred to a microscopic stage and the width of the uncalcified cartilage plate, which does not stain and remains white, measured under low power with a calibrated micrometer eyepiece. Ten individual readings are made across the epiphysis.

Evaluation

Mean values are obtained from a total of 20 readings for each bone specimen. They are averaged for each dose group. With a 2 + 2-point assay, the potency ratio with confidence limits versus the standard is calculated.

Critical Assessment of the Method

The test has been firmly established as a standard method for assessing growth-promoting activity. For epiphyseal measurement, morphometry may be applied.

Modifications of the Method

Bentham et al. (1993) described a double-staining technique for the detection of growth hormone and insulin-like growth factor-1 binding to rat tibial epiphyseal chondrocytes that were incubated with biotinylated ligands with or without an excess of unlabeled ligands, followed by incubation with Vectastain ABC complex, which was then reacted with diaminobenzidine. Double staining was accomplished by carrying out the first reaction with diaminobenzidine in the presence of nickel ammonium sulfate to give a black precipitate, followed by incubation with the second ligand, then the ABC complex, and finally diaminobenzidine in the absence of nickel ammonium sulfate to give a brown stain.

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^{35}S Uptake

Purpose and Rationale

Cartilage contains glucosaminoglycans, e.g., chondroitin sulfate. The uptake of labeled sulfate into cartilage is greatly reduced after hypophysectomy and restored after growth hormone application. This phenomenon can be used as the basis of a bioassay of growth hormone activity (Collins and Baker 1960; Papkoff and Li 1962).

Procedure

Female Sprague–Dawley rats are hypophysectomized at 21 days of age and used for experimentation 3 weeks later. The animals are given intraperitoneal injections of growth hormone together with radioactively labeled sulfate once

daily for 4 days. Eight to ten animals are used for at least two doses of test preparations (growth hormone derivatives) and standard. The rats are sacrificed 24 h after the last injection and the amount of radiosulfate present in the seventh rib cartilage determined. A linear relationship exists between the uptake of radiosulfate and the hormone given over a range of 3–20 μg per day for 4 days.

Evaluation

Mean values for each group and the potency of the test preparation versus standard calculated with confidence limits are calculated in a 2 + 2-point assay.

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Inhibition of Glucose Uptake in Adipocytes in Vitro

Purpose and Rationale

The conversion of glucose to lipid in murine adipocytes is dose-dependently inhibited by human growth hormone (hGH). A sensitive in vitro bioassay was developed by Foster et al. (1993).

Procedure

3 T3-F442A embryonic murine fibroblasts (preadipocytes) are grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.1 μg/ml fungizone. The cells are plated in 60- or 100-mm plastic culture dishes at a density of 200 cells/cm^2 and grown to confluence at 37 °C under a humidified atmosphere of 90 % air/10 % CO_2 in medium supplemented with 25 mM glucose and 10 % calf serum. Medium is replaced every 2–3 days. Once confluent, the fibroblasts are converted to cells with the characteristics of adipocytes by incubation for 48 h in medium supplemented with 25 mM glucose, 10 % fetal bovine serum, 0.5 mM methylisobutylxanthine, 2 μg/ml insulin, and 250 nM dexamethasone. This medium is then replaced with medium containing 10 % fetal bovine serum and 2 μg/ml insulin with changes made every 2 days for 5–8 days until at least 70 % of the cells have the characteristics of adipocytes as assessed by phase contrast microscopy.

For bioassays, the medium consists of DMEM containing 5.5 mM glucose, 2 % BSA, 25 nM dexamethasone, 37 nM estradiol, 10 μg/l insulin, and 0.1 μCi/ml uniformly labeled [^{14}C] D-glucose. Cultures are coincubated with increasing concentrations of 22-kDa human growth hormone (0.313–40 μg/l) as standard or test substance or medium (controls) for 24 h. For determination of hGH in patients, 100 μl or 200 μl serum is added. After incubation, the medium is removed and discarded. The cells are treated with Doles reagent (one part heptane, four parts isopropanol, 0.1 part 1 N H_2SO_4), the plates scraped, and the contents transferred to a glass tube. Lipids are extracted by the method of Dole and Meinertz (1969), and radioactivity of the lipid is determined by scintillation counting in a liquid scintillation spectrophotometer. Results are expressed as ^{14}C counts per minute per dish. Lipid accumulation in controls without hGH is taken as 100 %. Logarithmic doses of 0.313–40 μg/l hGH result in a linear decrease of lipid accumulation. The assay is rather specific for 22-kDa hGH.

Evaluation

From dose–response curves, activity ratios can be calculated.

Modifications of the Method

Xu et al. (1995) studied the effects of GH antagonists on 3 T3-F422A preadipocyte differentiation. The antagonists not only failed to induce adipose differentiation, including late marker gene expression (adipocyte protein 2), immediate early gene expression (c-fos), and tyrosine phosphorylation of intracellular proteins, but also antagonized GH-induced c-fos expression and phosphorylation of proteins of apparent molecular mass 95 kDa.

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Eluted Stain Bioassay for Human Growth Hormone

Purpose and Rationale

Ealey et al. (1988, 1995) and Dattani et al. (1993, 1995) developed an eluted stain bioassay (ESTA) for human growth hormone (hGH). This assay is based on the production of MTT–formazan [MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] by quiescent Nb2 cells under the influence of the hormone.

Procedure

Rat Nb2 lymphoma cells are grown in suspension culture which consists of RPMI medium (Gibco) containing 50 U penicillin/ml, 50 µg streptomycin/ml, 2×10^{-3} M L-glutamine, 10 % fetal calf serum, and 10 % horse serum. The cells are incubated in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C.

Prior to the bioassay of hGH, the cells are transferred to a quiescent medium, which is identical in composition to the growth medium described above except that the FCS is reduced from 10 % to 1 %. This slows down the rate of cell division and reduces the optical density of the unstimulated control in the subsequent ESTA bioassays by about 50 %. Incubation is continued in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C for 24 h.

For the bioassay, the cells are transferred to the bioassay medium, which is the same as the growth medium but without FCS. The cells are plated out into 96-well microtiter plates, such that a final density of 2×10^5 cells/ml is obtained. Usually, 50 µl of cell suspension at 4×10^4 cells/ml is added to each well. This is followed by the addition of 50 µl of various concentrations of test compound or standard (0.1–10 mU GH/l). The cells are incubated in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C for 96 h.

At the end of the bioassay incubation, the colorimetric endpoint is determined by the addition of 10 µl of MTT bromide solution (5 mg/ml in phosphate-buffered saline containing 0.1 mg/ml $CaCl_2$ and $MgCl_2 \cdot 6H_2O$, pH 7.3) to each well: incubation is continued for 40 min at 37 °C in a dry incubator. During this time, activated cells reduce the yellow MTT salt to its purple formazan. After the 40-min incubation, 50 µl of 10 % Triton X-100 in 0.1 M HCl is added to each well and the plate gently shaken for 30 min at room temperature. Bioassay responses of the 96 wells are quantified with a Biorad microtiter plate reader at optical densities at a test wavelength of 595 nm and a reference wavelength of 655 nm to correct for differential scattering.

Evaluation

The determinations for all experiments are made from triplicate or quadruplicate microcultures and the results expressed as means and standard deviations. Dose–response curves for standard and test compounds are established.

Modifications of the Method

The sensitivity of the assay can be increased by addition of ionic zinc (Dattani et al. 1993, 1995; Strasburger and Dattani 1997).

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Reverse Hemolytic Plaque Assay for Growth Hormone

Purpose and Rationale

The reverse hemolytic plaque assay as described by Neill and Frawley (1983), Luque et al. (1986), and Smith et al. (1986) can be used to determine growth hormone (GH) secretion by dispersed rat pituitary cells (Niimi et al. 1994a, b).

Procedure

Anterior pituitary lobes of male Sprague–Dawley rats weighing 200–250 g are collected and minced into approximately 1-mm^3 fragments. The pituitary cells are dispersed and transferred to a siliconized flask containing 3 mg/ml trypsin in Dulbecco's phosphate-buffered saline containing 0.1 % BSA and antibiotics and incubated in a water bath for 5 min at 37 °C. After gentle trituration by a siliconized pipette, the cells are separated by centrifugation and rinsed with phosphate-buffered saline containing 2 mg/ml DNase (type I; Sigma, St. Louis, Mo., USA). The dispersed cells are separated by centrifugation and rinsed once in phosphate-buffered saline with 1.5 mg/ml trypsin inhibitor (type II-L, Sigma). The cells are washed five times with Dulbecco's modified Eagle's medium (DMEM) containing 0.1 % BSA and resuspended. Ovine erythrocytes are coupled with staphylococcal protein A (Sigma) in the presence of 0.9 % chromium chloride in normal saline. Pituitary cells (10^6 cells/ml) are combined with an equal volume of a 30 % solution of protein-A-coupled erythrocytes in DMEM containing 0.1 % BSA and antibiotics. The cell mixture is infused into a poly-L-lysine-coated Cunningham slide chamber and preincubated at 37 °C, 95 % air/5 % CO_2, for 50 min. After preincubation, the chamber is rinsed in DMEM-BSA and placed in Petri dishes containing DMEM and 10 % horse serum containing 1 % nonessential amino acids before performing the reverse hemolytic plaque assay.

After 24 h of coincubation, the chambers are rinsed with DMEM-BSA. Monkey antirat GH serum diluted 1:150 in assay medium is then infused into the chambers alone or with different secretagogues and incubated for 2 h. Plaque development is initiated by infusion of guinea pig complement (Gibco) at a final dilution of 1:40. The reaction is terminated after 0.5 h by the infusion of B-5 fixative (6 g $HgCl_2$ and 1.25 g sodium acetate in 90 ml distilled water, add 10 ml 37 % formaldehyde immediately before use). The pituitary cells are stained with 0.5 % toluidine blue to facilitate observation of the hemolytic plaques.

Evaluation

In each experiment, each concentration of secretagogue or vehicle is run in duplicate, and 150–200 cells/slide are counted. Two separate experiments have to be performed. The plaque area is measured by using a calibrated ocular reticule. The area of 50 plaques per slide is measured. Statistical analysis is performed by Student's *t*-test and one-way analysis of variance.

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Determination of Growth Hormone Isoforms by 22-kDa GH Exclusion Assay

Purpose and Rationale

Human growth hormone exists in variety of isoforms. In the pituitary, the most abundant isoform is 22-kDa GH, while other isoforms (non-22-kDa GH) are present in variable amounts. Boguszewski et al. (1996) and Strasburger and Dattani (1997) described an analytical approach that focuses on the isoforms differing from monomeric and oligomeric 22-kDa GH.

Procedure

Reagents

- Recombinant 22-kDa human GH (Genotropin, Pharmacia, Uppsala, Sweden)
- 22-kDa GH-specific monoclonal antibody (MCB), (Genentech, San Francisco, Calif., USA)
- Magnetic polystyrene beads coated with rat antimouse IgG 1 (Dynabeads M-450), (Dynal, Oslo, Norway)
- Polyclonal antibody-based IRMA (Pharmacia, Uppsala, Schweden)
- Assay buffer containing phosphate-buffered saline, 5 g/l BSA, 5 ml/l Tween-20, 0.1 g/l thiomerosal

Assay Procedure

A 100-µl aliquot of serum or test solution is mixed with either 10 µl of assay buffer containing MCB (final concentration 0.3 µmol/l) or 10 µl of assay buffer without MCB. The samples are incubated for 24 h at room temperature. A 160-µl aliquot of magnetic beads coated with rat antimouse IgG (concentration 4×10^5 beads/µl) is added to the samples. After further incubation for 2 h at room temperature with gentle agitation in a rotator, the tubes are put in contact with a magnetic device, Dynal MPC-E (Dynal, Oslo, Norway), for 1 min. The magnetic beads with the 22-kDa GH-MCB complexes are attracted by the magnet. While the tubes are in the magnetic device, 50-µl aliquots of the supernatant are transferred to new tubes for measurement of non-22-kDa GH levels in duplicate by the polyclonal antibody-based IRMA. The same procedure is performed for samples incubated with assay buffer (without addition of MCB) to determine total GH concentration.

Evaluation

The amount of non-22-kDa GH isoforms is expressed as a percentage of total GH concentration. The Mann–Whitney *U* test (two-tailed) is used to compare the percentage of non-22-kDa GH isoforms between the groups. The method is an example of the increasing use of physicochemical methods to characterize the heterogeneity of hormone preparations obtained by rDNA methods.

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Steroid Regulation of Growth Hormone Receptor and GH-Binding Protein

Purpose and Rationale

Gabrielsson et al. (1995) studied steroid regulation of growth hormone receptor (GHR) and GH-binding protein (GHPB) mRNAs in the rat. GHR and GHBP, arising from alternative splicing of the same gene, show a sexually dimorphic and GH-dependent expression pattern. Multiple alternative 5′-untranslated regions are present in GHR and GHBP transcripts in the rat, one of which, GHR_1, has been shown to be liver specific and found at higher levels in females.

Procedure

Animal Treatment

For human GH treatment, groups of Wistar rats are implanted subcutaneously with osmotic minipumps delivering recombinant hGH at 200 μg/day for 7, 12, or 14 days. Steroid-treated animals are implanted subcutaneously with slow-release pellets delivering estradiol (E_2, 25 ng/day), testosterone propionate (12.5 μg/day), or corticosterone (3.5 μg/day) for up to 2 weeks. At the end of each study, animals are sacrificed and samples of blood and liver rapidly removed.

Protein Binding (GHBP) Assay

Plasma GHBP levels are measured by radioimmunoassay (RIA). Recombinant rat GHBP is used for iodination and reference preparation (Carmignac et al. 1992).

GH-Receptor-Based Assays

Liver samples are homogenized in 0.3 M sucrose containing 3 mM imidazole HCl, pH 7.4 (Carmignac et al. 1993). Samples of 100 μl (~2 mg protein) are incubated in duplicate at 22 °C with radioiodinated bovine GH (100 μl,20,000 cpm) and 100 μl buffer (25 mM Tris–HCl, pH 7.4, containing 10 mM $CaCl_2$ and 0.1 % BSA). After 2 h, 3 ml cold (4 °C) buffer is added, the tubes centrifuged at 2,700 *g* for 30 min, and the radioactivity in the pellets determined. Nonspecific binding is estimated in the presence of 1 μg unlabeled bovine GH and is subtracted from total binding to derive percentage specific bovine GH binding per milligram protein.

Generation of RNA Probes

Using an in vitro transcription kit, probes are labeled with [^{35}S]uridine triphosphate for solution hybridization assay and [^{32}P]uridine triphosphate for Northern blots and RNase protection assays. Total GHR coding region transcripts are measured using an antisense probe corresponding to a 423-nucleotide (nt) *Nco*I-*Kpn*I fragment (nt 989–1411) of the λ1 clone of the rGHR inserted into the pT7T318U vector. This sequence spans the transmembrane domain and part of the intracellular domain of the GHR and is not present in GHBP mRNA. Total GHBP transcripts are determined using a 46-nt oligonucleotide probe complementary to the alternate splice sequence encoding the GHBP hydrophilic tail. This probe does not detect GHR transcripts. GHR_1-containing transcripts are measured using a probe complementary to this 5′-UTR alternative exon sequence.

Northern Blots

Total RNA is prepared using the method of Chomczynski and Saachi (1987). RNA is run overnight on a 2.2 M formaldehyde/3-*N*-morpholine propane-sulfonic acid agarose gel, transferred to a Nylon membrane, and cross-linked using a Stratalinker (Strategene, La Jolla, Calif., USA). Membranes are prehybridized for 2–4 h at 60–65 °C in 50 % formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 5 × Denhardt's solution, 50 mM Na_2HPO_4, 0.2 % sodium dodecyl sulfate (SDS), 250 μg/ml salmon sperm DNA, and 100 μg transfer RNA/ml and incubated with ^{32}P-labeled probe [(1–3) × 10^6 cpm/ml] at the same temperature in 50 %

formaldehyde, 5 × SSC, 1 × Denhardt's solution, 20 mM Na_2HPO_4, 0.2 % SDS, 10 % dextran, 100 µg/ml salmon sperm DNA, and 100 µg tRNA/ml. After washing (60 °C and 20 min/wash, starting at 3 × SSC/0.1 % SDS and reducing to 0.3 × SSC/0.1 % SDS), membranes are exposed to X-ray film with intensifying screens at −80 °C or are detected by phosphorimaging.

Solution Hybridization Assays

Hepatic mRNAs are quantified by solution hybridization after the method of Möller et al. (1991). Liver is homogenized in 10 mM Tris–HCl, pH 7.5, containing 1 % SDS and 5 mM EDTA, after proteinase K digestion. Total nucleic acids (TNAs) are extracted with phenol/chloroform, precipitated with ethanol, and the pellets dissolved in 0.2 × 10 mM Tris–HCl, pH 7.5, containing 1 % SDS and 5 mM EDTA. For each assay, 10–100 µg TNAs is hybridized in duplicate with ^{35}S-labeled RNA probes (20,000–30,000 cpm) at 50 °C overnight in 40 µl, 21 mM Tris–HCl buffer, pH 7.5, containing 600 mM NaCl, 4.5 mM EDTA, 7.5 mM dithiothreitol, 0.1 % SDS, and 25 % formamide. Standard tubes contain known quantities of target RNA, transcribed from the sense strand of the appropriate plasmid and quantified by absorption at 260 nm.

After digestion with RNase A (40 µg) and RNase T_1 (2 µg), protected fragments are precipitated with 10 % trichloroacetic acid, are collected by filtration (GF/C paper, Whatman), detected by scintillation counting, and expressed as specific mRNA levels (attomoles per µg TNA).

RNase Protection Assay

Total RNA (20 µg) is hybridized under the same conditions as above, except that a ^{32}P-labeled probe (500,000 cpm) is used. After overnight incubation at 50 °C, the samples are treated with 300 µl of a solution of 10 mM Tris, pH 7.5, 5 MM EDTA, and 300 mM NaCl, containing RNase A (40 µg/ml) and RNase T_1 (2 µg/ml), for 30 min at room temperature. After incubation with 50 µg proteinase K and 20 µl 10 % SDS for 15 min at 37 °C, samples are extracted with phenol/chloroform and ethanol precipitated with 10 µg tRNA as carrier. The pellets are dissolved in gel-loading buffer (10 mM EDTA and 1 mg/ml bromophenol blue in 80 % formamide), denatured at 85 °C for 4 min, and run on an 8 M urea/6 % polyacrylamide gel. Detection is by autoradiography or phosphorimaging.

Evaluation

Data are calculated as mean ± SEM. Differences between treatment groups are assessed using Student's *t*-test or analysis of variance followed by Student-Newman-Keul's or Dunnett's tests.

Modifications of the Method

Nilsson et al. (1995) described the expression of functional growth hormone receptors in cultured human osteoblast-like cells.

To measure the absolute number of mRNA molecules encoding the growth hormone receptor in human tissue, Martini et al. (1995) developed a quantitative polymerase chain reaction assay.

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Adrenocorticotropin (ACTH)

Adrenal Ascorbic Acid Depletion

Purpose and Rationale

This is now a historical assay, which, however, has been used extensively for standardization of ACTH preparations. The administration of pituitary adrenocorticotropic hormone (ACTH) is followed by a decrease in the amount of ascorbic acid present in the adrenals. The depletion of adrenal ascorbic acid is a function of the dose of ACTH administered. This relationship was used for a quantitative assay of ACTH by Sayers et al. (1948). The method has been selected for standardization of ACTH by several Pharmacopoeias, e.g., the United States Pharmacopeia USP 23 (1995), Deutsches (1986), and British Pharmacopoeia (1988). Furthermore, the test has been used for evaluation of synthetic corticotropin analogues (Geiger et al. 1964; Vogel 1965, 1969a, b). A similar test is used for luteinizing hormone action in the rat ovary.

Procedure

Male Wistar rats weighing between 100 g and 200 g are hypophysectomized 1 day prior to the test. The range of weights in any one test should not exceed 15 g. For one test with three doses of test preparation and standard, at least 36, preferably 60, hypophysectomized rats are necessary.

Solutions

Five units of the international standard for corticotropin (Bangham et al. 1962) or an amount of test preparation supposed to contain about five units are dissolved in 0.25 ml of 0.5 % phenol solution and diluted with 8.1 ml of 15 % gelatin solution. In this way, 0.5 ml contains 300 mU ACTH. Then, 3 ml of this solution is diluted with 6.0 ml gelatin solution (to prevent adsorption to glassware), resulting in 100 mU ACTH per 0.5 ml. Then, 3 ml of this solution is again diluted with 6.0 ml gelatin solution, resulting in a content of 33 mU ACTH per 0.5 ml.

The hypophysectomized rats are randomly distributed to six groups. Each rat receives subcutaneously 0.5 ml of one of the various concentrations of test preparation or standard. Three hours after injection, the animals are anesthetized, both adrenals removed, freed from extraneous tissue, and weighed. The rats are sacrificed and the skull opened to verify completeness of hypophysectomy.

The adrenals are homogenized in 4 % trichloroacetic acid and the ascorbic acid determined according to the method of Roe and Kuether (1943). Other methods have been described.

Ascorbic Acid Determination

Reagents

First, 100 mg L-ascorbic acid is dissolved in 100 ml 4 % trichloroacetic acid and 20 ml of this solution diluted with 4 % trichloroacetic acid to achieve a 0.2 % ascorbic acid solution; 2 ml of this solution is diluted with 4 % trichloroacetic acid to achieve a 0.02 % ascorbic acid solution. Sulfuric acid (85 %) is obtained by adding 900 ml concentrated sulfuric acid to 100 ml distilled water. Then, 2 g dinitrophenylhydrazine is dissolved in 100 ml of 9 N H_2SO_4 (75 ml distilled water and 25 ml concentrated sulfuric acid), and 6 g thiourea is dissolved in 100 ml distilled water.

Calibration

Trichloroacetic acid (4 %) is added to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 ml of the 0.02 % ascorbic acid solution and 1.0, 1.5, and 2.0 ml of the 0.2 % ascorbic acid solution to reach a final volume of 8.0 ml. Then, 100 mg charcoal is added to each sample and thoroughly mixed by shaking for 1 min. After 5 min, the solutions are

filtered. An aliquot of 0.1 ml of the 6 % thiourea solution is added to 2.0 ml of the filtrate followed by 0.5 ml dinitrophenylhydrazine solution. The mixture is shaken and heated for 45 min at 57 °C in a water bath. The solutions are placed in an ice-cold water bath and with further cooling 2.5 ml of the 85 % sulfuric acid added. The calibration curve is established at a wavelength of 540 μm using the solutions without ascorbic acid as blank.

Preparation of the Adrenals

Both adrenals are homogenized in glass tubes containing 200 mg purified sand and 8.0 ml of 4 % trichloroacetic acid. The reagents are added as described for the calibration curve.

Evaluation

The potency ratio including confidence limits is calculated with the 3 + 3-point assay.

Modifications of the Method

The original method, as described by Sayers et al. (1948), used intravenous administration of ACTH and the difference of ascorbic acid in the left adrenal before injection and the right adrenal 1 h after injection as endpoint. Different values of the activity of synthetic peptides versus the international standard resulted from different ways of administration (Vogel 1965).

The ascorbic acid depletion test can also be performed in dexamethasone-blocked rats. However, different potency ratios of synthetic corticotropin analogues have been found than in hypophysectomized rats (Vogel 1969a). The difference most likely depends on the dexamethasone blocking dose.

Other authors, including British Pharmacopoeia (1988) and Deutsches Arzneibuch (1986), use the 2,6-dichlorophenol-indophenol method for determination of ascorbic acid.

The glands are homogenized in 2.5 % metaphosphoric acid with the addition of a small quantity of washed sand. With additional 2.5 % metaphosphoric acid, a final volume of 10 ml is reached. Then, 5 ml of the filtrate is added to 5 ml indophenol acetate solution, and the absorbance of the mixture is read immediately in a photometer with a 520 μm filter. The indophenol acetate solution is prepared by dissolving 15 mg of 2,6-dichlorophenol-indophenol in 500 ml distilled water and dissolving 22.65 g sodium acetate · $3H_2O$ in 500 ml distilled water and mixing equal volumes.

A cytochemical bioassay of corticotropin was described by Chayen et al. (1976).

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Corticosterone Blood Levels in Dexamethasone-Blocked Rats

Purpose and Rationale

Corticotropin activity can be measured by the increase of corticosterone in venous blood of hypophysectomized rats (no longer required) or dexamethasone-blocked rats. The test can be used to measure time–response curves of corticotropin analogues or depot preparations (Vogel 1969a, b). The sensitivity can be increased by determining corticosterone in adrenal venous blood after cannulation of the adrenal vein (Retiene et al. 1962).

Procedure

Male Sprague–Dawley rats weighing 150–200 are injected subcutaneously 24 h and 1 h prior to subcutaneous injection of the ACTH preparation or the standard with 5 mg/kg dexamethasone in oily solution. Eight rats are used for each dose of test preparation or standard. At increasing time intervals after ACTH injection, the rats are anesthetized with 60 mg/kg pentobarbital i.p. and blood withdrawn by venipuncture (alternatively by retro-orbital puncture). Next, 1 ml plasma is diluted with 2 ml distilled water and extracted (washed) with 5 ml petrol ether to remove the lipids. The petrol ether is discarded and 2 ml of the water layer extracted twice with 5 ml methylene chloride by vigorous shaking for 15 min. The methylene chloride phase is separated by centrifugation. Both methylene chloride extracts are unified and shaken with 1 ml ice-cold 0.1 N NaOH. The water phase is immediately removed and the methylene chloride extracts dried by addition of dry sodium sulfate. A 5-ml aliquot of the methylene chloride extract is mixed with 5 ml of the fluorescence reagent (seven parts concentrated sulfuric acid, three parts 96 % ethanol, v/v). After vigorous shaking, the methylene chloride phase is removed and fluorescence measured with primary filters of 436 μm and secondary filters of 530–545 μm. For calibration, concentrations of 0, 20, 50, 100, and 250 μg/ml corticosterone are treated identically and measured in each assay.

Evaluation

Using three doses of test compound and standard, potency ratios with confidence limits can be determined for each time interval with the 3 + 3-point assay giving evidence for the duration of action (Vogel 1969a, b).

Modifications of the Method

Pekkarinen (1965) used fluorometric corticosteroid determinations in guinea pigs resulting in highly deviating activity ratios of synthetic and commercial corticotrophins as compared with the international working standard of ACTH.

The contemporary method for the determination of the corticosterone concentration in serum is radioimmunoassay, requiring much smaller blood samples and reducing the stress to the animals very markedly.

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In Vitro Corticosteroid Release

Purpose and Rationale

An in vitro assay of corticotropin was described by Saffran and Schally (1955). This test has been modified by Van der Vies (1957) and subsequently used by several authors (Staehelin et al. 1965; Vogel 1969a, b).

Procedure

Solutions

"Double Ringer" solution is prepared as follows:

4.50 %	(w/v) Sodium chloride	200.0 ml
5.75 %	Potassium chloride	8.0 ml
6.10 %	Calcium chloride	6.0 ml
10.55 %	Monopotassium phosphate	2.0 ml
19.10 %	Magnesium sulfate	2.0 ml
	Double distilled water up to	545.0 ml

Final solution:

54.0 ml	Double ringer
26.0 ml	1 % Glucose solution (freshly prepared)
29.0 ml	Double distilled water
21.0 ml	1.3 % $NaHCO_3$ solution which has been gassed with carbon dioxide at room temperature for 1 h

The final solution is gassed with a mixture of 95 % O_2 and 5 % CO_2 for 10 min.

Preparation of Adrenals

Male Sprague–Dawley rats ($n = 20$) weighing 150–200 g are anesthetized with 50 mg/kg pentobarbital sodium i.p. applying as little handling stress as possible. The adrenals are removed and freed of connective tissue, taking care that the adrenals are not damaged. Each adrenal is carefully cut into four quarters with fine scissors. The eight quarters from each rat are randomly distributed to 20 preweighed incubation vessels filled with 1.5 ml of final solution. The flasks are mounted on Warburg manometers or placed into a suitable shaking water bath and are gassed under continuous shaking for 1 h with a mixture of 95 % O_2 and 5 % CO_2 at 38 °C (= preincubation period).

The flasks are removed from the bath at the end of the preincubation period. The medium is aspirated as much as possible by means of a small tube attached via a collection bottle to the vacuum line. A 1.4-ml aliquot of fresh medium is added to each flask.

To five vessels, each of the following solutions are added:

- 10.0 mU/0.1 ml ACTH standard
- 50.0 mU/0.1 ml ACTH standard

- 10.0 mU/0.1 ml test preparation
- 50.0 mU/0.1 ml test preparation
- 50.0 mU/0.1 ml medium (control)

The vessels are again incubated and gassed with a mixture of 95 % O_2 and 5 % CO_2 at 38 °C under continuous shaking for 2 h. Then, 1-ml aliquots of the medium in each vessel are transferred to carefully cleaned glass-stoppered tubes containing 2 ml methylene chloride. The tubes are vigorously shaken for 1 min and centrifuged for 5 min. The methylene chloride phase is transferred with a long needle and a syringe to a quartz microcuvette and readings taken at 225, 240, and 255 μm.

Dry weight of the adrenals is determined by heating the incubation vessels to 150 °C for 2 h.

Evaluation

Extinction values are calculated for the maximum of absorption (Allen 1950) according to the formula

$$E = E_{240} - \frac{E_{225} + E_{255}}{2}$$

The potency ratios are calculated with the 2 + 2-point assay.

Modifications of the Method

Saffran et al. (1971) described a flow-through system for the study of adrenocortical function by rat tissue in vitro, in which the fluorometric measure of corticosterone is completely automated.

Corticosterone is now conveniently determined by RIA or by a high-performance liquid chromatography (HPLC) method.

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Thymus Involution

Purpose and Rationale

This is a classical indirect bioassay based on the steroid response of the target organ and its effect. Administration of corticotropin decreases thymus weight (due to inducing corticosterone secretion), followed by an increase of adrenal weight (Hayashida and Li 1952; Thing 1953; Thompson and Fisher 1953; Fischer 1962). Young rats respond with an involution of the thymus gland to graded doses of corticotropin (Rerup 1958). A similar assay is used for adrenal steroids.

Procedure

Sprague–Dawley or Wistar rats of either sex, 7–10 days of age, weighing 10–15 g are used. Littermates are preferred. The animals are distributed at random to three groups of the standard and three to five groups of the unknown sample. The ACTH standard or test compounds are injected at different doses once daily subcutaneously for 3 days. Twenty-four hours after the last injection, the animals are sacrificed, the thymus dissected out, and weighed to the nearest 0.1 mg. The response is expressed as the average of the individual values for each dose level.

Evaluation

Dose–response curves are established and potency ratios calculated using a 3 + 3-point assay.

Modifications of the Method

The numerical sensitivity of the assay can be increased using the quotient between the increase of the weight of the adrenals and the decrease of the weight of the thymus gland (Hohlweg et al. 1960) (ratio adrenal weight increase/thymus weight decrease).

Male Wistar rats at an age of about 3 weeks weighing 18–22 g are injected three times daily at 4-h intervals over 3 days with the test preparation or the standard dissolved in 10 % gelatin solution. The animals are sacrificed 18 h after the last injection and the adrenals and the thymus gland removed and weighed. The ratio of adrenal weight to thymus weight provides a steep dose–response curve.

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Receptor Binding Assay for ACTH

Purpose and Rationale

As for other hormones, receptors have been used as the test preparations for comparison of the binding affinities of adrenocorticotropin peptides using a cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line (Kapas et al. 1996). Such assays measure the initial membrane binding but not strictly the biological activation, e.g., of adrenal cells or melanocytes.

Procedure

Hela cells are seeded into 12-well culture plates at a density of 10^6 cells/well. On the 2nd day of culture, the cells are washed as follows: 2 × in 1 ml of ice-cold 0.9 % NaCl, 1 × in 1 ml of ice-cold glycine (50 mM glycine, 100 mM NaCl, pH 3.0) for 5 min, 2 × in 0.5 ml of ice-cold 0.9 % NaCl. Cells are then incubated for 60 min at 20 °C with increasing concentrations of nonradioactive ACTH or various ACTH analogues and the

reactions initiated on the addition of [^{125}I-iodotyrosyl^{23}]ACTH[1–39] (2000 Ci/mmol; final concentration 0.1 pmol/l) in DMEM. At the end of the incubation, the medium is removed and the cells washed three times with 0.9 % NaCl and then dissolved in 0.5 M NaOH/0.4 % sodium deoxycholate. Each point is determined in triplicate, and the radioactivity is measured using a gamma counter. Specific binding is determined by subtracting from the total binding the radioactivity associated with cells in the presence of 10^{-5} M nonradioactive ACTH.

Evaluation

Binding parameters are determined using a computer-assisted calculation, e.g., the LIGAND program (Munson and Rodbard 1980).

Modifications of the Method

Penhoat et al. (1993) reported the identification and characterization of corticotropin receptors in bovine and human adrenals by covalent cross-linking of radiolabeled ACTH with the bifunctional cross-linking agent disuccinimidyl suberate to cultured bovine adrenal fasciculata reticular cells and to crude plasma membrane fractions prepared from both human and bovine adrenals.

Lebrethon et al. (1994) and Penhoat et al. (1995) studied the regulation of ACTH receptor mRNA and binding sites by ACTH and angiotensin II in cultured human and bovine adrenal fasciculata cells.

Picard-Hagen et al. (1997) found that glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells.

Zavyalov et al. (1995) described the receptor binding properties of peptides corresponding to the ACTH-like sequence of human pro-interleukin-1$_\alpha$.

Naville et al. (1996, 1997) developed a stable expression model in order to characterize the human ACTH receptor by binding studies and functional coupling to adenylate cyclase.

Schioth et al. (1996) described the pharmacological distinction of the ACTH receptor from other melanocortin receptors in the mouse adrenocortical cell line Y1.

Moreover, melanocortin receptors do not have a binding epitope for ACTH beyond the sequence of alpha-melanocyte-stimulating hormone (Schioth et al. 1997).

Critical Assessment of the Method

Binding affinities are determined for structure–activity studies, and the results need to be assessed and confirmed for biological relevance using an assay for the biological response of tissues in vitro and/or the biological effects in animals. These methods are also useful for assessing the sensitivity of hormone-responsive tissues from animals previously treated (adaptive response).

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Thyrotropin (TSH)

General Considerations

The thyroid-stimulating hormone (TSH) can be determined by bioassays (effects on the thyroid gland and secretion of thyroid hormones) and its concentration measured by immunoassays, as with other polypeptide hormones (Utiger 1979; Meinhold et al. 1994; Spencer 1994). TSH receptors have been identified and their functional regulation described (Vassart and Dumont 1992).

Large-scale synthesis of recombinant human thyrotropin has been reported (Cole et al. 1993; Hussain et al. 1996). The role of the thyrotropin receptor has been reviewed by Vassart and Dumont (1992). Castagiola et al. (1992) described a binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. A brain-derived TSH receptor has been cloned and expressed (Bockmann et al. 1997), in accordance with the developmental effects of TSH in the fetus and newborn. Binding characteristics of antibodies to the TSH receptor were described by Oda et al. (1998).

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Thyroid Histology

Purpose and Rationale

Hypophysectomy results in atrophy of the thyroid gland, which is reversed by administration of thyrotropin. In the thyroid of normal young guinea

pigs (Junkmann and Schoeller 1932; McGinty and McCullough 1936) or chicks (Jones 1939), characteristic histological changes are observed after administration of thyrotropin, associated with an increase of thyroid weight (goitrogenic response). In classical bioassays, these findings were the basis for standardizing by biological units (Turner 1950, 1969).

Procedure

Male guinea pigs weighing 180–200 g are injected once daily on 4 days successively. Thyroids are removed on the 6th day, weighed, and embedded for histological examination. Administration of thyrotropic hormone is followed by colloid resorption, increased vascularity, and increased epithelial cell height. Several regions of the thyroid are examined histologically. Alternatively, computer-assisted morphometry may be used. Rating scores are defined between +1 and +4.

Evaluation

The rating scores are averaged and compared between test preparation and standard.

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Iodine Uptake

Purpose and Rationale

This is a bioassay for thyroid activation related to the initial step of thyroid-stimulating hormone (TSH) action (Turner 1950, 1962, 1969). The uptake of iodine (trapping) as well as the release of the newly formed iodinated thyroid hormones is under the control of thyrotropin (and in animal assays, under control of hypothalamic thyrotropin-releasing hormone, TRH). As a consequence, the uptake of ^{131}I and the release of ^{131}I-labeled thyroxin are increased after administration of TSH. A method using ^{131}I release in mice has been described by McKenzie (1958) and modified by Sakiz and Guillemin (1964).

Procedure

Female mice weighing 10–15 g are kept in a temperature-controlled room and fed a low-iodine diet for 10 days. They are then injected intraperitoneally with 1.5 μC ^{131}I, followed 5 h later by 10 μg l-T_4 subcutaneously. After 24 h, they receive a second injection of 5 μg L-T_4 and are used 48 h after the last injection. Under ether anesthesia, 0.25 ml blood is withdrawn from the jugular vein into a heparinized syringe. Various doses of the test preparation or standard TSH are injected by the same route in 0.3 ml volume. Two hours later, again under ether anesthesia, a second 0.25-ml sample is taken and the radioactivity measured. The increase of radioactivity in the blood samples is dependent on the dose of TSH.

Evaluation

A four-point assay technique is used with six observations for each of two doses of the standard and of the unknown preparation.

Modifications of the Method

Depletion of ^{131}I from the thyroids of chickens was used as the endpoint for a TSH assay by Bates and Cornfield (1957).

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TSH Bioassay Based on cAMP Accumulation in CHO Cells

Purpose and Rationale

Indirect cell assays measure the activation of protein kinase A (PKA) through adenylcyclase. Several cell assays for thyrotropin using cultured FRTL-5 cells have been described (Vitti et al. 1986; Nissim et al. 1987; Horimoto et al. 1989). Persani et al. (1993) reported a cell assay for human thyrotropin using measurement of cAMP accumulation on Chinese hamster ovary (CHO) cells transfected with the recombinant TSH receptor.

Procedure

Cells of the CHO-R strain JP-09 are cultured in Petri dishes in RPMI-1640 medium supplemented with 1 mM glutamine and 10 % fetal calf serum. In these cells, TSH biological activity is evaluated by measuring cAMP production. Cells are harvested from Petri dishes using a Trypsin-EGTA mixture and seeded in 96-well plates (10,000 cells/well). Cells are fed with fresh RPMI-1640 medium 24 h after seeding. The assay is run after 48 h. After washing, 100 µl of TSH standard or samples diluted in hypotonic or isotonic medium containing 0.4 % BSA, 10 mM HEPES, and 0.5 mM isobutylmethylxanthine are incubated for 2 h at 37 °C. Three different dilutions of immunoconcentrated TSH are bioassayed in triplicate, as are TSH preparations. cAMP is measured in nonacetylated samples by an RIA method using a commercial polyclonal anti-cAMP antibody (Vitti et al. 1986).

Evaluation

Dose–response curves or single-point comparisons are used for potency or activity estimates.

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Hormones Related to TSH

General Considerations

These hormonal factors are now characterized by molecular cloning. One of the characteristic symptoms of thyroid activation (Graves' or

Basedow disease) is exophthalmos. A dissociation of the exophthalmos-producing activity from the TSH activity has been found (Dobyns and Steelman 1953). Moreover, time–response curves for TSH activity from the serum of patients with hyperthyroidism differed from those of the standard TSH, giving evidence for the presence of an abnormal factor, long-acting thyroid-stimulating factor (LATS). Bioassays for these factors have been developed (Ludgate 1999). TSH receptor stimulating antibodies have also been identified and suitable animal models investigated (Di Cerbo and Corda 1999; De Felice et al. 2004).

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Assay of Exophthalmos-Producing Substance (EPS) in Fishes

Purpose and Rationale

Some TSH fractions were reported to produce more exophthalmos than others (Dobyns and Steelman 1953). A second fraction containing exophthalmos-producing substance (EPS) could be separated (Brunish et al. 1962). The activity of this substance was demonstrated in fishes.

Procedure

Fundulus heteroclitus Linn., the common Atlantic minnow, has been found to be the suitable animal model (Albert 1945; Sobonya and Dobyns 1967; Turner 1969). Other fish species, such as *Carassius auratus* (the common gold fish; Haynie et al. 1962) and *Cyprinus carpio* (der Kinderen et al. 1960), were also used but gave less reliable results (Sobonya and Dobyns 1967). *Fundulus heteroclitus* Linn., common Atlantic minnows, 8–10 cm in length, are kept in a tank of running tap water at 10 °C in winter and 18 °C in summer. Before treatment, the intercorneal distance is measured with Vernier calipers to the nearest of 0.1 mm. The test compound and the standard are injected intraperitoneally at various, but at least two, doses. To avoid loss of fluids by the intraperitoneal injection route, the needle is inserted into the cloaca, through the rectum, over the pelvic girdle, and into the peritoneal cavity for a distance of about 1.2 cm. The volume of fluid injected should be 0.1–0.5 ml. The effect of the hormone is to cause protrusion of the eyeballs. The intercorneal distance is measured again after 24, 48, and 72 h. The increase in proptosis is expressed as a percentage of the intercorneal distance found at the beginning of the test.

Evaluation

The increases of intercorneal distance after each dose are averaged and activity ratios with confidence limits calculated from the 2 + 2-points assay.

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Assay of Long-Acting Thyroid-Stimulating Factor (LATS) in Mice

Purpose and Rationale

This is a modification of the TSH assay to assess the late onset of action of LATS. In the assay of McKenzie (1958) (see section on "Iodine Uptake"), mice previously injected with ^{131}I show a maximum increase in serum ^{131}I after an interval of 2–3 h when TSH is administered. In the assay of serum from thyrotoxic patients, Adams (1958) noted that the maximum response in guinea pigs did not occur until after 16 h. The abnormal responses to thyrotoxicosis sera suggested the presence of an additional factor different from TSH.

Procedure

Mice maintained on a low-iodine diet for 10 days are injected with 15 μC ^{125}I and 10 μg Na l-T_4. Four days later, 0.1 ml of blood is obtained by retro-orbital puncture immediately before the injection of the test substance 2 and 9 h later. Radioactivity in the blood is then measured. By definition, radioactivity which is maximal after 2 h is indicative of TSH, whereas LATS causes a maximal increase at 9 h.

Evaluation

The increases of radioactivity after 2 and 9 h are compared and evaluated by statistical methods.

Modifications of the Assay

Ikeda and Nagataki (1983) and Ikeda et al. (1984) used male DDY mice weighing 15 g. They were fed a low-iodine diet for 14 days and then injected daily with 1 μg of 3,5,3′-triiodothyronine (T_3) s.c. and given T_3 (5 μg/ml) ad libitum in drinking water until sacrifice. From the 5th day of T_3 treatment, they were injected i.p. with 0.25 ml of LATS-positive serum for 9 days. Groups of five mice were sacrificed before, 1, 3, 5, 7, and 9 days after the first injection of LATS. Then, 1 μCi (0.5 ml) of Na [^{131}I] was administered i.p. 1 h before sacrifice. Thyroid lobes were excised, weighed, and radioactivity measured by a gamma counter. Immediately before injection of Na [^{131}I], approximately 60 μl of blood was collected from the orbital plexus with heparinized capillary tubes and centrifuged at 12,000 rpm for 3 min. T_4 concentrations in serum were determined by radioimmunoassay.

Ealey et al. (1984, 1985) developed a sensitive cytochemical bioassay for thyroid stimulators, using reference preparations of thyrotropin and LATS. Thyroid stimulators cause changes in lysosomal membrane permeability within the thyroid follicular cells of guinea pigs, which can be monitored by measuring increased intralysosomal enzyme activity (in the case of this assay, naphthylamidase), with a chromogenic substrate leucine-2-naphthylamide, which itself does not readily permeate the lysosomal membrane in unstimulated cells.

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Posterior Pituitary Hormones

General Considerations

Oxytocin and vasopressin were the first hormones isolated from the posterior pituitary lobe (Dale and Laidlaw 1912; Hogben et al. 1924; Fromherz 1926; Schaumann 1937). Vasopressin has antidiuretic activity and, at higher doses, hypertensive effects as well as endocrine functions (Hedge and Huffman 1987) and effects on the central nervous system (Gash et al. 1987), which are however outside the scope of this chapter. In terms of bioassays, the antidiuretic activity is the main parameter. Many analogues of vasopressin have been synthesized resulting in selective agonists and antagonists (Vogel and Hergott 1963; Allison et al. 1987; Mah and Hofbauer 1987; Manning et al. 1987). Different types of vasopressin receptors have been identified: V_1 (V_{1a}, V_{1b}) and V_2-receptors (Jard et al. 1976, 1986; Fahrenholz et al. 1988; Walker et al. 1988; Burnatowska-Hledin and Spielman 1989). Research on the vasopressin analogues has provided several compounds of clinical utility, and antagonists of vasopressin are now increasingly explored for several indications, including nonpeptide vasopressin antagonists (Mayinger and Hensen 1999; Serradeil-Le Gal et al. 2002; Greenberg and Verbalis 2007; Urban et al. 2007).

The use of bioassays in the evaluation of these vasopressin and oxytocin analogues has been reviewed (Liard 1988; Chan et al. 2000).

Oxytocin receptors have been described in several organs, such as uterus, mammary gland, and CNS (Soloff 1976; Hruby and Chow 1990).

The synthesis and development of several orally active, nonpeptide oxytocin antagonists have been reported (Bell et al. 1998; Kuo et al. 1998).

Radioimmunoassays are available for both oxytocin (Kagan and Glick 1978) and vasopressin (Glick and Kagan 1978).

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Posterior Pituitary Hormones

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Contents

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Oxytocin

Isolated Uterus

Purpose and Rationale

Several authors, such as Dale and Laidlaw (1912), Fromherz (1926), Glaubach and Molitor (1932), Lipschitz and Klar (1933), and Simon (1933), used the isolated uterus of virgin guinea pigs as a sensitive test for determination of oxytocin activity. The isolated uterus of the rat (Holton 1948) is less sensitive, but, in contrast to the guinea pig, the rat uterus shows no spontaneous contractions in solutions with low calcium and glucose concentrations. Historically, the method has been adopted by several pharmacopoeias, e.g., by the British Pharmacopoeia (1988). The United States Pharmacopeia 23 (1995) uses the isolated guinea pig uterus for determination of oxytocin activity in vasopressin preparations. Physicochemical assays are now used for standardizing drug content, instead of the biological responses.

Procedure

Female Sprague-Dawley or Wistar rats weighing 120–200 g are used. At 18–20 h prior to the assay, the rat is injected i.m. with 100 μg of estradiol benzoate for priming (receptor induction). Immediately before the assay, the rat is tested for estrogen-induced epithelial proliferation by vaginal smear. One horn of the uterus is suspended in

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an organ bath containing a solution of the following composition:

Sodium chloride	6.62 g/l
Potassium chloride	0.45 g/l
Calcium chloride	0.07 g/l
Sodium hydrogen carbonate	2.56 g/l
Disodium hydrogen orthophosphate	0.29 g/l
Sodium dihydrogen orthophosphate	0.03 g/l
Magnesium chloride	0.10 g/l
D-Glucose	0.50 g/l

The bath temperature is maintained at 32 °C, a temperature at which spontaneous contractions of the uterus are abolished and the preparation maintains its sensitivity. The solution is bubbled with a mixture of 95 % O_2 and 5 % CO_2. The preparation is loaded with 1–2 g, and the contractions recorded using a Statham transducer and a polygraph. Two doses of the standard preparation are added, ranging usually between 10 and 50 μU/ml organ bath. The preparation examined is diluted in such a way to obtain responses on the addition of two doses similar to those obtained with the standard preparation. The ratio between the two doses of the preparation being examined should be the same as that between the two doses of the standard preparation. The two doses of the standard preparation and the two doses of the preparation being examined are given according to a randomized block or a Latin square design, and at least six to eight responses to each are recorded. The doses should be recorded at regular intervals of 3–5 min depending on the rate of recovery of the muscle.

Evaluation

The activity and potency ratios with confidence limits are calculated from the 2 + 2-point assay.

Modifications of the Method

The isolated rat uterus is also used to test spasmolytic activity of various drugs against oxytocin as spasmogen. Liebmann et al. (1993) used the rat uterus to test the pharmacological and molecular actions of the bradykinin B_2 receptor antagonist, Hoe 140.

The method described by Schübel and Gehlen (1933) using the **uterus of cats** 2–4 days after partum is of historical interest only.

In addition to the isolated rat uterus, Berde et al. (1957) used the **rat uterus in situ**, the **cat uterus in vitro**, and the **cat uterus in situ** for evaluation of synthetic analogs of oxytocin.

Murray and Miller (1960) observed characteristic postural changes in rats following administration of oxytocin to unanesthetized rats described as "cramping" which was dose dependent in estrogen-pretreated animals.

An in vitro **hen oxytocic assay** was designed by Munsick et al. (1960). Muscle strips of the uterine portion of the oviduct of laying hens are dissected and suspended in a van Dyke–Hastings solution containing 0.15 mM calcium, 0.5 mM magnesium, and 100 mg% glucose. The strips are 2–3 cm long and 2–3 mm wide. The solution is gassed with oxygen containing 5 % carbon dioxide and maintained at a temperature of 43 °C. These conditions are necessary to prevent spontaneous contractions.

The **isolated uterus from immature guinea pigs** was used for evaluation of oxytocin activity by Fromherz (1926) and by Vogel and Hergott (1963).

Guissani et al. (1995) and Pettibone et al. (1996) reported the effect of oxytocin antagonists in **pregnant rhesus monkeys** in vivo.

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Chicken Blood Pressure

Purpose and Rationale

Oxytocin induces a transient fall in blood pressure in chicken and other birds. This effect can be used as an assay for oxytocin (Coon 1939). The method has been modified by Munsick et al. (1960) and accepted by pharmacopoeias; for example, the United States Pharmacopeia 23 (1995a, b) uses the chicken blood pressure method for determination of oxytocin activity with three doses of standard and test preparation and calculation of the activity ratio with confidence limits.

Procedure

White Leghorn chickens weighing 1.2–2.0 kg are anesthetized by intravenous injection with 200 mg/kg sodium phenobarbital via the brachial vein. The ischiadic artery is exposed by removing the feathers from the outer surface of the left thigh, and an incision 7–8 cm long is made in the skin, parallel to and about 1.5 cm below the femur, exposing the gluteus primus muscle. The lower edge of this incision is retracted to expose the edge of the gluteus primus muscle overlying the semitendinosus muscle. The edge is then freed for the length of the incision, and when the free edge is lifted, the ischiadic artery, the ischiadic vein, and the crural vein can be seen lying along the edge of the semitendinosus muscle. The gluteus primus muscle is cut at right angles near the proximal end of the incision and the resulting flap deflected and secured to the upper thigh. A length of the ischiadic artery and the crural vein are dissected free and the artery is cannulated. The cannula is connected to a Statham pressure transducer. Blood pressure should be between 100 and 120 mmHg. The crural vein is cannulated for injections of the test preparations. Intravenous injection of oxytocin induces in chickens an immediate, transient fall in blood pressure. Doses of the standard are chosen which are followed by a decrease of blood pressure of between 20 and 40 mmHg. The required doses normally lie between 20 and 100 mU.

Two doses of the standard and two doses of the test preparations are injected according to a randomized block or to a Latin square design, and at least six to eight responses to each should be recorded. The interval between injections should be constant and lies between 3 and 10 min, depending on the rate at which the blood pressure returns to normal.

Evaluation

The responses to each dose are averaged and potency ratios with confidence limits calculated from the 2 + 2-point assay.

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Milk Ejection in the Lactating Rabbit or Rat

Purpose and Rationale

A sensitive bioassay for the estimation of oxytocin was described by van Dyke et al. (1955). This test makes use of the milk-ejecting properties of oxytocin.

Procedure

A rabbit in the first or second week of lactation is anesthetized with urethane and pentobarbital. Usually, artificial respiration is not necessary. One jugular vein is cannulated for injections. One of the six ducts in a nipple of the rabbit is cannulated with a hypodermic needle and connected with a Statham strain gauge transducer. Two doses of the standard and the test preparation are injected according to a randomized block or to a Latin square design, and at least six to eight responses to each should be recorded. The interval between injections should be constant at 3 min.

Evaluation

The responses of each dose are averaged and potency ratios with confidence limits calculated from the 2 + 2-point assay.

Modifications of the Method

The British Pharmacopoeia (1988) recommends the measurement of milk-ejection pressure in the lactating rat. A lactating rat weighing about 300 g in the 3rd to 21st day after parturition is anesthetized by pentobarbitone sodium. The trachea is cannulated. One jugular or femoral vein is cannulated for injection of the test preparations. The tip of one lower inguinal teat is excised, and a polyethylene tube with an external diameter of 0.6 mm is inserted to a depth sufficient to obtain appropriate measurement of pressure into the primary teat duct which opens onto the cut surface and is tied firmly in place with a ligature. The cannula is connected with a suitable strain gauge pressure transducer for recording on a polygraph.

Tindal and Yokoyama (1962) recommended the use of guinea pigs using essentially a similar procedure, but the injection is made into the internal saphenous artery after ligation of the main branches supplying the limb. In the guinea pig, the mammary glands are supplied with blood from the external pudendal arteries, which branch from the internal saphenous artery just as they enter the legs.

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Oxytocin Receptor Determination

The family of the vasopressin and oxytocin receptors has been studied very intensely (Peter et al. 1995) in order to obtain evidence for new indications and to determine the specific regions

in each receptor subtype and the associated effect or systems for the G-protein-linked subtypes (Thibonnier et al. 1998).

Purpose and Rationale

Premature labor and preterm delivery is an important cause of death among infants and a major cause of newborn and child morbidity. Strong evidence exists that oxytocin and catecholamines are involved in the spontaneous uterine contractions which bring about premature delivery. Development of oxytocin antagonists is believed to be of therapeutic value for the prevention of preterm labor (Manning et al. 1995; Chan et al. 1996).

In vitro binding to the oxytocin receptor is used as the first step in the characterization of potential oxytocin antagonists (Pettibone et al. 1990, 1991, 1993a, b, 1996; Evans et al. 1993; Manning et al. 1995; Freidinger and Pettibone 1997; Pettibone and Freidinger 1997).

Procedure

Uterine tissue is taken from nonpregnant adult Sprague-Dawley rats pretreated (18–24 h) with diethylstilbestrol (300 µg/kg, i.p.) and mammary tissue from lactating rats (4–14 days of lactation). The tissues are homogenized in 10 mM Tris containing 1 mM EDTA and 0.5 mM dithiothreitol, pH 7.4 and centrifuged at 48,000 *g* for 30 min at 4 °C. The resulting pellets from mammary/uterine tissue are resuspended in 50 mM Tris/5 mM $MgCl_2$/0.1 % BSA (pH 7.4) and centrifuged again to produce the final pellet. Competition studies are conducted at equilibrium for 60 min at 22 °C using 1 nM [^{3}H]OT (30–60 Ci/mmol, New England Nuclear, Boston, Mass., USA) in the following buffer: 50 mM Tris/5 mM $MgCl_2$/0.1 % BSA (pH 7.4). Nonspecific binding (5 %–10 % of total binding) is determined using 1 µM unlabeled oxytocin. IC_{50} values are calculated from the linear regression analysis of log concentration of inhibitor versus percentage inhibition of specific binding. Saturation binding studies are conducted at equilibrium using a 100-fold range of radioligand concentrations (i.e., 0.1–10 nM) and analyzed by a nonlinear regression program (McPherson 1985a, b). The binding reactions are initiated by the addition of the tissue preparation (final protein concentrations, 100–200 µg protein/ml) and terminated by rapid filtration through Skatron glass fiber filters using a Skatron cell harvester system (Model 7019, Skatron, Sterling, Va., USA).

Evaluation

Inhibition constants (K_i) are calculated for each compound from three to six separate IC_{50} determinations ($K_i = IC_{50}/[1 + c/K_d]$) using mean dissociation constants (K_d) obtained from saturation binding assays.

Modifications of the Method

Maggi et al. (1994) used cultured Hs 805.Ut (corpus uteri, normal, human) cells or cells obtained from women in the early follicular or late luteal phase to study binding of antagonists at the human oxytocin receptor.

Pak et al. (1994) compared the binding affinity of oxytocin antagonists to human and rat oxytocin receptors and correlated the results with the rat oxytocic bioassay.

Species differences in central oxytocin receptor gene expression were investigated by Young et al. (1996).

Cloning and expression of the rhesus monkey oxytocin receptor was reported by Salvatore et al. (1998).

Molecular cloning and functional characterization of the oxytocin receptor from a rat pancreatic cell line (RINm5F) was reported by Jeng et al. (1996).

Elands et al. (1987) and Klein et al. (1995) recommended selective radioligands for the oxytocin receptor for the structure–activity research directed to receptor subtypes.

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Vasopressin

Hereditary Vasopressin Deficiency in Rats (Brattleboro Strain)

This bioassay is based on an animal strain with genetic deficiency of vasopressin synthesis. Patients with diabetes insipidus excrete large amounts of very diluted urine and need a high fluid intake. This disorder is due to a lack of vasopressin. A similar syndrome has been found in Brattleboro rats. In the rare nephrogenic form, the vasopressin receptors are defective (Sokol and Valtin 1982; Nyunt-Wai and Laycock 1990; Szot and Dorsa 1992). Vasopressin is also considered to be a modulator in the central nervous system in particular learning and memory processes. Therefore, many studies on learning and memory have been performed with this strain of rats. Valtin et al. (1965) found very little vasopressin in hypothalami and pituitaries of homozygous Brattleboro rats.

Schmale and Richter (1984) and Schmale et al. (1984) found a single-base deletion in the vasopressin gene as the cause of diabetes insipidus in Brattleboro rats. The mutant vasopressin gene is transcribed, but the message is not efficiently translated. Spontaneous hypertensive rats crossbred with Brattleboro rats inherit the mutated vasopressin gene (McCabe et al. 1988). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus can be reversed by a vasopressin agonist (Laycock et al. 1994)

For testing aquaretic effects in Brattleboro rats, see chapter "► Diuretic and Saluretic Activity".

Modifications of the Method (Other Diabetes Insipidus Models)

Similar studies were reported by Byrnes et al. (1953), Randall and Selitto (1958), Eviatar et al. (1961), Turner (1965), Dorfmann (1969), Neri et al. (1972), Neumann et al. (1977), Furr et al. (1987), Snyder et al. (1989), Christiansen et al. (1990), Shibata et al. (1992), Neubauer et al. (1993), and Tagekawa et al. (1993).

Several other animal models of diabetes insipidus have been developed and characterized (Herman et al. 1986; Grant 2000; Lloyd et al. 2005; Petersen 2006) for the understanding of pathophysiology, for explanation of genetic deficiencies, and to validate the animal models based on substitution of vasopressin deficiency.

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Vasopressor Activity

Purpose and Rationale

Vasopressin activity can be determined by the increase of blood pressure observed after surgical or pharmacological elimination of the central and peripheral nervous regulation of the cardiovascular system. Dekansky (1952) described a quantitative assay of vasopressin in anesthetized rats after blockade of other pressor substances by dibenamine (pithed rat).

Procedure

A male Wistar rat weighing about 300 g is anesthetized by subcutaneous injection of 1.75 g/kg urethane. After 45–60 min, the trachea is cannulated with a polyethylene tube of 2.5 mm diameter. One femoral vein is cannulated for injections and the carotid artery for measuring blood pressure with a Statham transducer. The central and peripheral nervous systems, including both vagi and associated sympathetics, are left intact. No artificial respiration is necessary. Heparin (2,000 U/kg) is injected through the venous cannula and washed through with saline. Dibenamine *N*-(2-chloroethyl)dibenzylamine hydrochloride is injected twice intravenously with an interval of 10 min at a dose of 1 mg/kg. The blood pressure stabilizes at a basal level of about 50 mmHg. Small doses of vasopressin induce an increase in blood pressure which is dependent on the dose. Two doses of vasopressin standard (approximately 3–5 mU) and two doses of test preparation are injected repeatedly (usually six times) using a Latin square design. The doses are injected at intervals of 10–15 min.

Evaluation

The responses of each dose are averaged and potency ratios calculated from the 2 + 2-point assay.

Modifications of the Method

One of the first recommendations for standardizing posterior pituitary extract was by measuring blood pressure in anesthetized dogs (Hamilton 1912).

Vogel and Hergott (1963), studying the properties of a synthetic vasopressin analogue, described a method in decerebrate rabbits previously used in this laboratory for standardization of posterior pituitary extracts. Rabbits weighing 2–3 kg were anesthetized by slow intravenous injection of butallylonal. The trachea is cannulated and the cannula connected with a respiration pump. One femoral vein is cannulated for injection of the test compounds. One carotid artery is cannulated for measurement of blood pressure with a Statham transducer. For chemical decapitation, the head of the anesthetized rabbit is bent forward and a needle introduced into the foramen occipitale magnum, and then 0.5 ml of 30 % trichloroacetic acid is injected. Artificial respiration is started immediately. Blood pressure is

stabilized at a level of 30–40 mmHg. Two doses of standard and of test preparation are injected according to a Latin square design. The method has been proven to be very sensitive.

The British Pharmacopoeia (1988) recommends the intravenous injection of 10 mg/kg of the α-adrenoreceptor blocking agent phenoxybenzamine hydrochloride 18 h prior to the experiment to stabilize the blood pressure at a low level. USP 23 (1995a, b) uses two doses of standard and test preparation in the phenoxybenzamine-blocked rat for calculation of vasopressin activity with confidence intervals in vasopressin and oxytocin preparations.

Knape and van Zwieten (1988) used the pithed rat to study vasoconstrictor activity of vasopressin after pretreatment with various drugs.

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Antidiuretic Activity in the Conscious Rat

Purpose and Rationale

For some period of time, it was uncertain whether vasopressin and adiuretin are the same or separate hormones. Special tests for antidiuretic activity in water-loaded rats were developed (Burn 1931) and adopted by pharmacopoeias. Vasopressin analogues show considerable dissociation of vasopressive and antidiuretic activities (Vogel and Hergott 1963).

Procedure

Wistar or Sprague-Dawley rats weighing between 140 and 250 g of either sex are used. The range of weights in any one test should not exceed 50 g. Not less than 2 days before testing the preparation, the rats have to be accustomed to the metabolism cage by carrying out a preliminary test. All animals are injected with 0.1 ml saline solution per 100-g body weight instead of the test preparations given in the main test. Any rat that shows signs of stress or undue excitement or that has abnormally low or high rate of urine excretion should not be used in the main test. Food and water are withheld during each test but access is allowed between the tests. The rats are assigned at random to four groups, each no less than four animals, and are weighed and marked for identification purposes. By stomach tube, each rat receives a volume of water warmed to approximately 37 °C and equivalent to 5 % of the animal's body weight. Each rat is placed in a separate cage for collection of urine. Then 30 min later, the

volume excreted by each rat is recorded, and a second volume of tap water equal to the volume of urine together with a further volume equivalent to 3 % of the animal's body weight is administered. This provides a total water load equivalent to 8 % of the animal's body weight.

Using a different group of rats and two dilutions of the preparation to be examined and of the standard preparation, immediately after administration of the second dose of water, each rat is injected subcutaneously with a volume of the appropriate dilution equivalent to 0.1 ml per 100-g body weight. The urine passed during the first 5 min after injection is discarded, and the volumes collected at intervals of 15 min are noted until a volume greater than 30 % of the total water load is excreted.

Evaluation

The responses of each dose are averaged, and activity ratios with confidence limits are calculated from the 2 + 2-point assay.

Modifications of the Method

Hydrated **conscious dogs** have been used to test the antidiuretic activity of vasopressin by van Dyke et al. (1955).

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Antidiuretic Activity in the Rat in Ethanol Anesthesia

Purpose and Rationale

This is a sensitive bioassay often reported for vasopressin analogs, which has been applied extensively but is no longer required due to the availability of other methods (e.g., Brattleboro rats). Ethanol suppresses the secretion of endogenous antidiuretic hormone (van Dyke and Ames 1951). Sensitive methods measuring urine output in ethanol-induced anesthesia were described (Dicker 1953; Dettelbach 1958; Munsick et al. 1960; Berde and Cerletti 1961), whereby the water load is automatically kept constant.

Procedure

Female Sprague-Dawley rats weighing 200–250 g are spayed 7–10 days prior to use in order to eliminate the effect of gonadal steroids on the response to a water load. Each animal is given a 5-ml gavage of tepid tap water. At 3-day intervals thereafter, the water load is increased by 1-ml increments until a 10-ml level is reached. The day before an assay is to be performed, two or three animals are selected and given a 10-ml gavage of 2 % ethanol. The animals are fasted overnight but are allowed free access to water. On the test day, they are placed into individual metabolism cages for water loading and anesthetizing. Each rat receives 5 % of its body weight of warm 12–15 % ethanol by stomach tube. This induces anesthesia within a few minutes. After 45 min, the same volume of 2 % ethanol is again given by gavage. After 1 h, the bladder is emptied by suprapubic pressure, and the total urine output is measured.

The animal with the largest volume is selected and the water load calculated as the total volume administered less the volume excreted. The load on this animal is now increased to between 6 % and 8 % of its body weight with warm 2 % ethanol. Then the urinary bladder is catheterized. Polyethylene tubing is inserted into the rat's stomach. A femoral vein is cannulated for injections. The hind legs are secured to the operation table, while the front legs are left free. Assays are started when urine flow reaches a steady level of at least 50 µl/min. The catheter from the bladder is connected to an apparatus consisting of two Woulff bottles. Urine is directed through the catheter into the first Woulff bottle, displacing fluid into the second Woulff bottle. The second bottle is filled with 0.5 % NaCl solution in 50 % ethanol to reduce the size of the drops. Each drop activates an impulse counter. The drops are collected in a small glass reservoir and led by means of polyethylene tubing to the urine metering pipette. When the fluid level in this tube rises and makes contact with an adjustable needle electrode near the top, another pipette which is calibrated to the same volume empties a solution of 5 % glucose in 2 % ethanol through the gavage tube into the rat's stomach. By these means, a constant water load is maintained.

The animals produce urine at a relatively steady rate for 3–5 h. Intravenous injections of vasopressin produce characteristic changes (decreased rate of urine flow), which begin usually within 2 min and subside in most instances within 15 min. The range in which the assay of antidiuretic activities is useful is between 2 and 64 µU/injection.

Evaluation

Antidiuretic potency is calculated as the log ratio of the volume excreted in 5 min preceding injection to the volume excreted in the 5 min beginning 1 min after injection. Dose–response relationships are established for the standard, and the test preparation and potency ratios are calculated.

Modifications of the Method

Berde and Cerletti (1961) placed the rat anesthetized with ethanol on a balance. The water loss due to urine excretion and perspiratio insensibilis is replaced automatically using a solution of 5 % glucose in 2 % ethanol.

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Spasmogenic Activity of Vasopressin in the Isolated Guinea Pig Ileum

Purpose and Rationale

The ileum of the guinea pig has been found to be quite sensitive to vasopressin (Simon 1933; Schaumann 1937; Vogel and Hergott 1963). This is a direct effect on smooth muscle similar to the pressor effect. This test is not specific for vasopressin.

Procedure

Pieces of guinea pig ileum are suspended in an organ bath according to the Magnus' technique (see chapter "► Pharmacological Effects on Intestinal Functions"). Two different doses of the test preparation and of the standard are applied according to a Latin square design, and the contractions measured using a strain gauge transducer. Four to six doses of each solution are measured.

Evaluation

The responses of each dose are averaged, and activity ratios with confidence limits are calculated from the 2 + 2-point assay.

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Vasopressin Receptor Determination

Purpose and Rationale

As in other areas of drug research, when specific receptor preparations became available for in vitro research, those identifying new vasopressin-like compounds (in structure–activity studies) turned to new methods with high specificity and capacity, replacing the cumbersome bioassays. Tests for receptor affinity are also used in "receptor screens" for the general pharmacology of new drug candidates, to predict their spectrum of activities.

Arginine vasopressin exerts its action through three membrane-bound G-protein-coupled receptor subtypes (V_{1a}, renal V_2, and V_3). The vasopressin-induced antidiuresis (via V_2 receptors coupled to aquaporins) helps maintain plasma osmolality and salt (NaCl) balances. The human V_1, V_2, and V_3 (V_{1b}) receptors and water-selective membrane proteins (aquaporins) in the kidney have been cloned, and receptor signaling has been characterized in detail (Thibonnier et al. 1998).

Pharmacological characterization of the human vasopressin receptor subtypes stably expressed in Chinese hamster ovary cells was reported by Tahara et al. (1998).

Selective nonpeptide vasopressin antagonists (without agonistic activity) have been shown to be aquaretic agents in animals and humans. The development and therapeutic indications of orally active vasopressin receptor antagonists were reviewed by Thibonnier (1998), as were the postreceptor signaling mechanisms for the vasopressin receptor subtypes.

Serradeil-Le Gal et al. (1993, 1994a) studied the biochemical and pharmacological properties of a nonpeptide antagonist on rat and human vasopressin V_{1a} receptors, to explore the possibility of using centrally acting derivatives for indications in neuropharmacology.

Procedure

Tissue samples from the human uterus, adrenals, kidneys, and pituitaries are collected in conformity with national ethical rules. Uterus, adrenal, and kidney samples are immediately chilled in cold saline. Membranes are prepared within 3 h after collection. Pituitaries are collected within 6 h after death and immediately frozen in liquid nitrogen. Bovine kidneys can be obtained from a local slaughterhouse. Rat mammary tissue is taken from 19-day Sprague-Dawley pregnant rats.

For human adrenal membrane preparations, pieces of adrenal glomerulosa zone are suspended in a cold buffer (10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and fragmented using a glass/glass Dounce homogenizer. The homogenate is filtered through glass wool and centrifuged for 15 min at 1,500 *g* at 4 °C. The pellet is incubated for 15 min in ice-cold hypotonic buffer (10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF) and kept on ice to allow cell lysis. Lysed cells are recovered by centrifugation at 1,500 *g* for 15 min at 4 °C, homogenized using a loose-fitting Dounce homogenizer in hypotonic buffer supplemented with 40 % glycerol, and stored at −20 °C. Before experiments, glycerol is eliminated by washing the membranes in glycerol-free hypotonic buffer.

For preparation of hypophyseal membranes, frozen entire pituitary glands are rapidly thawed at 37 °C in isotonic buffer supplemented with

0.1 mM PMSF, and the adenohypophyses are separated. Adenohypophyseal membranes are prepared as described above for adrenal membranes.

For rat mammary gland membrane preparations, tissues are minced and homogenized in 50 mM 10 % (wt/vol) buffer A Tris–HCl, pH 7.4, 320 mM sucrose, and 0.5 mM dithiothreitol and centrifuged at 900 *g* for 15 min; the pellet is resuspended in buffer A and centrifuged as above. The two 900-*g* supernatants are filtered through cheesecloth and centrifuged at 70,000 *g* for 20 min. The pellet is washed with buffer B containing 50 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$. Finally, the 70,000-*g* washed pellet is suspended in buffer B at a final concentration of ~8 mg protein/ml and stored in aliquots in liquid nitrogen.

V_{1a} binding assays using ^{125}I linear AVP antagonist on human adrenal membranes, myometrial membranes from nonpregnant uterus, or platelet membranes are performed using an incubation medium (200 µl) containing 10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA), 0.05 mg/ml soybean trypsin inhibitor, 0.5 mg/ml bacitracin, 0.1 mM PMSF, 0.5 mM EDTA, 10–60 pM ^{125}I-AVP linear antagonist, and increasing amounts of test compound. The reaction is started by the addition of membranes (10–40 µg/assay) that are incubated at 30 °C for 45 min. The reaction is stopped by adding 3 ml of ice-cold filtration buffer (10 mM Tris–HCl, pH 7.4, and 3 mM $MgCl_2$) followed by filtration through GF/C Whatman glass microfiber filters that have been soaked for at least 5 h in a solution containing 10 mg/ml BSA. Filters are washed five times with 3 ml of filtration buffer and counted for radioactivity by gamma spectroscopy. Nonspecific binding is determined in the presence of 0.3 µM unlabeled iodinated AVP linear antagonist or 1 µM AVP.

Evaluation

The IC_{50} value is defined as the concentration of inhibitor required to obtain 50 % inhibition of the specific binding. Inhibition constant (K_i) values are calculated from the IC_{50} values using the Cheng and Prusoff equation. Data for equilibrium binding (K_d, B_{max}), competition experiments (IC_{50} n_{Hill}), and kinetic constants (k_{obs}, k_1) are analyzed using an iterative nonlinear regression program (Munson and Rodbard 1980).

Modifications of the Method

A receptor assay for arginine vasopressin is described by Gopalakrishnan et al. (1986).

Pearlmutter et al. (1985) characterized a specific binding site for arginine vasopressin in particulate membranes from rat aorta.

Pávó et al. (1993) reported the synthesis and binding characteristics of two sulfhydryl-reactive probes for vasopressin receptors.

Serradeil-Le Gal et al.(1994b) tested the effect of a nonpeptide vasopressin V_{1a} vasopressin antagonist on the binding and mitogenic activity of vasopressin in Swiss 3T3 cells.

Yatsu et al. (1997) tested the vasopressin antagonistic activities of a nonpeptide dual vasopressin V_{1a} and V_2 receptor antagonist on V_{1a} receptors in dog platelets and on V_2 receptors in dog kidney homogenates.

Ogawa et al. (1996) and Tahara et al. (1997a) tested V_{1a} receptor binding in homogenates of rat liver and V_2 receptor binding in rat kidney.

Barberis et al. (1995) characterized a linear radioiodinated vasopressin antagonist as an excellent radioligand for vasopressin V_{1a} receptors.

Radioligands for vasopressin V_1 receptors were described by Elands et al. (1988) and by Kelly et al. (1989).

Ala et al. (1997) reported the properties of a radioiodinated antagonist for human vasopressin V_2 and V_{1a} receptors and recommended this ligand for further studies on human vasopressin V_2 receptor localization and characterization, when used with a selective vasopressin V_{1a} ligand.

Howl and Wheatley (1995) found species heterogeneity in the characteristics of V_{1a} receptors and in the expression of hepatic V_{1a} receptors.

Thibonnier et al. (1994) reported molecular cloning, sequencing, and functional expression of a cDNA encoding the human V_{1a} vasopressin receptor.

Carnazzi et al. (1997) described photoaffinity labeling of the rat V_{1a} vasopressin receptor using a linear azidopeptidic antagonist.

Phalipou et al. (1997) studied the peptide-binding domains of the human V_{1a} vasopressin receptor with a photoactivatable linear peptide antagonist.

DDAVP (1-desamino-8-D-arginine vasopressin), which is considered to be a standard V_2 vasopressin receptor-selective agonist, was found to act also as an agonist at the V_{1b} vasopressin receptor (Saito et al. 1997).

Tahara et al. (1997b) investigated the effects of a nonpeptide V_{1a} and V_2 vasopressin receptor antagonist in binding and functional studies of rat vascular smooth muscle cells.

Tahara et al. (1998) expressed three subtypes of human arginine vasopressin receptors, hV_{1a}, hV_{1b}, and hV_2, in Chinese hamster ovary cells and characterized them by [^{3}H]AVP binding. In addition, the coupling of the expressed receptor protein to a variety of signal transduction patterns was investigated.

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Hypothalamic Hormones

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Thyrotropin-Releasing Hormone (TRH)

General Considerations

Hypothalamic regulatory hormones are used in diagnostic procedures and for therapy, usually modified by chemical synthesis to enhance activities (Reichlin et al. 1976). The existence of regulators of anterior pituitary function, postulated many years ago (Bargmann 1949; Scharrer and Scharrer 1954), was experimentally demonstrated for the first time by Saffran and Schally (1955) in experiments utilizing hypothalamic and neurohypophyseal extracts. TRH was the first hypothalamic hormone whose chemical structure was elucidated (Bøler et al. 1969; Schally et al. 1970). Its main use is as a diagnostic instrument in thyroid disorders, pituitary tumors, and infertility.

Cloning, characterization, and transcriptional regulation of the TRH gene are reviewed by Wilber and Xu (1998).

Pekary (1998) discussed the physiological role of TRH-enhancing peptide (Ps4) which results from the proteolytic processing of prepro-TRH.

The effects of TRH and its analogs in the CNS not related to the release of thyroid-stimulating hormone (TSH) (extrapituitary effects) have been found, indicating perhaps other therapeutic indications (Metcalf 1983; Flohé et al. 1983; Nemeroff et al. 1984; Horita et al. 1986; Horita 1998).

Radioimmunoassays for TRH are available (Bassiri et al. 1978).

Furukawa et al. (1980) reported the local effects of TRH on the isolated small intestine and tenia coli of the guinea pig. This effect could be related to the local release of catecholamines.

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TRH Receptor Binding Assays

Purpose and Rationale

Thyrotropin-releasing hormone (TRH) receptors from mouse (Straub et al. 1990; Jones et al. 1996), from rat (Sellar et al. 1993), and from men (Duthie et al. 1993; Matre et al. 1993; Yamada et al. 1993; Hinuma et al. 1994) have been expressed and characterized. Two isoforms have been identified (de la Peña et al. 1992; Lee et al. 1995). Constitutive activity of native TRH receptors has been demonstrated by Jinsi-Parimoo and Gershengorn (1997). The molecular and cellular biology of TRH receptors has been reviewed by Vassart and Dumont (1992) and by Gershengorn and Osman (1996).

TRH receptors have been localized in the pituitary gland (hypothalamic control of thyroid-stimulating hormone secretion) and in other brain regions (extrapituitary effects), which have been extensively explored but not resulted in therapeutic applications (Burt and Taylor 1983). TRH receptors can be determined by the binding of [^{3}H]MeTRH (Taylor and Burt 1981; Sharif and Burt 1983; Jarowska-Feil et al. 1995; Yamada et al. 1995) or [^{3}H](3-Me-His^2)TRH (Simasko and Horita 1982).

TRH receptor affinity methods can be applied to the investigation of molecules which might interact with these receptors (Faden et al. 2005; Colson and Gershengorn 2006) and to the quantitation of the description of receptor changes ex vivo after therapy.

Procedure

Pooled tissue samples are homogenized in a sodium phosphate buffer (20 mM, pH 7.4) and centrifuged at 30,000 *g* for 30 min. The resultant pellets are washed twice by means of resuspension and centrifugation. The washed membranes are dispersed in fresh buffer and are used for the TRH receptor binding assay.

The membranes are incubated in 250 µl of the total volume with 0.5–8 nM of [^{3}H]MeTRH (NEN, sp. act. 62.8 Ci/mmol), in the presence or absence of 10 µM of TRH for 5–6 h at 0 °C (in a water-ice bath). The receptor-bound and free [^{3}H]MeTRH is separated by rapid filtration through a glass fiber filter GF/B (Whatman) under reduced pressure (Brandel harvester). The trapped receptor-bound radioactivity is determined by liquid scintillation spectrometry (Beckman). The amount of specifically bound [^{3}H]MeTRH is expressed as fmoles per mg of protein.

Evaluation

The data are subjected to the six-point Scatchard analysis. The receptor density (B_{max}) and apparent dissociation constant (K_d) are determined.

Modifications of the Method

Sun et al. (1998) described the cloning and characterization of the chicken TRH receptor.

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Release of ^{131}I from Thyroid Glands of Mice

Purpose and Rationale

An animal bioassay method for TRH utilizes iodine-deficient mice, treated with ^{131}I, codeine, and 1 μg thyroxine (Redding et al. 1966; Bowers

et al. 1967; Redding and Schally 1969). This is a historical test based on the effect of thyroid hormone release by TRH (indirect bioassay). The rise in radioactivity in blood 2 h after injection of the TRH preparations is proportional to the effect of TSH released by TRH. It needs to be assessed whether the extract being tested is free of TSH contamination.

Procedure

Weanling mice of the Swiss Webster strain weighing 15 g are fed a low-iodine diet and given distilled water for 10 days. At time zero, 4 μC $Na^{131}I$, 1 μg thyroxine, and 1 mg codeine phosphate are injected subcutaneously. Codeine is given again subcutaneously at 24, 30, and 48 h after time zero and then 2 h after the fourth injection of codeine, blood is taken from the orbital venous sinus. The test preparation or TRH standard is injected intravenously at increasing doses (0.01, 0.03, 0.09 μg/mouse), and 2 h later, a second blood sample is taken from the orbital venous sinus. The response is obtained by the increase of radioactivity from the first to the second blood sample (Δcpm).

Evaluation

Dose–response curves are established for the test preparation and standard in order to calculate potency ratios with confidence limits.

Modifications of the Method

TRH also stimulates the release of TSH and subsequently of ^{131}I in rats pretreated with $Na^{131}I$ and 5 μg thyroxine (Yamakazi et al. 1963).

TRH increases plasma TSH levels in thyroidectomized rats pretreated with 1 μg of T_3 (Bowers et al. 1965). This assay is based on enhanced TSH secretion after eliminating the feedback of thyroid hormones. The baseline is then lowered by T_3 pretreatment to provide a wider range for stimulation by TSH. Rats weighing 350–400 g are surgically thyroidectomized. After 1–3 months, 1 μg of L-triiodothyronine Na is given intraperitoneally; 2 h later urethane is given subcutaneously; approximately 1 h later, 1.5 ml blood is removed from the jugular vein and the TRH preparation is administered intravenously. Another 1.5 ml blood is removed 15 min later. Heparin is added to the blood and TSH levels in plasma are assayed by the release of ^{131}I from the mouse thyroid (McKenzie 1958) (see chapter "► Anterior Pituitary Hormones"). Results are recorded as a mean change in blood ^{131}I levels in five mice.

TRH produces a significant depletion of pituitary TSH content in mice (Bowers et al. 1967). Swiss Webster strain mice are treated as described for the determination of blood ^{131}I radioactivity. Two hours after the administration of TRF, the animals are decapitated and the pituitaries immediately removed. The pituitaries of each group of five mice are combined and homogenized in 5 ml of 0.01 M acetic acid which contains 0.9 % NaCl. TSH is determined according to McKenzie (1958) (see "► Anterior Pituitary Hormones"). Today, this has been replaced by radioimmunoassay determination of pituitary TSH content.

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Release of TSH from Rat Anterior Pituitary Glands In Vitro

Purpose and Rationale

The in vitro bioassay method of Saffran and Schally (1955a, b), developed for detecting corticotropin-releasing factor (CRF) activity, has been modified to measure TRH activity in vitro (Guillemin et al. 1963; Bowers et al. 1965; Schally and Redding 1967).

Procedure

Male Sprague–Dawley rats weighing 150–200 g serve as donors. After removal, each pituitary is cut in half, transferred to a 15-ml beaker containing 1.5 ml Krebs–Ringer bicarbonate medium with 200 mg% glucose and incubated for three 60-min periods. The media used in the first two incubations are discarded. At the beginning of the third incubation period, various amounts of the test preparation or TRH standard are added to individual beakers. At the end of the third incubation period, the media from both control and experimental beakers are carefully freed of pituitary tissue. The media are then assayed by RIA for content of TSH.

Evaluation

Dose–response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

Modifications of the Method

For the assay of TRH analogs, cultures of enzymatically dispersed anterior pituitary cells from rats can be used instead of pituitary halves (Vale et al. 1972).

Barros et al. (1986) studied the effect of TRH on cultured GH3 rat anterior pituitary cells using the whole-cell voltage-clamp technique.

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TSH and Prolactin Release by TRH in Rats

In bioassays for TRH activity based on the release of TSH measured by radioimmunoassay, it was found in many species investigated, and also in clinical testing of humans, that the injection of TRH causes the dose-proportional release of prolactin. This test is suitable for biological activity of TRH, e.g., in domestic animals, and also for the clinical investigation of prolactin secretion in humans.

Procedure

The TRH test in rats is performed in a minimally invasive manner, and when animals have been previously treated, it is done 24 h after the last dosing on the following morning. The test dose is a subcutaneous injection of 1 μg/kg body weight synthetic TRH followed by blood sampling in unanesthetized rats 30 min later. Blood sampling may be performed by retro-orbital puncture.

For extended studies on pituitary responsiveness, it is also possible to use a very specific TRH test blocking basal TSH by the injection of levothyronine (L-T_3, 1 μg/rat s.c.) 18 h before the test. The following morning, the TRH dose is injected intravenously followed by blood sampling at 15 min or (in anesthetized rats) after 15 and 30 min.

Evaluation

The usual procedure for evaluation is to compare the TSH concentration in control animals and treated animals (drug treatment or physiological manipulation), to calculate group means, and to evaluate the significance of difference by appropriate statistical tests, where necessary by performing an analysis of variance (ANOVA).

The individual response of the animals may be assessed by comparing the basal TSH concentration before the TRH test injection and the TSH increments at increasing dose intervals after TRH stimulation.

Modifications of the Method

The TRH test is useful in rats and in other animal species. The test dose may be administered by intravenous injection or preferably by subcutaneous injection; nasal application should also be considered (a considerably higher TRH dose is required, and this test can be performed in dogs).

Several analytical methods are available to determine the serum concentrations of TSH and prolactin, both from commercial suppliers and for research applications (Faglia 1998; Markianos et al. 1996; Katznelson et al. 1998; Fail et al. 1999; Hashizume et al. 2005; Koch et al. 2006). For human diagnostic applications, highly sensitive TSH tests based on measuring only the basal concentrations have replaced the diagnostic TRH test in many instances. Methods are increasingly sensitive, and, for a review, refer to the web site "thyroidmanager" (Spencer 2004).

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Luteinizing Hormone-Releasing Hormone (LH-RH)

General Considerations

Gonadotropin secretion is controlled by the hypothalamic peptide luteinizing hormone-releasing hormone (LH-RH), which stimulates the release of FSH and LH. The discovery of the structure of LH-RH then led to the synonym of gonadotropin-releasing hormone (Gn-RH) and identification of its receptors followed (Conn et al. 1986). The search for a specific FSH-releasing factor has continued (McCann et al. 1993) but so far has remained elusive. LH-RH is secreted in a pulsatile fashion (Levine et al. 1991). This is not relevant to the bioassays, which use LH-RH injections as well as infusion for several hours. Radiolabeling and photoaffinity labeling of Gn-RH receptors have been described (Perrin et al. 1982; Hazum and Keinan 1983). LH-RH bioactivity is determined in vitro on pituitary cells and in vivo by its effects on ovulation, spermatogenesis, and other gonadal parameters (McCann 1970; Steelman 1970). Radioimmunoassays and radioreceptor assays are available (Nett and Niswender 1979). Many studies of rats and primates showed that prolonged administration of LH-RH agonists results in a decrease of LH and gonadal hormones to castrate levels (Sandow et al. 1978, 1980; Akhtar et al. 1983; Weinbauer et al. 1987, 1990).

Short- and long-term effects on pituitary–gonadal function in neonatal and adult female rats treated with gonadotropin-releasing hormones have been analyzed by Trimino et al. (1993).

The Food and Drug Administration (FDA) recommendations for preclinical testing of Gn-RH analogs, including pharmacology, pharmacokinetics, and toxicology, are published by Raheja and Jordan (1994).

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LH-RH Receptor Assays

Purpose and Rationale

LH-RH (Gn-RH) interacts with a membrane receptor which belongs to the G-protein-coupled receptor family. The LH-RH-R is encoded by a single-copy gene consisting of three exons and two introns. Consistent with its site of action, LH-RH-R mRNA has been found in the brain, pituitary, gonads, and placenta, as well as in tumor tissue and tumor cell lines (Jennes and Conn 1994). Binding to LH-RH receptors in rat pituitary membranes has been studied for LR-RH agonists and antagonists. Furthermore, the time course of downregulation of LH-RH receptors was followed (Halmos et al. 1996).

Procedure

The receptor binding of LH-RH is determined using a sensitive in vitro ligand competition assay based on the binding of radiolabeled buserelin or [D-Trp^6]LH-RH to rat anterior pituitary membrane homogenates (Halmos et al. 1993, 1996; Szöke et al. 1994). Membrane homogenates containing 40–80 μg of protein are incubated in triplicate with 60,000–75,000 cpm (≈0.15 nM) [^{125}I][d-Trp^6]LH-RH as radioligand and with increasing concentrations (10^{-12}–10^{-6} M) of nonradioactive peptides in a total volume of 150 μl of binding buffer. At the end of the incubations, 125-μl aliquots of suspension are transferred to the top of 1 ml of ice-cold binding buffer containing 1.5 % bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma). The tubes are centrifuged at 12,000 *g* for 3 min at 4 °C. Supernatants are aspirated,

and the bottoms of the tubes containing the pellet are cut off and assayed in a gamma counter. Protein concentration is determined by the method of Bradford (1976) using a BioRad protein assay kit.

Evaluation

Specific ligand-binding capacities and affinities are calculated by the computerized curve-fitting program of Munson and Rodbard (1980) as modified by McPherson (1985). To determine the types of receptor binding, dissociation constants (K_d values), and the maximal binding capacity (B_{max}), LH-RH binding data are also analyzed by the Scatchard method. Statistical significance is assessed by Duncan's new multiple range test.

Modifications of the Method

Flanagan et al. (1998) recommended ^{125}I-[His^5, Tyr^6]-Gn-RH as the radioligand for analysis of mutant Gn-RH receptors.

Perrin et al. (1982) compared the binding of radiolabeled antagonists and antagonists of gonadotropin-releasing hormone to rat anterior pituitary membrane homogenates.

Fekete et al. (1989) reviewed the role of receptors for LH-RH, somatostatin, prolactin, and epidermal growth factor in rat and human prostate cancers and in benign prostate hyperplasia.

The cloning, sequencing, and expression of the human gonadotropin-releasing hormone receptor are published by Kakar et al. (1992).

Marheineke et al. (1998) characterized the human gonadotropin-releasing hormone receptor heterologously produced using the baculovirus/insect cell and the Semliki forest virus systems.

The binding kinetics of a long-acting gonadotropin-releasing hormone antagonist to rat LH-RH receptors were studied by Li et al. (1994).

Lovejoy et al. (1995) determined the receptor binding of gonadotropin-releasing hormone analogs in bovine pituitary membrane preparations.

The cDNA encoding the receptor for LH-RH was isolated from a human pituitary cDNA library and heterologously expressed in the murine fibroblast cell line LTK$^-$ by Beckers et al. (1995).

Tsutsumi et al. (1995) investigated the role of altered receptor biosynthesis in agonist-induced receptor downregulation in αT_3-1 cells, a mouse gonadotrope cell line.

Beckers et al. (1997) characterized gonadotropin-releasing hormone analogs by a cellular luciferase reporter gene assay. The assay is based on a fusion of the c-fos immediate-early gene promoter to *Photinus pyralis* luciferase (LUC) as reporter gene, stably transfected in murine LTK$^-$ cells expressing the human Gn-RH receptor. The transcription of endogenous c-fos and fos–Luc fusion gene is transiently induced quite similarly by fetal calf serum and a superagonistic Gn-RH analog. The reporter gene was used to monitor agonist-induced signaling via the human Gn-RH receptor. Whereas Luc activity was induced in a dose-dependent manner by Gn-RH or an agonistic analog, different antagonistic peptides completely inhibited this stimulation.

The current state of knowledge is reviewed by Cheng and Leung (2005), Kakar et al. (2004), and Millar et al. (2004) for domestic animals and for the human, including the description of two separate types of the gonadotropin-releasing hormone receptor (Gn-RH-I and Gn-RH-II) and the increasing complexity of Gn-RH receptor-mediated signaling.

Critical Assessment of the Methods

There is an ever-increasing complexity of the extensive results of the application of LH-RH (Gn-RH) receptor methods, which may be due to structure–activity studies with LHRH analogs, to elucidating species-specific regulation in domestic animals and in humans, or to the measurement of adaptive changes of the LH-RH receptor concentrations in physiology, in pharmacological experiments, and in the pathophysiology of human disease. Numerous approaches have been described based on ligand binding to receptors extracted from tissue to cloned receptor proteins and based on direct measurement of the receptor protein concentrations.

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LH Release in the Ovariectomized Estrogen–Progesterone-Blocked Rat

Purpose and Rationale

Ramirez and McCann (1963) recommended the ovariectomized, estrogen–progesterone-blocked rat (OEP rat) as a highly sensitive test model for LH-releasing activity. The gonadotropin content of the pituitary is increased after ovariectomy, due to reduced feedback inhibition through the absence of gonadal steroids. Basal secretion is then acutely lowered to a stable baseline by estrogen–progesterone blockade, whereas the amplitude for the stimulation of LH secretion is augmented. The increase in plasma LH levels in donor rats after injection of LH-RH previously determined by bioassay in recipient rats is now conveniently measured by RIA. Since natural and synthetic LH-RH releases both gonadotropins (Schally et al. 1971b), merely the stimulation of LH is necessary for routine LH-RH assay.

Procedure

Female Sprague–Dawley rats weighing 150–200 are ovariectomized. They are kept in a light- and temperature-controlled animal room at 24 °C and are fed commercial rat chow and tap water ad libitum. Tests are performed 1–3 months after ovariectomy. Three days prior to the assay, the rats receive 50 μg estradiol benzoate and 25 mg progesterone in sesame oil by the transmuscular-subcutaneous route. For the assay, the rats are anesthetized by subcutaneous injection of 0.6 ml/100 g of 25 % urethane solution. Changing to a new anesthetic will require validation of the effect of that anesthetic on sensitivity of the test. Various doses of the test compound or the standard are injected intravenously into the jugular vein. After 10 min, 4–6 ml blood is withdrawn by cardiac puncture from each donor allowing the separation of 2 ml plasma.

In the original method, bioassayable LH was measured by the ovarian ascorbic acid depletion (OAAD) method according to Parlow and Reichert (1963) (see also chapter "▶ Anterior Pituitary Hormones"). Immature female Sprague–Dawley rats are injected on day 1 at 3:00 p.m. with 50 IU pregnant mares' serum gonadotropin (PMSG) in 0.2 ml saline subcutaneously. On day 3, the rats receive at 9:00 a.m. 25 IU human chorionic gonadotropin (hCG) subcutaneously. On day 7, three different doses of the standard (e.g., NIH LH-S-1, National Institutes of Health, Bethesda, Md., USA) or the 2 ml of plasma from the OEP rats is injected intravenously. Eight animals are used per group. Three hours later, the animals are sacrificed; both ovaries are prepared, weighed, and homogenized for determination of ascorbic acid content. The LH activity in OEP plasma, as measured by OAAD, is expressed in terms of NIH-LH-S16 standard.

Furthermore, LH activity in the plasma of OEP rats is measured by radioimmunoassay. OEP rats are anesthetized with 25 % urethane intraperitoneally. Blood is withdrawn by retro-orbital puncture. Then various doses of the test preparation or the standard are injected intravenously. After 10 min, blood is withdrawn by cardiac puncture. Radioimmunoassays of LH are carried out by the double-antibody method of Niswender et al. (1968).

Evaluation

Dose–response curves are established for the standard and the test preparation of LH-RH measured by bioassayable LH in OEP plasma as well as for RIA of the LH level in plasma, allowing the calculation of potency ratios with confidence limits. Furthermore, time–response curves can be established by RIA determination of plasma LH levels in OEP rats.

Modifications of the Method

Instead of OEP rats, normal Male Sprague–Dawley rats can be used for the measurement of the time course of release of LH after injection of LH-RH or LH-RH derivatives (Arimura et al. 1972). Male Sprague–Dawley rats weighing 120–150 g are anesthetized with 0.6 ml/100 g body weight of 25 % urethane solution subcutaneously. After 30 min, 0.8 ml blood is withdrawn from the jugular vein and immediately substituted by the same volume of Haemaccel solution. The LH-RH preparation is injected subcutaneously in 1 % gelatin/saline. Blood is withdrawn at hourly intervals up to 6 h, each time being substituted with Haemaccel solution. LH in plasma is determined by the double-antibody method of Niswender et al. (1968) and follicle-stimulating hormone according to the method of Daane and Parlow (1971).

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Gonadotropin Release from Anterior Pituitary Cells

Purpose and Rationale

Anterior pituitaries can be used directly, kept in culture, or used for cell lines (Mittler and Meites 1964, 1966; Mittler et al. 1970; Sandow et al. 1972b) in order to study the synthesis and release of gonadotropins in response to LH-RH.

Procedure

Female Sprague–Dawley rats weighing 100–150 g are used as donors. Each anterior pituitary is removed and cut into four to six pieces of approximately equal size. The cultures are performed in sterile disposable plastic Petri dishes each containing 3 ml medium consisting of 9 parts Difco Medium 199 and 1 part of newborn calf serum; 25 U/ml penicillin and 25 μg/ml streptomycin are added. In each dish, the explants are supported at the gas interface. An atmosphere of 95 % oxygen and 5 % carbon dioxide and a temperature of 36 °C are maintained. The opposite sides of the same pituitaries provide matched control and experimental preparations. The pituitaries are incubated for a total time of 5 days. After the first 2 days, the medium is removed and discarded. Fresh medium is then added with the LH-RH solutions. Approximately 12 h after the first change of medium and addition of LH-RH, media are removed and frozen. Fresh medium with LH-RH is again added; this procedure is repeated until six samples of medium representing the last 3 days of culture are obtained. Media are assayed for LH content by radioimmunoassay according to Niswender et al. (1968) and for FSH content according to Parlow and Reichert (1963).

Evaluation

Using various concentrations of test preparation and LH-RH standard, dose–response curves are obtained allowing the calculation of potency ratios with confidence limits.

Critical Assessment of the Method

These methods do not reflect the time course of release found in vivo but are useful for potency estimates.

Modifications of the Method

Instead of pituitary halves for the assay of LH-RH as well as for the assay of TRF and its analogs, cultures of enzymatically dispersed anterior pituitary cells from rats can be used (Vale et al. 1972; Martin and Sattler 1979).

Loughlin et al. (1981) used perifused pituitary cultures as a model for LH-RH regulation of LH secretion.

O'Connor and Lapp (1984) studied the effect of pulse frequency and duration of exposure to LH-RH in anterior pituitary cells attached to Cytodex I beads.

The functional integrity of anterior pituitary cells separated by a density gradient has been studied (Scheikl-Lenz et al. 1985).

The receptor binding ability of different agonists and antagonists of LH-RH to rat pituitary and human breast cancer membranes was studied by Fekete et al. (1989).

Vigh and Schally (1984) and Csernus and Schally (1991) described in detail a cell superfusion system consisting of a Sephadex column with dispersed pituitary cells. The LH response of anterior pituitary cells to a 3-min exposure to various concentrations of LH-RH at 30-min intervals as well as the growth hormone (GH) response to human GH-releasing hormone resulted in excellent dose–response curves. The effect of GH-releasing hormone was inhibited by somatostatin. Likewise, the effect of LH-RH was inhibited by pretreatment with LH-RH antagonists.

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Radioimmunoassay of Rat LH

Purpose and Rationale

Luteinizing hormone is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin-releasing hormone activity, a homologous assay is necessary. The reagents are

provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

Procedure

Reagents

Standard	NIH-rat-LH-RP 1
Antiserum	Rabbit-anti-rat-S 9
Tracer	^{125}I-rat-LH-I 6
Second antibody	Behring goat-anti-rabbit-gamma-globulin (Behring, Cat. No. OTOP 14/15)
Buffer	0.01 M phosphate-buffered saline pH 7.4

Standard and samples are dissolved in 1 % bovine serum albumin, tracer in 0.1 % bovine serum albumin, and antiserum in EDTA–PBS (1:350 normal rabbit serum as carrier).

Assay

Standards	0.25–62.5 ng/tube, 200 µl/tube
Antiserum	1:20,000 100 µl/tube
Tracer	Specific activity 120 µCi/µg, 8,000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum and tracer for 48 h at +4 °C, and the second antibody 1:50 (200 µl/tube) is added and incubated for 24 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline pH 7.4, the vial spun at 1,300 *g* for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma counter.

Evaluation

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

Quality Control Parameters

Limit of detection	0.36 ng/tube
Standard curve (ED_{80}–ED_{50}–ED_{20})	1.31–4.56–15.74 ng/tube
Inter-assay CV (15 assays)	20.9 %
Intra-assay CV	lt, 15 %

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Radioimmunoassay of Rat Follicle-Stimulating Hormone (FSH)

Purpose and Rationale

Follicle-stimulating hormone (FSH) is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin-releasing hormone activity, a homologous assay is necessary. The reagents and the procedure are provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

Procedure

Reagents

Standard	NIAMDD-rat-FSH-RP-1
Antiserum	Rabbit-anti-rat-FSH (NIAMDD-S-9)
Tracer	^{125}I-rat-FSH (e.g., NIAMDD-I-4)
Second antibody	Behring goat-anti-rabbit-gamma-globulin (Behring, Cat. No. OTOP 14/15)
Buffer	0.01 M-phosphate-buffered saline/0.1 % bovine serum albumin, pH 7.4

Assay

Standards	6.25–1,600 ng/tube, 200 µl/tube
Antiserum	1:2,000 100 µl/tube
Tracer	Specific activity 200 µCi/µg, 10,000 cpm in 100 µl/tube

Standards (or sample) are incubated with antiserum and tracer for 72 h at +4 °C, and the second antibody 1:50 (200 µl/tube) is added and incubated for 48 h at +4 °C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline pH 7.4, the vial spun at 1,300 g for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma counter.

Evaluation

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

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Measurement of Ascorbic Acid Depletion in Ovaries of Pseudopregnant Rats

Purpose and Rationale

The assay for LH-RH and LH-RH analogs can be performed in one step in pseudopregnant immature female rats using the biological response of the luteinized ovaries to gonadotropins released by the test compounds. Pseudopregnancy is induced in immature female Sprague–Dawley rats by treatment with gonadotropins. Numerous corpora lutea are formed after ovulation. On days 7–8 after the start of treatment, the corpora lutea are very sensitive to endogenous or exogenous gonadotropins. The stimulation of steroid synthesis, mainly progesterone, is seen, associated with dose-dependent ascorbic acid depletion in the ovaries. The activity of LH-RH and LH-RH analogs can be determined by the decrease of ovarian ascorbic acid concentration or the increase in progesterone secretion. Ascorbic acid depletion is a sensitive parameter for the endogenous gonadotropin release in the animals.

Procedure

Immature female Sprague–Dawley rats weighing 35–45 g are pretreated with 50 IU pregnant mares' serum gonadotropin (PMSG) followed by an injection of 25 IU human chorionic gonadotropin (hCG) on the third day. On day 7 or 8, they are injected intramuscularly or subcutaneously with the test preparation or the LH-RH standard in 0.1 ml 1 % gelatin/saline. Eight animals are used for each of three doses of the test preparation and standard. One hour later, the ovaries are dissected out and homogenized, and ascorbic acid is determined according to the method of Mindlin and Butler (1938) by photometry.

Evaluation

Dose–response curves of ascorbic acid depletion are obtained for the test preparation and standard allowing the calculation of potency ratios with confidence limits.

Modification of the Method

The method has been adopted as a biological assay of gonadorelin with some modifications by pharmacopeias, e.g., the British Pharmacopoeia (1988).

Critical Assessment of the Method

Classical bioassays of the kind described here are now rarely applied for standardizing LH-RH (gonadorelin) due to the complexity of the biological method. They are being replaced by analytical methods of high specificity and sensitivity, e.g., high-performance liquid chromatography (HPLC) which has been compared with the biological assay and found to be suitable for standardization (see current versions of the British Pharmacopoeia). Standard preparations for gonadorelin (LH-RH, Gn-RH) and for LH-RH analogs may be obtained from the suppliers (pharmaceutical companies); they are no longer prepared by the National Institute for Biological Standards and Control (NIBS, UK).

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Progesterone Production in Pseudopregnant Rats

Purpose and Rationale

Pseudopregnancy is induced in immature rats by pretreatment with gonadotropins. Numerous corpora lutea are formed after ovulation that are sensitive to endogenous gonadotropins released by LH-RH or LH-RH analogs on days 6–8 after treatment. By measurement of plasma progesterone, the steroidogenic activity of LH-RH analogs can be determined (Sandow et al. 1976).

Procedure

Immature female Sprague–Dawley rats weighing 35–45 g are pretreated with 50 IU pregnant mares' serum gonadotropin (PMSG) followed by an injection of 25 IU human chorionic gonadotropin (hCG) on the third day. They are injected intramuscularly with LH-RH or the LH-RH analog on days 6, 7, or 9 between 8:00 and 10:00 a.m. Eight animals are used for each of three doses of test preparation and standard. Blood samples are collected 1 h after treatment. Plasma samples of equal volume are extracted with peroxide-free diethyl ether. The ether phase containing progesterone is evaporated and the sample redissolved in BSA/phosphate buffer. Tritium-labeled progesterone and a specific antiserum against progesterone are added and incubated over a period of 24 h at 4 °C. Bound hormone and free hormone are separated by absorption on dextran-coated charcoal. The activity of the sample is determined in a scintillation cocktail containing Triton X.

Evaluation

Dose–response curves for progesterone concentrations are established for the test preparation and standard, allowing the calculation of potency ratios with confidence limits.

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Induction of Ovulation in Rabbits

Purpose and Rationale

Ovulation can be induced in mature rabbits by injection of LH-RH after initial priming. This is a modified assay for LH release, which was applied to hypothalamic extracts containing LH-RH activity (Sandow et al. 1972).

Procedure

Six unmated, mature female rabbits (Himalayan strain) weighing 1.0–1.2 kg are used for each dose of standard or test preparation. Follicular maturation is induced by eight subsequent daily

subcutaneous injections of LH-RH or the LH-RH analog to be tested in 0.2 ml 1 % gelatin/saline. Then 48 h after the last injection, ovulation is induced by administering a fourfold to tenfold higher dose of the peptide in 0.2 ml subcutaneously. After 24 h, the animals are sacrificed, the ovaries are weighed, and ovulation is determined by counting the numbers of follicles ovulated in the ovaries, as defined by local bleeding.

Evaluation

At least two doses of test preparation and standard are used in order to establish dose–response curves, allowing the calculation of potency ratios with confidence limits.

Modifications of the Method

Schröder et al. (1972) and Sandow and Hahn (1973) described changes in the ovary transplanted to the anterior eye chamber in the rabbit enabling the direct observation of ovulation. This method is also applicable to steroids and prostaglandins.

Critical Assessment of the Methods

Classical assays such as the eye–ovary transplant have provided excellent morphological information about changes of the ovary during drug treatment. They are however becoming increasingly obsolete because of other methods which are much less invasive, and their use should be considered only as an exception.

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Induction of Superovulation in Immature Rats

Purpose and Rationale

Immature female rats (24–26 days of age) do not show vaginal cycles before the onset of puberty. Treatment with pregnant mares' serum gonadotropin (PMSG) induces follicular maturation, followed by spontaneous ovulation 2 days later. Spontaneous ovulation can be blocked by barbiturates (e.g., phenobarbital) acting on the hypothalamus and is overcome by exogenous LH-RH. The rats ovulate due to the release of endogenous gonadotropins. After hypophysectomy, no ovulatory activity is observed in the absence of the pituitary (Sandow et al. 1972).

Procedure

Immature female Sprague–Dawley rats weighing 55–65 g are injected on day 1 with 10 IU PMSG subcutaneously. Following this pretreatment, the animals will ovulate spontaneously on day 3 between 2:00 and 4:00 p.m. Spontaneous ovulation is blocked by phenobarbital 4 mg/kg at 1:00 p.m. One hour later, the ovulatory peptide, dissolved in 1 % gelatin/saline, is injected intravenously. Controls are treated with 1 or 2 IU human chorionic gonadotropin (hCG); negative controls, with gelatin/saline only. Eight rats are used for control groups and various doses of LH-RH or the LH-RH analog. On the next day, the rats are sacrificed at 11:00 a.m. and the oviducts are dissected and stained with Patent Blue. The number of ova is counted under a microscope.

Evaluation

A dose-dependent increase in the number of ova per rat is observed after LH-RH due to LH release and after hCG by direct action on the ovary. The effect of LH-RH analogs is compared with the effects of hCG. Minimal effective doses for LH release can be calculated.

References and Further Reading

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Inhibition of Experimentally Induced Endometriosis

Purpose and Rationale

This is an example of a disease model in animals designed to mimic treatment of an experimentally induced pathological condition. Endometriosis-like lesions can be induced in female rats by autotransplantation of endometrium under the renal capsule (Sakata et al. 1990; Mizutani et al. 1995). This method was used to compare the effect of steroid-induced suppression by a LH-RH analog after chronic administration (pituitary inhibition).

Procedure

Under anesthesia, laparotomy is performed in 9-week-old female Sprague–Dawley rats. The left uterine horn is resected and opened by a longitudinal incision. The endometrium is dissected from the myometrium. Then a 5 × 5 mm section of the endometrium is grafted under the capsule of the left kidney of the same animal. Two weeks later, the attachment and the viability of the endometrial explant are examined by a second laparotomy; the length, width, and height of the explant are measured; and the volume is then calculated. The criterion for a viable graft is fluid accumulation around the lesion. The rate of induction of endometriosis is more than 80 %. Animals with endometriosis (with body weights of approximately 250 g) are randomly divided into treatment groups of ten animals each. Gonadotropin-releasing hormone agonists are injected at doses of 15 or 30 μg/kg subcutaneously daily for 3 weeks. A third laparotomy is performed 3 weeks after the beginning of the experiment. The presence of fluid accumulation and the size of the endometrial explant are examined. The explant is excised and fixed in 10 % formalin for histological evaluation.

Evaluation

The evaluation of treatment on the regression of lesions in experimental endometriosis is analyzed statistically with the χ^2 test.

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LH-RH Antagonistic Activity

Testosterone Suppression in Rats

Purpose and Rationale

LH-RH antagonists suppress the endogenous secretion of LH and testosterone in adult male rats after a single subcutaneous injection and after repeated dosing (Loy 1994).

Procedure

Groups of eight adult male Wistar rats weighing 200–250 g are injected subcutaneously with various doses of the test compound or a reference LH-RH antagonist dissolved in 5 % mannitol solution. Four hours later, rats are decapitated and blood is collected from the trunk. Serum is separated in a refrigerated centrifuge (10 min at 3,000 *g*) and stored frozen at −20 °C until assay.

Radioimmunoassay for Testosterone

Serum testosterone is measured by radioimmunoassay in serum extracts using a specific antiserum without prior chromatography or by direct assay in serum depending on the method. Serum samples of 0.5 ml are extracted with 2 ml of freshly purified, peroxide-free diethyl ether by shaking for 60 s on a vortex-type mixer. The aqueous phase is frozen at −70 °C, and the ether phase containing testosterone is transferred to conical test tubes and evaporated under a stream of dry nitrogen. The dry residue

is redissolved in BSA/phosphate buffer (BSA = 1 % bovine serum albumin) for RIA. [1,2,6,7-^{3}H] Testosterone (New England Nuclear NET 367) and a specific antiserum (AS-781, Behringwerke, Marburg, Germany) are added and incubated over a period of 24 h at +4 °C under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal by incubation for 30 min at +4 °C and centrifugation at 3,000 *g* for 15 min. Then 500 µl of supernatant is transferred into minivials and scintillation cocktail is added. Radioactivity is determined in a beta counter (Falvo and Nalbandov 1974).

Evaluation

The hormone levels in the sample are calculated from a standard curve by means of a computer program, using appropriate control sera. Using various doses of standard and test preparation, dose–response curves can be established, allowing the calculation of potency ratios with confidence limits. Alternatively, minimum effective doses can be calculated from comparisons with controls. Using different time intervals, e.g., 4, 8, and 24 h, the duration of the effect can be evaluated.

Modifications of the Method

Testosterone suppression can be measured in several animal species, e.g., dogs and marmoset monkeys or cynomolgus monkeys (Habenicht et al. 1990).

Ayalon et al. (1993) tested the potency of the LH-RH antagonist on the pituitary–gonadal system of female castrated and intact ovulating rats.

Reissmann et al. (1996) investigated the antitumor and hormone-suppressive effect of the LH-RH antagonist cetrorelix in the model of DMBA-induced mammary carcinoma (DMBA is 7,12-dimethylbenz[a]anthracene) in female rats and by testosterone determination in normal male rats.

Danz (1995) studied the effect of a gonadotropin-releasing hormone antagonist on androgen-binding protein production and its distribution among the epididymis, seminiferous tubule fluid, testicular interstitial fluid, and blood in rats.

Fallest et al. (1995) studied the transcriptional regulation of the rat luteinizing hormone β (rLHβ) gene through the use of **transgenic mice** bearing a region of the rLHβ gene linked to a luciferase (LUC) reporter gene. The postgonadectomy rise in pituitary rLHβLUC activity in females and males was blocked by daily administration of the Gn-RH antagonist antide.

Rivier et al. (1996) synthesized and evaluated many Gn-RH analogs and established a dose–response relationship between Gn-RH antagonists and pituitary suppression.

Critical Assessment of the Method

Structure–activity studies on LH-RH antagonists are based on screening for receptor affinity and receptor-associated signaling for high-throughput assays. They need to be followed up by confirmation of biological activity, and for the quantitation of structure–activity assessment, the assays described here have been eminently suitable for extended compound screening, although the estimates obtained need to be checked for their relevance against the results from intact animals. Suppression of testosterone secretion is one of the preferred methods of confirmation.

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Antiovulatory Activity in Rats

Purpose and Rationale

The rat antiovulatory assay (AOA) was widely used for LH-RH antagonists. Immature female rats (24–26 days of age) are primed with pregnant mares' serum gonadotropin (PMSG) to induce follicular maturation, followed by spontaneous ovulation 2 days later. The endogenous hypothalamic LH-RH discharge can be blocked by barbiturates (e.g., phenobarbital) to prevent spontaneous ovulation (see section "Induction of Superovulation in Immature Rats"). For antiovulatory activity, a test dose of 800 ng exogenous LH-RH is administered together with increasing doses of the LH-RH antagonist. The antagonist will then inhibit the LHRH-induced gonadotropin release. The test measures the dose of LH-RH antagonist required to inhibit the effect of a standard dose of LH-RH.

Procedure

Immature female Wistar rats weighing 55–65 g are injected on day 1 at 9:00 a.m. with 10 IU PMSG subcutaneously. This priming induces follicular maturation and estradiol secretion. A spontaneous, endogenous LH discharge is observed on day 3 between 2:00 and 4:00 p.m. The release of LH and spontaneous ovulation is blocked by intraperitoneal injection of phenobarbital 4 mg/kg on day 3 at 1:00 p.m. The LH-RH antagonist is administered in various doses subcutaneously, intraperitoneally, or intravenously 30 min before phenobarbital injection. Two hours later, a standard dose of 800 ng LH-RH is injected subcutaneously to induce ovulation. Control groups receive phenobarbital only (negative control) or phenobarbital and 800 ng LH-RH (positive control). Six to eight rats are used for control groups and various doses of the LH-RH antagonist. On the next day, the rats are sacrificed at 9:00 a.m. Both ovaries are prepared and weighed. The oviducts are dissected and stained with Patent Blue. The number of ova is counted under a microscope. An effective dose of antagonist prevents LH-RH-induced ovulation.

Evaluation

A dose-dependent suppression of induced ovulation is observed after the LH-RH antagonist. ID_{50} values can be calculated for various LH-RH antagonists according to the procedure of Litchfield–Wilcoxon. Minimal effective doses which fully suppress ovulation in each animal are calculated.

Modifications of the Method

De la Cruz et al. (1975) described the blockade of the preovulatory LH surge in **hamsters** by an inhibitory analog of LH-RH.

Kovács et al. (1993) found that in ovariectomized and normally cycling rats, antiovulatory doses of antagonists of LH-RH inhibit LH and progesterone but not follicle-stimulating hormone (FSH) and estradiol release.

Evaluation of biological activities of new LH-RH antagonists in male and female rats was reported by Pinski et al. (1993).

Rivier et al. (1995) tested a series of gonadotropin-releasing hormone antagonists in the rat antiovulatory assay.

Pinski et al. (1995) evaluated the optical isomers of the LH-RH antagonist cetrorelix in the

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Effect of Repeated Administration of LH-RH Antagonists in Rats

Purpose and Rationale

Repeated administration of LH-RH antagonists reduces the testosterone secretion in serum and the tissue content in the testes, and it also decreases the weight of testosterone-dependent organs, such as prostate and seminal vesicles. Moreover, pituitary LH content is decreased and the secretory capacity of the testes for testosterone is diminished. The effects are similar to those after

supraphysiological doses of LH-RH agonists (paradoxical antifertility effects).

Procedure

Male Wistar rats with an initial weight between 150 and 200 g are housed under standard conditions. They are treated with daily injections of the LH-RH antagonist for a period of 7 days up to 4 weeks. Alternatively, the LH-RH antagonist can be administered subcutaneously by infusion via minipumps or other routes of administration. Animals receiving the vehicle serve as controls, and rats castrated at the beginning of the experiment may be included for maximum inhibition. At the end of the treatment period, the animals are sacrificed 4 h after the last administration. Blood is collected for testosterone determination by radioimmunoassay. The androgen-dependent organs, testes, epididymis, ventral prostate, and seminal vesicles are dissected and weighed to the nearest 0.1 mg. The testes are decapsulated and incubated with 250 mU human chorionic gonadotropin (hCG) in order to determine the secretory capacity for testosterone. The testosterone tissue content is determined in the supernatant fraction of a testicular homogenate in the absence of hCG (unstimulated testis) and after 3 h of incubation of the contralateral testis with hCG (stimulated testis). Pituitary glands are dissected and the anterior lobe is frozen at −20 °C for the determination of LH by a rat-specific radioimmunoassay (section "Radioimmunoassay of Rat LH"). LH receptors in anterior pituitary homogenate are measured by the binding of ^{125}I-buserelin in vitro.

Evaluation

The effects of various doses of the LH-RH antagonist after various time intervals on the different parameters mentioned above are compared with values from intact controls and castrated animals. Significant differences compared to controls at the 95 % level are calculated by Dunnett's test.

Modifications of the Method

These are mainly models for contraceptive applications and tumor suppression using LH-RH antagonists.

Kangasniemi et al. (1996) used a combined treatment with a Gn-RH antagonist and an antiandrogen (flutamide) to suppress spermatogenesis in mice. Despite this effect, the treatment did not enhance recovery from spermatogenesis produced by a 10-Gy dose of radiation.

Sinha-Hikim and Swerdloff (1993, 1994) examined the time course of suppression and recovery of spermatogenesis and its relationship to the temporal changes in circulating levels of gonadotropin and testosterone and intratesticular testosterone levels after cessation of treatment with a potent Gn-RH antagonist.

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Inhibition of Gonadotropin Release from Anterior Pituitary Cultures

Purpose and Rationale

The same methods as described for studying the stimulation of gonadotropin release by LH-RH agonists (sections "LH Release in the Ovariectomized Estrogen–Progesterone-Blocked Rat" and "Gonadotropin Release from Anterior Pituitary Cells") can be used for the assessment of LH-RH antagonists.

Procedure

Anterior pituitaries of young adult Sprague–Dawley rats are digested with collagenase for 1 h followed by a mechanical dispersion. The resulting cell suspension from 1.5 pituitaries, containing mostly small clusters of cells, is then sedimented together with a suspension of Sephadex G-10 (Sigma) and packed into 6.6-mm columns. Tissue culture Medium 199 (Sigma) with supplements, equilibrated with 95 % air/5 % carbon dioxide, is perfused through the columns at a flow rate of 0.33 ml/min. After an overnight recovery period, during which the baseline stabilizes and the cells regain their full responsiveness, the samples to be tested are introduced through a four-way valve. During a 9-h experimental period, 180 1-ml fractions are collected. The system is standardized with 3-min exposures to 100 mM potassium chloride or 3 nM LH-RH. The compounds are introduced in various concentrations, generally for 3–9 min (time of one to three fractions), at 30-min intervals. Rat LH levels are measured from aliquots (50 µl) of the collected medium effluent by radioimmunoassay. As a standard, rat LH-RP2 reference standard is used. Repeated stimulation with 3 nM LH-RH for 3 min at 30-min intervals results in a pulsatile LH release.

Evaluation

The suppression of pulsatile LH release due to repeated administration of LH-RH by antagonists is evaluated.

Modifications of the Method

Krummen et al. (1991) assessed the direct effects of testosterone in primary cultures of pituitary cells.

A special long-term superfusion system was developed by Rékási et al. (1993) in order to evaluate the effects of cytotoxic compounds linked to LH-RH analogs on different types of rat pituitary cells. LH, growth hormone, and prolactin were determined simultaneously in the effluent.

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Antitumor Effect of LH-RH Antagonists

Purpose and Rationale

LR-RH antagonists were found to inhibit experimental tumors in rats and mice.

Procedure

To induce mammary carcinomas, female rats are each given a single dose of DMBA (dimethylbenzanthracene) at the age of 50 days. The first mammary tumors can be detected 20–30 days later. The tumor weight is determined by palpation, comparing the volume of each tumor to that of preformed Plasticine models. The tumor weight is calculated by multiplication of the model weight by a factor which takes account of the specific weights of Plasticine and tumor tissue. After the total tumor mass per animal has reached about 1 g, the animals are randomly divided into treatment groups. At least eight animals are used per group. The rats are treated for 3 weeks with different subcutaneous doses of LH-RH antagonists or vehicle. The experiment is terminated at the end of the 3rd week from the initiation of the experiment. Histological examination of the tumors is performed.

Evaluation

The change in tumor volume is calculated on the basis of individual responses. For the determination of the mitotic index, 4,000 cells are considered in each tumor.

Modifications of the Method

Reduction of tumor weight in female BDF_1 mice bearing MXT mammary adenocarcinomas after treatment for 3 weeks with a LH-RH antagonist was reported by Szende et al. (1990).

Inhibition of MIA PaCa-2 human pancreatic xenografts in nude mice by a LH-RH antagonist was reported by Radulovic et al. (1993).

The involvement of insulin-like growth factors in growth regulation of the Ishikawa endometrial tumor cell line and the possible interference by LH-RH analogs was evaluated by Kleinman et al. (1993).

Pinski et al. (1994) investigated the effects of treatment with a LH-RH antagonist and a LH-RH agonist in Copenhagen rats bearing the anaplastic androgen-independent Dunning R-3327-AT-1 prostatic adenocarcinoma implanted orthotopically into the ventral lobes of the prostate glands.

Vincze et al. (1994) tested the antitumor effect of the gonadotropin-releasing hormone antagonist MI-1544 in vitro on human breast cancer lines and in vivo on xenografts in immunosuppressed mice.

Manetta et al. (1995) reported the in vitro and in vivo inhibitory effects of a LH-RH antagonist against a panel of human ovarian carcinomas.

The effects of LH-RH and a LH-RH antagonist on cell growth and production of hCG and cAMP in JAR human choriocarcinoma cells were examined in vitro by Horváth et al. (1995).

Critical Assessment of the Method

In many instances, research is now based on assessing and confirming the antiapoptotic

activity of tumor-inhibiting compounds, e.g., methods based on thymidine incorporation to assess tissue proliferation may be considered. In the case of LH-RH antagonists, the use of biological systems based on transplanted tumors in animals reflects the suppression of pituitary hormone release followed by reduced secretion of gonadal steroids. Testing the effect on experimental tumors in animals is a recommended procedure for obtaining confirmation of the clinical potential of application.

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Corticotropin-Releasing Hormone (CRH)

General Considerations

A hypothalamic factor inducing the release of corticotropin (ACTH) from pituitaries was the first of the hypothalamic releasing hormones identified using in vitro bioassays (Saffran and Schally 1955; Guillemin and Rosenberg 1955). A common preprohormone, opiomelanocortin, was identified as the source of corticotropins and endorphins. Much later, the structure of a 41-residue ovine hypothalamic peptide stimulating the secretion of corticotropin and β-endorphin was identified (Vale et al. 1981), followed by elucidation of the structure of human CRH (Shibahara et al. 1983) and of other species, such as porcine corticotropin-releasing factor (CRF; Patthy et al. 1985) and equine CRF (Livesey et al. 1991). Reviews are given by Brodish (1979), Rivier and Plotsky (1986), Taylor and Fishman (1988), Vigh et al. (1982), and Koob (1999). Conformational differences between ovine and human CRH were detected using circular dichroism, Fourier transform infrared spectroscopy, nuclear magnetic resonance (NMR), and dynamic light scattering (Dahte et al. 1996).

The binding sites of immunoreactive CRH in the rat ovary and its potential physiological role were studied by Mastorakos et al. (1993).

Studies on CRF receptors in the pituitary have been performed (Wynn et al. 1983; Millan

et al. 1987). Investigations into the general pharmacological properties of human CRH did not reveal any considerable side effects (Andoh et al. 1994).

Derivatives with agonistic and antagonistic properties have been synthesized and tested (Rivier et al. 1984; Kornreich et al. 1992; Chen et al. 1996; Schulz et al. 1996; Webster et al. 1996; Arai et al. 1998).

The role of CRH in inflammatory processes was investigated by Webster et al. (1998).

Nonmammalian peptides sauvagine (from frog) and **urotensin** 1 (from fish) have approximately 50 % sequence homology with CRF and share in vitro and physiological actions characteristic of CRF (Rivier et al. 1983). A mammalian urotensin-like peptide (named **urocortin**) with partial sequence identity with urotensin 1 and CRF has been identified in rat (Vaughn et al. 1995) and human (Donaldson et al. 1995) tissues. The mouse and human urocortin genes have been isolated and characterized (Zhao et al. 1998).

A **corticotropin-releasing factor binding** *protein* (*CRFBP*) was isolated and is thought to be an important modulator of CRF in both the CNS and the periphery (Behan et al. 1995; Petraglia et al. 1996; Cortright et al. 1997; Hobel et al. 1999). CRFBP levels rise at 30–35 weeks of pregnancy and dramatically decrease at 38–40 weeks. In vitro, CRFBP inhibits the ACTH-releasing properties of CRF.

Cortright et al. (1995) described a mouse brain CRH-binding protein (CRH-BP) highly homologous to human and rat CRH-BPs but distinct from the CRH receptor.

Stenzel-Poore et al. (1996) described **transgenic mice** with CRH overproduction that induces behavioral changes that parallel the stress syndrome. Van Gaalen et al. (2002, 2003) reported anxiety-like behavior and reduced attention in mice overproducing CRH in an operant five-choice serial task, which taxes sustained and divided attention.

Bale et al. (2002) found that mice deficient for both CRF receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior.

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Central Effects and Actions of CRH

In some contrast to the initial conception of a hypothalamic factor regulating adrenocortical function, research on CRH, related CRF peptides, and nonpeptidic compounds interacting with the CRH receptors has established for CRH both a central role and important peripheral effects. In keeping with knowledge about the effects of neurohypophyseal hormones and steroids on brain functions, memory and learning, a similar role has been established for CRH (Croiset et al. 2000). In the process of establishing these functions, the presence of CRF receptors in the central nervous system and other tissues has been a starting point for investigations (reviewed by De Souza 1995; Perrin and Vale 1999; Eckart et al. 2002). As in other areas, specific animal models are now focusing on mutant mice for the prediction and confirmation of function (Contarino et al. 1999). Bruijnzeel and Gold (2005) have reviewed the role of CRF-like peptides in various forms of drug dependence, both to elucidate mechanisms and to explore potential therapies (Chatzaki et al. 2006). In keeping with the functional effect on the pituitary–adrenal system, involvement of CRH in the stress response has been addressed by Smith and Vale (2006), and the synthesis of CRF receptor antagonists may offer therapeutic potential in central disorders related to anxiety, psychiatric disorders, stress, and drug dependence (Valdez 2006).

Effects on the gastrointestinal tract of CRH have been described by several groups (Martinez and Tache 2006, 2007; Tache and Bonaz 2007).

Vergoni and Bertolini (2000) and Doyon et al. (2004) have suggested a functional role in the central regulation of appetite and body weight, by interaction with the melanocortin system.

Based on its interaction with CRH receptors, indications for modulation of synaptic transmission have also been found with CRH (Orozco-Cabal et al. 2006), which has parallels with the newly discovered functional role of LH-RH (GnRH),. Cardiovascular effects have been described and assigned to CRH receptors (Nazarloo et al. 2006). The existence of a CRH-like placental corticotropin-releasing factor

has been confirmed even though the functional role remains elusive (Fadalti et al. 2000).

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In Vitro Assay for CRH Activity

Purpose and Rationale

The first in vitro assay procedures to detect CRF activity (Saffran and Schally 1955; Saffran et al. 1955) were based on incubation of rat anterior pituitary halves with the test substances in bicarbonate-buffered, oxygenated medium at 38 °C for 1 h in the presence of 0.0004 M DL-arterenol and 0.004 M ascorbate followed by measurement of ACTH release into the medium by incubation with adrenal tissue and determination of corticosterone (a two-stage bioassay where a biological product of CRH action is determined by a quantitative biological response). Guillemin and Rosenberg (1955) used pituitary tissue culture from rat and dog and determined ACTH activity in the medium by the in vivo ascorbic acid depletion method in the rat according to Sayers (1954) (see chapter "▶ Anterior Pituitary Hormones"). The assay was later considerably simplified by using ACTH radioimmunoassay (RIA). Several modifications of these procedures have been used (Schally et al. 1968; Yasuda et al. 1982). Most investigators used rat pituitary cell cultures

(Giguère and Labrie 1982; Giguère et al. 1982; Aguilera et al. 1983; Bilezikjan and Vale 1983; Vale et al. 1983a, b; Patthy et al. 1985), while pituitary segments have been used by Antoni et al. (1983) and by Widmaier and Dallman (1984).

Procedure

Anterior pituitaries are obtained from adult Sprague–Dawley rats and are cut into small pieces and incubated in a Dubnoff incubator for 45 min at 37 °C in 10 ml of oxygenated Medium 199 (GIBCO) containing 0.5 % collagenase, 0.25 % BSA, and 50 µg/ml gentamicin. The fragments can be easily dispersed mechanically into single cells by repeated suction and expulsion from a pipette. The cell suspension is centrifuged at room temperature for 10 min at 100 *g*. The cell pellet is then resuspended in 1.0 ml of medium and divided into four equal volumes. Each volume (containing about 5×10^6 cells) is mixed with 0.5 ml Sephadex G-15 which has been equilibrated previously with oxygenated medium. The mixture of pituitary cells and Sephadex is transferred into four chambers of a superfusion apparatus (Vigh and Schally 1984; Evans et al. 1988; Czernus and Schally 1991) consisting of a number of 1-ml plastic syringe barrels (modified by cutting off their distal end) mounted vertically in a Plexiglas holder which is kept at 37 °C by circulating water. Each barrel is fitted with plungers at both ends. Holes are drilled in the plungers to accommodate plastic tubing. The lower plunger is covered with a small piece of 30-µm-pore nylon net to keep the Sephadex beads from escaping. The "pores" between the beads are small enough to prevent the pituitary cells from escaping and large enough to allow unrestricted flow of medium through the column. The upper plunger tubing is used for directing the flow through the chamber from the medium reservoir. The flow through the system is controlled by a multichannel peristaltic pump which is placed after the superfusion chamber. Thus, the system is operated by suction with negative pressure.

The cells are perfused overnight with Medium 199 (Gibco) at a flow rate of 20 ml/h. The samples are administered every 30 min for 3 min and 1-ml fractions are collected at 3-min intervals. Synthetic CRF at 0.2 and 2 mM is used as standard. The corticotropin released into the medium is measured by a specific RIA for corticotropin.

Evaluation

Dose–response curves are established for test preparation and standard allowing the calculation of potency ratios with confidence limits.

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In Vivo Bioassay of CRH Activity

Purpose and Rationale

Munson and Briggs (1955) described an assay using rats pretreated with morphine and pentobarbital. Many modifications for a reliable in vivo bioassay of CRH activity have been tested including rats with hypothalamic lesions (Schally et al. 1968; Yasuda et al. 1982). Arimura et al. (1967) described an assay for corticotropin-releasing factor (CRF) using rats treated with morphine, chlorpromazine, dexamethasone, and pentobarbital. This assay has been further improved by Graf et al. (1985).

Procedure

Male Sprague–Dawley rats weighing about 200 g are maintained on a 12:12 h light:dark cycle at constant temperature for at least 5 days before use. On the day of the experiment, the rats are prepared with an initial single subcutaneous injection of a mixture of chlorpromazine (10 mg/kg) and morphine sulfate (20 mg/kg) followed after 75 min by pentobarbital (25 mg/kg) intraperitoneally. Then 60 min after the injection of pentobarbital, various doses of test substances or standard or vehicle (0.9 % NaCl, 1 % BSA, 0.1 % ascorbic acid) are injected into an exposed jugular vein. Blood samples (0.5 ml) are collected in heparinized syringes immediately before and 30 min after injection of test substances. The blood is transferred to chilled polypropylene tubes containing EDTA (1.0 mg) and aprotinin (Trasylol, 50 μl) and centrifuged (2,000 *g*, 15 min 4 °C). Plasma samples are stored at −20 °C for corticosterone assay using a cross-reacting cortisol RIA that also measures corticosterone, the main corticoid secreted by rats, or a corticosterone RIA.

Evaluation

Data for CRH activity of experimental groups are evaluated by analysis of variance or covariance followed by Duncan's multiple range test.

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Collection of Hypophyseal Portal Blood in Rats

Purpose and Rationale

Worthington (1966), Porter and Smith (1967), Gibbs (1984), and Sarkar and Minami (1991) described methods to collect hypophyseal portal blood in rats. These methods can be used to measure the changes in secretion of several hypothalamic hormones under the influence of various factors.

Gibbs and Vale (1982), Gibbs (1985a), and Plotsky and Sawchenko (1987) studied the effect of various conditions on hypophyseal portal plasma levels of corticotropin-releasing factor (CRF) and arginine vasopressin.

Luteinizing hormone-releasing hormone (LH-RH) was determined by Ching (1982), Sarkar (1987), and Petraglia et al. (1987); thyrotropin-releasing hormone (TRH) by Fink et al. (1982); growth-hormone-releasing hormone (GH-RH) by Plotsky and Vale (1985); β-endorphin by Sarkar and Yen (1985); neuropeptide Y by Sutton et al. (1988); vasoactive intestinal peptide by Sarkar (1989); vasopressin and oxytocin by Gibbs (1984); and dopamine and epinephrine by Ben-Jonathan et al. (1977) and Gibbs (1985b).

Procedure

Pentobarbital-anesthetized rats are placed on an isothermal heating pad in a stereotactic instrument. After cannulation of the trachea, the animals are mechanically ventilated. The base of the skull is exposed by a transpharyngeal approach, and the basisphenoid bone is drilled away. The dura is cut and deflected, the rat is heparinized, and, after the stalk is cut, a polyethylene cannula filled with saline is placed over the hypophyseal stalk for collection of blood. The cannula leads to an ice bath, and the distal end of the tubing ends in a fraction collector where portal blood is collected by gravity flow at a rate of about 10 µl/min.

Evaluation

Portal plasma concentrations of CRH, LH-RH, TRH, GH-RH, β-endorphin, neuropeptide Y, vasoactive intestinal peptide, vasopressin and oxytocin, and catecholamines were determined after various treatments and compared with control periods of spontaneous secretion.

Modifications of the Method

Cowell et al. (1991) described in vitro models for the examination of the mechanisms controlling the secretion of hypothalamic hormones.

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CRH Receptor Determination

Purpose and Rationale

CRH mediates its effects through high-affinity receptors identified and characterized by radioreceptor studies. Cellular signaling was measured by stimulation of adenylyl cyclase in membrane fractions of rat brain, pituitary, and spleen (Grigoriadis et al. 1993; Hauger and Aguilera 1993). Cloning studies indicate the existence of at least two types of mammalian receptor (De Souza et al. 1998; Steckler and Holsboer 1999; Dautzenberg and Hauger 2002). The CRF_1 receptor has been cloned from several species including human (Chen et al. 1993; Castro et al. 1996; Di Blasio et al. 1997; Grammatopoulos and Hillhouse 1998), mouse (Vita et al. 1993), and rat (Perrin et al. 1993). Species homologies are 98 % identical over the full length of 415 amino acids.

There are three isoforms of the CRF_2 receptor: the $CRF_{2(a)}$ receptor, which has been cloned from both rat (Lovenberg et al. 1995) and human (Liaw et al. 1996), is a 411-amino-acid protein which shares approximately 70 % identity with the CRF_1 receptor; the $CRF_{2(b)}$ receptor isoform

has been cloned from rat (Lovenberg et al. 1995), mouse (Stenzel et al. 1995), and human (Kostich et al. 1996) and is 431 amino acids in length, differing from the $CRF_{2(a)}$ receptor in that the first 34 amino acids in the N-terminus are replaced by 54 different amino acids; the $CRF_{2(c)}$ receptor has been identified in human brain (Sperle et al. 1997; Kostich et al. 1998).

[^{125}I]Sauvagine binding to CRH_2 receptors was characterized by Rominger et al. (1998).

CRH in plasma is mostly bound to the corticotropin-releasing hormone-binding protein (CRH-BP) and is therefore inactive except for the free fraction. CRH-BP is predominantly produced by the liver and distributed and expressed differently from the CRH receptors (Cortright et al. 1995; Zhao et al. 1997).

Rhode et al. (1996) used whole brains of Wistar rats for the CRH receptor assay.

Procedure

Whole brains of male Wistar rats weighing 220–250 *g* are homogenized with a Teflon-glass homogenizer (10 strokes at 800 rpm) in 0.32 M sucrose, 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, and 0.15 mM bacitracin at 50 mg wet weight per milliliter. After centrifugation at 1,000 *g* for 5 min, the supernatant is centrifuged at 26,000 *g* for 20 min. The pellet is resuspended in 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, adding 0.15 mM bacitracin and 0.0015 % aprotinin (assay buffer), and again centrifuged. The pellet is suspended in an assay buffer containing 0.32 M sucrose and stored at −20 °C. All steps are carried out at 4 °C. Protein concentrations are determined by the method of Bradford (1976) using BSA as standard.

Then, 100 µg of membrane preparation in 300 µl assay buffer is incubated in quadruplicate with 0.1 nM [^{125}I]Tyr-oCRH in the absence and presence of 12 different concentrations (0.2 nM up to 1 µM) of unlabeled peptides at 25 °C for 2 h. Nonspecific tracer binding is determined in the presence of 1 µM ovine CRH (oCRH). At the end of incubation,3 ml of ice-cold assay buffer without inhibitors (containing 0.01 % Triton X-100) is added to the assay tube and the samples are immediately filtered through GF/C filter disks (Whatman), presoaked for 2 h in 0.1 % polyethylenimine using a Brandel harvester. The incubation tubes and filters are then washed with 3 ml cold washing buffer. Triton X-100 in this buffer strongly reduces nonspecific tracer binding. Radioactivity retained on the filter is measured by gamma counting.

Evaluation

Receptor affinities (K_{ass}, $K_d = 1/K_{ass}$) and capacities (B_{max}) are estimated using the nonlinear least-squares curve-fitting program RADLIG (BIOSOFT, Cambridge, UK) and a K_d of 0.48 nM for the binding of the tracer peptide as determined from tracer saturation curves.

Modifications of the Method

Schulz et al. (1996) used P2 membranes from human neuroblastoma IMR32 cells to test the receptor binding of a selective nonpeptide antagonist of corticotropin-releasing factor (CRF).

Webster et al. (1996) used rat frontal cortex, pituitary, cerebellum, and heart tissue for in vitro characterization of antalarmin, a nonpeptide CRH receptor antagonist.

Grigoriadis et al. (1996) recommended ^{125}I-tyro-sauvagine as a high-affinity radioligand for the pharmacological and biochemical study of human CRF 2α receptors.

Differences between normal and adenomatous pituitary corticotrophs in the labeling characteristics of CRH receptors were reported by Abs et al. (1997).

Rabadan-Diehl et al. (1997) studied the role of glucocorticoids and hypothalamic factors in the regulation of pituitary CRH receptor mRNA and CRH during adrenalectomy in rats.

The differential regulation of hypothalamic pituitary CRH receptors during the development of adjuvant-induced arthritis in rats was reported by Aguilera et al. (1997).

Grammatopoulos and Hillhouse (1998) reported the solubilization and biochemical characterization of the human myometrial CRH receptor.

The interaction between glucocorticoids and CRH in the regulation of the pituitary CRH receptor has been studied in rats by Ochedalski et al. (1998).

The multiple actions of CRF on neuroendocrine and behavioral functions were examined using high-affinity, nonpeptide antagonists (Lundkvist et al. 1996).

Wei et al. (1998) described analogs of CRH and urocortin with selective activity at CRH_1 and $CRH_{2\beta}$ receptors.

Perrin et al. (1999) used an agonist, urocortin, and an antagonist, astressin, as radioligands for the characterization of CRF receptors.

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CRF Receptor Antagonists

Purpose and Rationale

Studies with CRF antagonists indicate a role for CRF in certain psychiatric diseases and drug addiction (Koob 1999). This is in line with the known clinical psychotropic effects of corticotropin and glucocorticoids at elevated doses.

Various truncations of agonist peptides with deletions of the first 8–11 amino-terminal amino acid residues have resulted in potent peptide antagonists (Rivier et al. 1984; Hernandez et al. 1993; Miranda et al. 1994; Schulz et al. 1996). Several nonpeptide CRF receptor antagonists have been described (Griebel et al. 1998; Okuyama et al. 1999; Habib et al. 2000; Gully et al. 2002; Griebel et al. 2002; Heinrichs et al. 2002; Grigoriadis 2003; Gutman et al. 2003). Receptor autoradiography was performed in treated rats by Heinrichs et al. (2002) and Gutman et al. (2003).

Procedure

Brains from treated rats are sectioned at the level of the prefrontal cortex and lateral septum to determine CRF_1 and CRF_2 receptor binding, respectively. The prefrontal cortex has been shown via in situ hybridization to express predominantly CRF_1 receptors, whereas the lateral septum expresses CRF_2 receptors in rodents (Van Pett et al. 2000). After the behavior experiments, 15-μm-thick rat brain sections containing the prefrontal cortex and the lateral septum are sectioned at approximately −20 °C, mounted on Superfrost Plus slides and stored at −80 °C until the assay. Ex vivo CRF receptor autoradiography is performed on 15-μm-thick rat brain sections. Sections are removed from the −80 °C freezer and allowed to warm up to room temperature in a desiccator. Brain sections are fixed for 2 min in 0.1 % paraformaldehyde, pH 7.5, followed by a 15-min incubation in assay buffer (50 mM Tris, 10 mM $MgCl_2$, 2 mM EGTA, 0.1 % bovine serum albumin, 0.1 mM bacitracin, and 0.1 % aprotinin, pH 7.5). Triplicate slides containing adjacent brain sections are incubated in one of three conditions:

1. 0.1 nM radiolabeled ^{125}I-sauvagine to determine total binding at both the CRF_1 and CRF_2 receptor subtypes
2. 0.1 nM radiolabeled ^{125}I-sauvagine + 1 μM of a specific CRF_1 receptor antagonist to be tested, to determine CRF_2 receptor-specific binding
3. 0.1 nM radiolabeled ^{125}I-sauvagine + 1 μM unlabeled sauvagine to determine nonspecific binding

After a 2-h incubation, unbound radioligand is removed by two 5-min rinses in ice-cold phosphate-buffered saline + 1 % bovine serum albumin on a rotating platform at 60 rpm, followed by two brief dips in ice-cold distilled, deionized H_2O. Slides are then rapidly dried using a cold-air blow dryer and exposed to Kodak Biomax MR film with ^{125}I-microscale standards (Amersham Biosciences) for 80–90 h.

Images from the receptor autoradiography films are digitized with a CCD-72 (Dage-MTI, Michigan City, Ind., USA) image analysis system equipped with a Nikon camera. Semiquantitative analysis is performed using AIS software (version 4.0, Imaging Research, St. Catharines, ON, Canada). Optical densities are calibrated against co-exposed ^{125}I-microscale standards and expressed in terms of nanocuries per gram of tissue equivalent.

Evaluation

CRF_1 receptor-specific binding is calculated as total binding minus CRF_2 receptor binding, and CRF_2 receptor-specific binding is calculated as CRF_2 receptor binding minus nonspecific binding. Receptor binding data are expressed as a percentage of the control values. The percentage of binding is subtracted from 100 to estimate percentage of occupancy by the antagonist relative to vehicle-treated rats. In all cases, three to four sections are matched at the rostrocaudal level and used to produce a single value for each animal. Significant differences are evaluated by one- or two-tailed *t* tests or one-way ANOVA followed by Student–Newman–Keuls post hoc analysis.

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Growth-Hormone-Releasing Hormone (GH-RH)

General Considerations

The discovery of the growth-hormone-releasing hormone (GH-RH) by physiological experiments and the isolation and purification of the hormonal principle are reviewed elsewhere (Reichlin et al. 1976; Krulich and Fawcett 1977; Ling et al. 1985; Frohman and Jansson 1986; Gelato and Merriam 1986; Grossman et al. 1986; Thorner et al. 1986; Frohman et al. 1986; Muller 1987; Vance 1990). The initial studies on analogs of GH-RH raised expectations that therapy by GH injections might be replaced by GH-RH therapy (Dean and Friesen 1986). It was however found that infusions and high-dose treatment with GH-RH reduced to the response of GH release, in a similar manner as described for the gonadotropin receptors, due to receptor adaptation (Catt et al. 1980).

The comparative endocrinology of hypothalamic control of GH secretion by GH-RH and the presence of GH-RH receptors in animal species have been explored for mammalian and nonmammalian animal species (Ukena et al. 2006; Lee et al. 2007).

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Radioreceptor Assay of Growth Hormone-Releasing Hormone

Purpose and Rationale

As for other polypeptide hormones, GH-RH receptors may be used for quantification and potency estimates of relative binding affinities. Several agonistic and antagonistic analogs of

human GH-RH (hGH-RH) have been synthesized by various groups of investigators. The clinical and basic aspects of GH-releasing peptides are reviewed by Argente et al. (1996).

In vitro assays with pituitary cells have been used for screening the biological activity of hGH-RH analogs prior to their evaluation in vivo (Heiman et al. 1985; Kovacs et al. 1988; Campbell et al. 1991). A careful determination of binding activities of the peptides to specific GH-RH receptors provides important data for the design of more active analogs. Several studies demonstrated that the radioligand, [His^1-, ^{125}I-Tyr^{10}, Nle^{27}]-hGH-RH(1-32)NH_2, binds specifically to a single class of receptors in rat pituitary cells and homogenates and can be used for radioreceptor assay (Campbell et al. 1991; Seifert et al. 1985a, b; Struthers et al. 1989). The use of a radioreceptor assay for in vitro screening of analogs of GH-RH was described by Halmos et al. (1993).

Procedure

Pituitaries from male Sprague–Dawley rats (250–300 g) are used to prepare crude membranes. Immediately after decapitation, anterior pituitaries are removed, rinsed with cold saline, and homogenized in five times their volume of homogenization buffer (50 mM Tris–HCl, 5 mM EDTA, 5 mM $MgCl_2$, 30 μg/ml bacitracin, pH 7.4) on ice using an Ultra-Turrax homogenizer at maximal speed. The homogenate is centrifuged at 500 *g* for 10 min at 4 °C. The supernatant containing the crude membrane fraction is again centrifuged at 70,000 *g* for 50 min at 4 °C. The pellet is washed twice by resuspending in ice-cold homogenization buffer and spinning. The final pellet is resuspended in homogenization buffer and stored at −70 °C until used for the receptor binding studies. Protein concentration is determined by the method of Bradford (1976) using a commercially available protein assay kit.

[His^1,^{125}I-Tyr^{10}, Nle^{27}]hGH-RH(1-32)NH_2 is iodinated by the chloramine-T method (Greenwood et al. 1963). The receptor binding assay is carried out for 60 min in GH-RH binding buffer (50 mM Tris–HCl, 5 mM EDTA, 5 mM $MgCl_2$, 1 % BSA, 30 μg/ml bacitracin, pH 7.4) at 24 °C. For saturation binding analyses, 50–150 μg membrane homogenates are incubated in duplicate with a least six concentrations of radioligand, ranging from 0.005 to 0.35 nM in the presence or absence of excess unlabeled peptide (1 μM) in a final volume of 300 μl. Incubation is terminated by immersing siliconized borosilicate tubes in ice water, transferring 270 μl of the suspension into cold siliconized polypropylene microfuge tubes, and centrifuging at 12,000 *g* for 2 min at 4 °C, and aspirating the supernatants. To reduce the nonspecific binding, 500 μl/tube of GH-RH binding buffer is added and the tubes are recentrifuged. The washing step is repeated and the supernatant again aspirated. Finally, the bottoms of the tubes, containing the pellet, are cut off and counted for radioactivity in a gamma counter.

In the competition experiments, which also include the specificity experiments, 50–100 μg of membrane homogenates are incubated in duplicate with 0.10–0.15 nM radioligand plus various concentrations of nonradioactive analogs and other peptides ($10^{-12} - 10^{-6}$ M).

Evaluation

Percent specific binding is plotted against the log concentration of competitors. The curves are compared at the 50 % specific binding levels (IC_{50}). The ligand-PC and McPherson computerized curve-fitting programs of Munson and Rodbard (1980) are used to analyze competition and saturation data. To determine the types of receptor binding, dissociation constants (K_d), and the maximal binding capacity of receptors (B_{max}), the saturation binding data are also analyzed by the Scatchard method.

Modifications of the Method

Carrick et al. (1995) described a rapid and sensitive binding assay for GH-releasing factor (GRF). Human embryonic kidney (HEK293) cells and rat pituitary tumor (GH_4C_1) cells were transfected with the porcine GRF receptor cDNA. Stably expressing cell lines are referred to as 293-P2 and GH_4-P1, respectively. GH_4C_1 cells transfected with somatostatin receptor subtype

2 are called GH_4-R2.20 and HEK293 cells transfected with somatostatin receptor subtype 5 are called 293-R5.2. GH_4-P1 and 293-R5.2 were derived by the calcium phosphate method of transfection and 293-P2 and GH_4-R2.20 using lipofectamine. For binding assays, cells were removed from culture dishes by the addition of phosphate-buffered saline containing 1 mM EDTA and cell membrane fractions prepared. Membrane pellets were suspended in 25 mM Tris, pH 7.4, plus protease inhibitors, aliquoted and stored at −80 °C. The binding assay consisted of approximately 25 pM radiolabeled ligand in the presence or absence of unlabeled ligand in assay buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA) and cell membranes (2–10 µg). The total assay volume of 200 µl included wheat-germ-agglutinin-coated SPA beads at a concentration of 1 mg/assay and for GRF binding, 0.05 mg/ml alamethicin (Sigma St. Louis, Mo., USA); 96-well plates were used for most assays. Assays were agitated on a plate shaker for 3 h. Samples were counted on a Wallac microbeta counter for 1 min per sample. The conventional binding assay was performed (Mayo 1992), except that following binding and centrifugation the membranes were washed in 0.5 ml binding buffer, centrifuged, and counted.

Abribat et al. (1990) and Gaudreau et al. (1992) developed a binding assay for GH-RH in rat pituitaries. Using this technique, Lefrancois and Gaudreau (1994) tried to identify the receptor-binding pharmacophores of GH-RH in rat pituitaries.

Hassan et al. (1995) developed a competitive binding assay using cloned porcine GH-RH receptors in order to study structure–activity relationships.

Kaijkowsi et al. (1997) investigated GH-RH receptor structure and activity using yeast expression technologies.

Muccioli et al. (1998) characterized specific binding sites for synthetic GH secretagogues (sGHS) on membranes from pituitary gland and different human brain regions using a peptidyl sGHS (Tyr-Ala-hexarelin) which has been radioiodinated to high specific activity at the Tyr residue.

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Growth Hormone Release from Rat Pituitaries in Vitro

Purpose and Rationale

For testing of pituitary cell stimulation, isolated pituitary glands or cell cultures may be used. Antagonism by adding somatostatin, for example, is tested in the presence of a standard concentration of GH-RH. Human GH-RH has the structure of a 44-amino acid amide, which was isolated from human endocrine tumors and later confirmed as a hypothalamic hormone. The effect of GH-RH analogs can be tested on isolated rat pituitaries measuring GH release. This test system avoids the interference of counter-regulatory somatostatin secretion, which limits the duration of GH release in vivo.

Procedure

The pituitaries of male Sprague–Dawley rats weighing about 100 g are quickly removed after decapitation. The posterior lobe is discarded and the anterior lobe is divided into two halves by a midsagittal cut. Five bisected hemipituitaries are incubated in plastic vials containing 4 ml TCM 199 with 0.1 % BSA, 15 μg/ml penicillin, and 25 μg/ml streptomycin. The vials are gassed with 95 % O_2 and 5 % CO_2. After 30 min of control incubation, the medium is changed and various doses of standard and test substances are added for an incubation of 90 min. GH content in the medium and in the pituitaries is determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

Evaluation

Dose–response curves are established for standard and test compounds measuring GH release into the medium and GH depletion from the pituitaries allowing calculation of potencies ratios with confidence limits.

Modifications of the Method

Superfused pituitary cells may be used measuring activity and duration of effect, as well as interaction of stimulatory and inhibitory factors. Growth-hormone-releasing factor from tumors in human pancreas and from rat hypothalami and analogs of GH-RH were evaluated in a superfused pituitary cell system (Vigh and Schally 1984; Czernus and Schally 1991; Halmos et al. 1993), see also section "In Vitro Assay for CRH Activity," in vitro assay for CRH activity. Anterior pituitaries of two young adult male Sprague–Dawley rats were digested with 0.5 % collagenase CLS2

(Worthington) for 50 min. After incubation, the fragments were digested into cell clusters (5–40 cells) by mechanical dispersion and then transferred onto two columns and allowed to sediment simultaneously with 0.8 ml Sephadex G-10. The dead volume of the system was set to 1 ml. Medium 199 containing BSA (2.5 g/l), $NaHCO_3$ (2.2 g/l) and gentamicin sulfate (85 μg/ml) was equilibrated with a mixture of 95 % air and 5 % CO_2 and used as the culture medium. The medium was pumped at a flow rate of 0.33 ml/min. During an overnight recovery period, the baseline stabilized and the cells regained their full responsiveness. The samples were then infused through a four-way valve at 5×10^{-10} M concentration for 3 min (1 fraction) at 45-min intervals. Rat GH was determined by double-antibody radioimmunoassay.

The same system was used by Rekasi and Schally (1993) and Kovács et al. (1996a) to evaluate the activity of GH-RH antagonists. For the determination of the antagonistic activity, the cells were exposed to 10^{-8}, 10^{-7}, and 10^{-6} M GH-RH antagonist simultaneously with 10^{-9} M GH-RH or to 10^{-6} M GH-RH antagonist combined with 100 mM KCl (controls for potassium-stimulated GH secretion) for 3 min. After 30 min, the duration of the inhibitory effect of GH-RH antagonist was also tested by repeated 3-min infusions of 10^{-9} M GH-RH.

Using this system with pituitaries of **transgenic mice** overexpressing the human GH-RH gene, Kovács et al. (1997) evaluated the effects of GH-RH antagonists.

In addition to growth-hormone release, Horváth et al. (1995) determined cAMP release from superfused rat pituitary cells stimulated by GH-RH.

GH release was determined using cultured rat pituitary cells (Brazeau et al. 1982; Perkins et al. 1983; Scheikl-Lenz et al. 1985). Pituitary cells were prepared by enzyme dispersion with collagenase, DNase, and pancreatin. The cells were cultured for 3 days in microbiological Petri dishes in Dulbecco's modified essential medium with 20 mM HEPES, 15 % fetal calf serum, 100 mU/ml penicillin-G, and 100 μg/ml streptomycin at 37 °C and 10 % CO_2.

Cheng et al. (1993) tested time- and dose-dependent GH release by a nonpeptidyl GH secretagogue in rat pituitary cells.

Sanchez-Hormigo et al. (1998) tested GH-releasing hexapeptide, one of several synthetic GH secretagogues, on GH secretion from cultured porcine somatotropes.

Kovács et al. (1996b) measured the effects of chronic administration of a GH-RH agonist on body weight, tibia length, and tail length in growth-hormone-deficient (monosodium glutamate-lesioned) rats.

Jacks et al. (1996) evaluated an orally active GH secretagogue in dogs. Serum GH levels were dose-dependently increased after oral and intravenous administration. Moreover, an increase of insulin-like growth factor and serum cortisol was found.

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Long-Term Pituitary Cell Culture

Long-term-cultured pituitary cells have been used extensively to study the response of GH secretion and synthesis. Roh et al. (2006) applied this principle to the investigation of priming by GH-RH and by synthetic secretagogues derived from ghrelin. They found that low doses of GHRP-2 and GH-RH were suitable for priming somatotrope cells in culture for subsequent stimulation by GH-RH and ghrelin.

Studies in vitro using long-term cell cultures have shown that priming with low doses of GH-RH enhances the subsequent response to the stimulation with GH-RH analogs. Fintini et al. (2005) have used a new animal model for the assessment of GH secretion, the GH-deficient GH-RH-knockout (GH-RH-KO) mouse model. The found that priming with the non-GH-RH secretagogue derived from the gastrointestinal peptide ghrelin augmented the subsequent response to a GH-RH analog.

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GH-RH Bioassay by Growth Hormone Release in Rats

Purpose and Rationale

Releasing activity can be determined by plasma GH stimulation after intravenous or subcutaneous injection of GH-RH in rats.

Procedure

Male Sprague–Dawley rats weighing about 100 g are anesthetized by fractionated subcutaneous injections of 0.7 ml/kg of 25 % urethane solution. The jugular vein is cannulated. Various doses of GH-RH test preparations or standard are injected intravenously in 0.2 ml 1 % gelatin/saline. After 15 min, blood samples are collected for determination of GH by a specific radioimmunoassay (Schalch and Reichlin 1966). The plasma concentration is expressed in terms of NIAMD-rat-GH-RP-1.

As a modification also suitable for testing other releasing hormones, e.g., by subcutaneous application, a double-barreled polyethylene catheter is implanted into the jugular vein, with one lumen being used for blood sampling and the second small lumen being used to keep the catheter open by heparin infusion into the tip of the collection catheter. Blood is continuously withdrawn by a peristaltic pump and fractions of 500 µl heparinized blood are collected at intervals of 10 min. To compensate for blood losses, the erythrocyte fraction of four to eight consecutive plasma samples is reinfused in a plasma expander (Haemaccel 3.5 %) via a femoral vein. Before treatment, two 2-min samples are collected, and, after injection, two 2-min samples are collected again, followed by sampling intervals of 10 min for up to 3 h. GH is determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

Evaluation

Using several doses of standard and test preparation, dose–response curves are established, allowing calculation of the potency ratio with confidence limits. Time–concentration curves after subcutaneous or, for example, intranasal application allow the duration of action to be evaluated.

Modifications of the Method

Wehrenberg and Ling (1983) and Wehrenberg et al. (1985) determined the in vivo biological potency of rat and human GH-releasing factor and fragments of human GH-releasing factor in swivel-cannulated, conscious, freely moving rats and in cannulated anesthetized rats.

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GH-RH Analogs

Many studies have been performed with synthetic GH-RH analogs, starting with the active sequence GHH (1–29) and proceeding to more powerful compounds, which were explored in domestic

animals (Bhatti et al. 2006) and tested for their therapeutic potential in clinical studies (Doi et al. 2004; Furuta et al. 2004; Veldhuis et al. 2005).

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Effects on Different Peptide Hormones

Jürgen Sandow

Contents

Other Peptide Hormones

Melatonin

General Considerations

The role of melatonin as a neurotransmitter, a hormone, and a possible therapeutic agent has been a matter of controversy (Brainard 1978; Reiter 1991; Hajak et al. 1996; Huether 1996; Jansen et al. 2006) ever since melatonin was isolated and identified as *N*-acetyl-5-methoxytryptamine by Lerner et al. (1958) and characterized by Axelrod and Wurtman (1966). Melatonin is synthesized and released by the pineal gland and has been shown to play a key role in the regulation of mammalian circadian rhythms and reproductive functions (Reiter 1991; Arendt et al. 1995; DiBella and Gualano 2006). Melatonin is also produced in extrapineal sites, such as the retina (Lundmark et al. 2006), the Harderian glands, and the gut (Huether 1993, 1994; Messner et al. 2001). It should however be noted that methods for the identification of peptides and neurotransmitters have reached extremely high levels of sensitivity; as a consequence, their presence in a tissue does not necessarily indicate that a relevant physiological function is subserved in that organ or tissue. Melatonin is synthesized from 5-hydroxytryptamine (5-HT) by a two-step biochemical pathway (Reiter 1991). Initially, 5-HT is acetylated to produce *N*-acetylserotonin, which is subsequently *O*-methylated to form melatonin.

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Melatonin Receptor Binding

Purpose and Rationale

The activity of melatonin is mediated through high-affinity G-protein-coupled receptors. Two human melatonin receptor subtypes, hMT_1 and hMT_2, have been cloned (Reppert et al. 1994, 1995; Browning et al. 2000; Dubocovich et al. 2001). A third melatonin-binding site named MT_3 has been described as the human homologue of cytoplasmic quinone reductase 2 (Nosjean et al. 2000, 2001).

Audinot et al. (2003) described the evaluation of selective ligands of human-cloned MT_1 and MT_2 receptors. Binding affinities were determined using [2-^{125}I]melatonin. Methods for the measurement of melatonin and melatonin derivatives (Middleton 2006) and for the melatonin receptor ligands (Zlotos 2005) have been reviewed.

Procedure

Membrane Preparations

HEK293 and CHO-K1 cell lines stably expressing the hMT_1 or hMT_2 receptor are grown to confluence, harvested in phosphate buffer containing 2 mM EDTA and centrifuged at 1000 *g* for 5 min (4 °C). The resulting pellet is suspended in 5 ml Tris–HCl, pH 7.4, containing 2 mM EDTA and homogenized using a Kinematica Polytron. The homogenate is then centrifuged (20,000 *g*, 30 min, 4 °C), and the resulting pellet is suspended in 75 mM Tris–HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM $MgCl_2$. Aliquots of membrane preparations are stored in binding buffer (Tris–HCl 50 mM, pH 7.4, 5 mM $MgCl_2$) at −80 °C until use.

[2-^{125}I]Melatonin-Binding Assay

Membranes are incubated for 2 h at 37 °C in binding buffer (Tris–HCl 50 mM, pH 7.4, 5 mM $MgCl_2$) in a final volume of 250 µl containing

[2-^{125}I]melatonin (200 pM) for competition experiments using CHO cells and 25 or 200 pM, respectively, for MT_1 and MT_2 receptors expressed in HEK cells. Nonspecific binding is defined with 10 μM melatonin. The reaction is stopped by rapid filtration through GF/B unifilters, followed by three successive washes with ice-cold buffer.

Evaluation

Data are analyzed using the program PRISM (GraphPad Software, San Diego, Calif., USA). For saturation assays, the density of binding sites (B_{max}) and the dissociation constant of the radioligand (K_D) are calculated according to the method of Scatchard. For competition experiments, inhibition constants are calculated according to the Cheng–Prusoff equation. For the correlation analysis of pK_i values, the Pearson product moment correlation coefficient is employed.

Modifications of the Method

Receptor binding assays have been used to evaluate new melatonin receptor ligands (Depreux et al. 1994; Dubocovich et al. 1997a; Charton et al. 2000a, 2000b; Nonno et al. 2000).

Dubocovich et al. (1997b) used **rabbit retina membranes** for binding studies with [2-^{125}I]melatonin.

Nonno et al. (2000) and Audinot et al. (2003) used the **[^{35}S]GTPγS binding assay** to evaluate the functional activity of agonists and antagonists. Membranes were prepared as for the [2-^{125}I]melatonin-binding assay. Together with test drugs, they were diluted in binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP) in the presence of 20 μg/ml saponin in order to enhance the agonist-induced stimulation level. For agonist tests, incubation was started by the addition of 0.1 nM [^{35}S]GTPγS to membranes and ligands and carried on for 60 min at room temperature in a final volume of 250 μl. To test for antagonistic activity, membranes were preincubated for 30 min with melatonin (30 nM or 3 nM for hMT_1 and hMT_2 receptors, respectively) and several concentrations of the tested compound. The reaction was started by the addition of 0.1 nM [^{35}S]GTPγS and followed by a 60-min incubation. Nonspecific binding was assessed using nonradiolabeled GTPγS (10 μM). All reactions were stopped by rapid filtration through GF/B unifilters presoaked with distilled water, followed by three successive washes with ice-cold buffer.

Data were analyzed using the program PRISM to yield EC_{50} and E_{max} values for agonists. Antagonist potencies were expressed as K_B with $K_B = IC_{50}/1 + ([ago]/EC_{50}ago)$, where IC_{50} is the inhibitory concentration of antagonist that gives 50 % inhibition of [^{35}S]GTPγS binding in the presence of a fixed concentration of agonist ([ago]) and the EC_{50}ago is the EC_{50} of the agonist when tested alone. I_{max} (maximal inhibitory effect) was expressed as a percentage of that observed with melatonin, 30 nM and 3 nM ([ago]) for hMT_1 and hMT_2 receptors, respectively.

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In Vitro Assay of Melatonin: Inhibition of Forskolin-Stimulated cAMP Accumulation

Purpose and Rationale

Reppert et al. (1995), Browning et al. (2000), and Charton et al. (2000a, 2000b) used the inhibition of forskolin-stimulated cAMP accumulation for characterization of melatonin receptors and evaluation of synthetic derivatives.

Procedure

Generation of CHO-mt_1 and CHO-MT_2 Cells

Clones representing the sequence of the human mt_1 receptor are amplified using degenerate primers based on the sequence of the *Xenopus laevis* melatonin receptor (Ebisawa et al. 1994). Cloning was in pBluescript (Stratagene, La Jolla, Calif., USA). The human MT_2 sequence is cloned in a similar manner using the sequence described by Reppert et al. (1995). The sequences encoding the receptor are cloned into the mammalian vector pcDNA3 (Invitrogen) and introduced into CHO cells by conventional calcium phosphate precipitation techniques, which are then placed under G418 selection (1 mg/ml). Cell lines, which give the greatest melatonin-induced inhibition of cAMP accumulation, are chosen for further studies.

cAMP Assays

The assay is performed using 96-well plates in a final assay volume of 200 µl. Confluent CHO-mt_1

and CHO-MT_2 cells are incubated at 37 °C with DMEM-F12, containing 300 μM isobutylmethylxanthine to inhibit phosphodiesterase activity. Following a 60-min incubation, agonist (0.01 pM to 100 μM) is added. After 60 min, forskolin (30 μM) is added and cells are incubated for a further 15 min. For antagonist experiments, melatonin is co-incubated with luzindole (0.1–100 μM) for 60 min prior to the addition of forskolin. The reaction is terminated by removal of media and addition of ice-cold ethanol (100 μl) for 30 min at 4 °C. Ethanol samples are evaporated and cAMP concentrations determined by [^{125}I] cAMP scintillation proximity assay (Amersham).

Evaluation

cAMP data are fitted with a four-parameter logistic equation to determine pIC_{50} values and Hill coefficients. Drug responses are expressed as percentage inhibition of forskolin-stimulated cAMP. The potency ratio is defined as the ratio of the EC_{50} of the drug relative to that of melatonin determined in the same experiment.

Modifications of the Method

Morgan et al. (1989) and Conway et al. (2000) studied inhibition of cAMP production in cultures of ovine pars tuberalis cells by melatonin.

Furthermore, Conway et al. (2000) characterized the human melatonin mt_1 and MT_2 receptors using the **CRE-luciferase reporter assay**. HEK293 cells expressing human melatonin mt_1 and MT_2 receptors were cultured to ~80 % confluence at which time they were co-transfected with the plasmids pGL3 (CRE2-TK) and pcDOR8 (Barrett et al. 1994) using the lipid transfection reagent FuGENE™ 6 (Boehringer Mannheim). Cells were cultured for 24 h post-transfection before being washed with phosphate-buffered saline (pH 7.4), recovered in phenol-red-free Dulbecco's modified Eagle medium (supplemented with 10 % fetal calf serum) and seeded as 50-μl aliquots (5×10^4 cells) into white 96-well tissue culture plates. Following a further 24-h incubation, drug treatments were added in a 5.6 μl volume to triplet wells, and plates were left to incubate overnight (16 h). The level of expressed luciferase was measured by the addition of 50 μl of Constant Light Signal Reagent (Boehringer Mannheim) to each well, and plates were briefly placed in the dark (30 min, room temperature) before being quantified on a Packard LumiCount™ Reader (1 s per well). Where experiments were designed to measure drug-mediated dose–response effects, a control melatonin dose–response study was always included. Data from the triplet wells were averaged and the control data normalized from a value of 100 (no melatonin) to a value of 0 (maximum response, 10^{-5} M melatonin). Data obtained from other drug treatments were normalized relative to the control values determined in that experiment. All experiments were performed on three or more separate occasions, and mean values ± SEM determined.

IC_{50} values and apparent efficacies were calculated by fitting a four-parameter logistic curve through the mean normalized data. For antagonists, pA_2 values were determined by performing Schild analysis.

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Dopamine Release from Rabbit Retina

Purpose and Rationale

Dubocovich et al. (1997) compared the pharmacological profile of the rabbit retina ML_1 presynaptic heteroreceptor that mediates the calcium-dependent release of dopamine to that of the human Mel_{1a} and Mel_{1b} recombinant melatonin receptors and determined potencies of melatonin receptor agonists and antagonists.

Procedure

Albino rabbits (2.5–3.5 kg) maintained on a 14-h/10-h light/dark cycle are sacrificed by decapitation during the light cycle. Retinal pieces are incubated in Krebs solution gassed with 95 % O_2 and 5 % CO_2 for 20 min at 37 °C in the presence of 0.1 μM [^{3}H]dopamine (specific activity 55 Ci/mol). The pieces of retina are washed in Krebs solution at 37 °C and placed in glass superfusion chambers between a pair of platinum electrodes 30 mm apart. Retinal tissue is superfused at 1 ml/min with Krebs solution (37 °C) containing ($S-R$)-sulpiride (0.1 μM) to block D_2 dopamine autoreceptors, beginning 60 min after the onset of superfusion, i.e., when the spontaneous outflow of radioactivity has leveled off. Samples of the superfusate are collected by means of a fraction collector at 4-min intervals. Tritium release from retinal tissue is elicited by field stimulation using 2-min-duration (3 Hz, 20 mA, 2 ms) pulses that are delivered from platinum electrodes by a Grass stimulator. During stimulation, pulses are monitored on an oscilloscope. In each experiment two periods of field stimulation are applied at 60 min and 100 min after the incubation with [^{3}H]dopamine. Samples of the superfusate are collected before, during, and after the period of stimulation. Drugs are added to the perfusion medium either 40 min before the first or 20 min before the second stimulation period. At the end of the experiment, the retinal tissue contained in each chamber is solubilized in 0.5 ml TS-1 (Research Products Intl., Elk Grove, Ill., USA), and the tritium content is determined by liquid scintillation counting.

Evaluation

The outflow of radioactivity in each sample is expressed as the percentage of total tissue radioactivity determined to be present at the beginning of each sample collection period: total tritium released per sample divided by total tritium present in the tissue all multiplied by 100 (Dubocovich 1985). Results are expressed as the ratio of the overflow during the second and first periods of field stimulation within the same experiments.

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Effect on *Xenopus* Melanophores

Purpose and Rationale

TeckTeh and Sugden (1998) examined the potency of melatonin receptor ligands using the

pigment aggregation response in a clonal line of *Xenopus laevis* melanophores.

Procedure

Culture of *Xenopus laevis* Melanophores

A clonal line of *Xenopus laevis* melanophores (M. Lerner, University of Texas) is grown in diluted (0.7 ×) L-15 medium (Sigma) containing 20 % heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The medium is first conditioned by *Xenopus laevis* fibroblasts (Danilolos et al. 1990).

Microtiter Plate Assays

Melanophores are seeded into flat-bottomed 96-well microtiter plates and grown in conditioned L-15 medium at 24 °C for 2–4 days until cell density is (6–8) × 10^4 cells/well. For the 4–18 h before using melanophores, condition medium is replaced with 0.7 × L-15 medium; pigmented granules remain fully dispersed in this medium. Agonist potency is determined by measuring the redistribution of pigment granules within the melanophores by quantitating the change in absorbance of the cells at 630 nm on a BioTek microtiter plate reader. The fractional change in absorbance ($1 - [A_f/A_i]$, where A_i is the initial absorbance before drug addition and A_f is the final absorbance 1 h after drug treatment) is calculated for each concentration of drug tested.

Evaluation

Concentration–response data are plotted and EC_{50} values are determined using the ALLFIT program (De Lean et al. 1978) with the four-parameter logistic equation:

$$Y = [A - D]/[1 + (X/C)B] + D$$

where X is the concentration of the analog, Y is the fractional change in absorbance, A is the maximal absorbance in the absence of the analog, B is the slope factor, C is the concentration of the analog producing half of the maximal response (EC_{50}), and D is the minimal absorbance. The response to each drug is measured in six independent experiments. Antagonistic potency (pA_2) is determined by constructing concentration–response curves to melatonin in the absence or presence of varying concentrations (1–100 μM) of each antagonist.

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Vasoconstrictor Activity of Melatonin

Purpose and Rationale

Evans et al. (1992), Ting et al. (1997), and Delagrange et al. (1999) used the vasoconstrictor action of melatonin in the tail artery of juvenile Wistar rats to evaluate melatonin agonists and antagonists. Two different recording systems, the Halpern pressure myograph (Halpern et al. 1984) and the Halpern–Mulvany wire myograph, were compared.

Procedure

Male juvenile (3–4 weeks old, weighing 55–100 g) Wistar rats are housed in a 12-h light/dark cycle. They are sacrificed 1–2 h after lights on by decapitation. The ventral artery of the tail is dissected and placed onto a dissecting disc in gassed, modified Krebs–Henseleit solution. The blood vessel is carefully cleaned from fat and connective tissues with the aid of a dissecting microscope and divided into ring segments of 2–3 mm length. Ring segments are suspended between two supporting jaws in a stainless steel chamber of a Mulvany–Halpern wire myograph and allowed to equilibrate for 30 min. The preparations are bathed in Krebs–Henseleit solution maintained at 34 ± 1 °C and gassed with 95 % O_2/5 % CO_2. Tension is applied by adjusting the micrometer connected to one of the supporting jaws, while an isometric transducer connected to the other jaw measures the force. An initial tension of 0.2–0.5 g is applied. Then the preparations are allowed to relax to 0.1–0.4 g weight. Vessels are

contracted with 60 mM KCl to assess tissue viability and provide a reference contracture for subsequent data analysis. A stimulator is used to deliver 5-s trains of electrical pulses (10–20 V, 3 ms pulse width) at a frequency of 2–3 Hz every 4–5 min. The voltage and the frequency of the field stimulation are adjusted at the beginning of each experiment to obtain 20 %–35 % of the KCl response. Concentration–response curves are constructed for melatonin and test compounds. For experiments with putative antagonists, these agents are added 20 min before the construction of a dose–response curve for melatonin.

Evaluation

Isometric tension responses are expressed as a percentage of the enhancement in response to the predrug of neurogenic contractions. The sensitivity of the preparations is assessed as the negative logarithm of the concentration required to produce 50 % of the maximum response (pEC_{50}) after the agonist concentration–effect ($E/[A]$) data are fitted to the formula:

$$E = \alpha[A]^n/([A]^n + [A_{50}]^n),$$

where E is the response, α is the asymptote, $[A]$ is the agonist concentration, n is the gradient of the $E/[A]$ curve, and $[A_{50}]$ is the midpoint of the $E/[A]$ curve (Black et al. 1985). $[A_{50}]$ values represent agonist concentration giving 50 % of the maximum responses and are shown as the negative logarithm (pEC_{50}). The maximum response of each analog is expressed as a ratio of the maximum response to melatonin (E_{max}) obtained in segments from the same animal. For the antagonistic experiments, the agonist concentration ratio (CR) is determined in each experiment. The CR is the ratio of EC_{50} values in the presence and absence of antagonist. Differences between mean values are compared using either paired or unpaired Student's t-test (two tailed) and are considered statistically significant if $P < 0.05$.

Modifications of the Method

Viswatanan et al. (1997) reported that melatonin receptors mediate **contraction of rat cerebral artery**. Male Sprague–Dawley rats (7–8 weeks old) were sacrificed by decapitation and their brains quickly removed and kept in ice-cold Krebs–Ringer buffer aerated with 95 % O_2/5 % CO_2. The posterior communicating artery, between the point at which the internal carotid artery joins and the middle cerebral artery originates, is dissected from the base of the brain, kept in ice-cold Krebs–Ringer buffer, cleaned of excess connective tissue, and transmitted to a vessel chamber filled with cold Krebs–Ringer buffer. Both ends of the artery are mounted on glass cannulas (100 μm diameter) and secured using 10–0 surgical silk sutures. The cannulas at the proximal and distal ends of the artery are connected to pressure transducers. The artery is perfused with oxygenated Krebs–Ringer buffer using a servo system. The intraluminal pressure of the artery is gradually increased to 60 mmHg by increasing the flow rate of the Krebs–Ringer buffer. The transmural pressure within the artery segment is maintained at 60–65 mmHg, a pressure chosen because it approximates the non-neurogenic pressure exerted on arterial blood vessels in rats. The chamber is superfused with oxygenated Krebs–Ringer buffer maintained at 37 °C. The lumen diameter of the artery is continuously measured using a video electronic analyzer. The preparation is equilibrated for 45–60 min and during this interval it develops the myogenic tone.

Increasing concentrations (10^{-12}–10^{-6} M) of melatonin are administered through the perfusion buffer, without washout, at regular intervals of 5 min, and the lumen diameter is recorded continuously. Changes in lumen diameter are measured and expressed as percentage contraction.

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Melatonin's Effect on Firing Rate of Suprachiasmatic Nucleus Cells

Purpose and Rationale

Ying et al. (1996) studied the effects of drugs related to melatonin on neuronal firing activity in the suprachiasmatic nucleus, intergeniculate leaflet, and other brain areas in anesthetized Syrian hamsters. The suprachiasmatic nucleus of the hypothalamus functions as the dominant pacemaker for behavioral and physiological circadian rhythms in mammals (Moore 1983; Meijer and Reitveld 1989). Photic information required for entrainment to lighting cycles reaches the suprachiasmatic nucleus through a direct projection from the retina and, via an indirect pathway, the geniculohypothalamic tract, originating in the retinorecipient intergeniculate leaflet of the geniculate nuclei (Zhang and Rusak 1989). The firing rates are suppressed by melatonin and its analogs, whereas antagonists reverse this effect (Ying et al. 1996; Liu et al. 1997).

Procedure

Male Syrian hamsters weighing 100–140 g are kept in a photoperiod with 14 h of light beginning at 05:00 a.m. for at least 2 weeks before being used. The animals are anesthetized with 25 % urethane (2 g/kg i.p.) and given subcutaneous injections of Robinul (3-hydroxy-1,1-dimethylpyrrolidinium bromide α-cyclopentylmandelate, A. H. Robins; 2 %,0.1–0.2 ml/animal) to reduce congestion of the respiratory tract during anesthesia. Hamsters are mounted in a stereotaxic apparatus and body temperature is maintained at 37 °C throughout the experiment. A hole is drilled in the skull overlying the suprachiasmatic nucleus or intergeniculate leaflet region, with the aid of a magnifier. Special care is taken to avoid bleeding caused by damage to the superior sagittal sinus. The eyelids contralateral to the recording site are retracted with sutures, and the eye is covered with mineral oil to prevent dehydration after the pupils are dilated with a topical application of 1 % atropine sulfate. Electrodes are aimed at the suprachiasmatic nucleus using stereotaxic coordinates (0.2–0.6 mm anterior to the bregma, 0.2–0.35 mm lateral to the midline, and 7.6–7.8 mm ventral to the cortical surface), with the upper incisor bar 2 mm below the interaural line. Electrodes aimed at the hippocampus, dorsal lateral geniculate nucleus, and intergeniculate leaflet are inserted 1.4–1.6 mm posterior to the bregma, 3.3–3.8 mm lateral to the midline, and 2.0–4.5 mm ventral to the cortical surface. The animal is maintained in a darkened room except during light pulses or while repositioning the electrodes.

Five-barrel glass micropipettes are prepared (Ying et al. 1993) with the recording barrel filled with fast green (Sigma) at a sub-saturated concentration in 2 M NaCl. One barrel is filled with 2 M NaCl for automatic current balancing. The other barrels are filled with melatonin hydrochloride, or metergoline, or test compound.

For intraperitoneal injections of doses between 0.5 and 10 mg/kg, melatonin and test compounds are dissolved in dimethyl sulfoxide (DMSO) and further diluted with saline. For iontophoretic studies, test compounds are used in 5 mM concentrations.

Single-unit extracellular recordings are made from 15:00 to 20:00 hours, overlapping both projected light and dark phases of the daily illumination cycle in the colony room. Contralateral

whole-retinal illumination is conveyed to the eye from a tungsten–halogen lamp using fiber optics and a computer-controlled shutter. Photically responsive neurons are identified and recorded during sustained light presentations (typically 1–2 min). Cells are identified as photically responsive or light sensitive if their firing rates are consistently increased or suppressed by >30 % by sustained, whole-retinal illumination.

Evaluation

A computer program controls data acquisition, light exposures, and iontophoretic applications of drugs. Agonist effects are defined as repeatable changes relative to predrug firing rates of >20 % at some dose of the drug. In order to compare the effects of melatonin and test compounds, agonist potencies are expressed as ED_{50} values, the doses required to produce half-maximal effects. The impact of an agonist is expressed as the proportion of the effect of an agonist that is reversed by the co-application of the antagonist, i.e., the difference between the agonist effects in the absence and the presence of the antagonist, divided by the agonist effect alone, and expressed as a percentage.

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Melatonin's Effect on Circadian Rhythm

Purpose and Rationale

Melatonin induces phase shifts of circadian rhythms through an action within the suprachiasmatic nucleus of the hypothalamus (Benloucif and Dubocovich 1996; MacArthur et al. 1997; Dubocovich et al. 1998; Delagrange et al. 1999). Competitive melatonin receptor antagonists block phase shifts of circadian rhythms of electrical activity and neuronal firing within the suprachiasmatic nucleus, suggesting that these physiological responses are mediated through activation of melatonin receptors. Dubocovich et al. (1998) tested phase shifts of running activity in C3H/HeN mice induced by melatonin and antagonists. From these data, the authors concluded that selective MT_2 melatonin receptor antagonism is necessary to block melatonin-mediated phase advances of circadian rhythms.

Procedure

Male C3H/HeN mice (5–6 weeks old) are housed in groups of five and maintained in temperature- and humidity-controlled rooms. Food and water are provided ad libitum. Animals are maintained for 2 weeks on a 12-h/12-h light/dark cycle (300 lux at the level of the cage) and are then transferred to constant and complete darkness in individual cages (18 × 30 × 12 cm) equipped with activity wheels. Mice are kept under constant darkness for 3 weeks to stabilize the free-running activity rhythm (TAU = mean duration of the free-running circadian period). The animals are treated 3 days consecutively at circadian time 10 (CT10) (CT12 = onset of activity). CT10 is estimated from the TAU value for each day. Each mouse is treated with vehicle or melatonin (0.3, 0.9, 3.0, 9.0, or 30 μg melatonin per animal) or the antagonist to be tested at various dosages. Each mouse receives one treatment every 3 weeks and a total of four treatments. All treatments are

performed under a dim red light (15 W, Kodak 1A filter) with illuminance of less than 3 lux.

Rhythms of wheel-running activity are measured with a magnetic microswitch, which detects revolutions of the running wheel and is on line with a computer (Benloucif and Dubocovich 1996). Data are collected using the Dataquest III hardware and software package and analyzed with the assistance of TAU software (Mini Mitter, Sunriver, Ore., USA). The exact circadian time of the pulse is determined after treatment from the onset of steady-state activity on the day of the pulse. Aligning the TAU guide by the eye over the steady-state activity onsets of free-running activity for 7–10 days before the pulse and from day 4 to 14 after the pulse assesses phase shifts.

Evaluation

Phase shifts induced by treatment are measured in hours as the difference between the steady-state pre- and post-pulse activity onsets, using the TAU program ruler. Differences between vehicle/melatonin and antagonist/melatonin-treated groups are assessed for each melatonin dose by analysis of variance, followed by pairwise comparison when indicated.

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Melatonin's Effect on Neophobia in Mice

Purpose and Rationale

Kopp et al. (1999a) studied the effects of melatonin on neophobic responses in different strains of mice. Melatonin counteracted avoidance responses to an unfamiliar environment in BALB/c and C3H/He mice but not in C5BL/6 mice. In this experimental situation, animals can move freely in simultaneously presented familiar and unfamiliar places (Hughes 1968).

Delagrange et al. (1999) and Kopp et al. (1999b) tested the effects of a melatonin antagonist on the neophobia-reducing properties of melatonin in BALB/c mice.

Procedure

Male BALB/c mice at an age of 10 weeks are housed in groups of five in standard cages with food and water available ad libitum under controlled conditions at a 12-h light/12-h dark cycle with lights on at 01:00 h, so that the animals can observed under dim red light in their active period.

The observation apparatus consists of a rectangular polyvinyl chloride box (30 × 20 × 20 cm), covered with Plexiglas and subdivided into six equal square units (10 × 10 × 20 cm), which are all interconnected by small holes located at the floor level. It can be divided in half lengthwise by closing a temporary partition. The apparatus is kept in the mouse room. The experimenter always stands next to the box at the same place. Approximately 24 h before testing, each animal is randomly placed in one-half of the apparatus with the temporary partition in place, to be familiarized with it. The floor of this is only half covered with sawdust, and the animal has unlimited access to food and water during 24 h of the familiarization period. At the end of this period, the temporary partition between the familiar and unfamiliar compartment is removed and the animal then observed for 10 min. Measures are taken of the number of approach responses followed by avoidance reaction toward the unfamiliar places (attempts), the time spent in the unfamiliar compartment (time), the total number of square units entered (locomotion), and the total number of rears made by the animals (rears).

Mice are treated either with saline (controls) or with 1 mg/kg melatonin, or with various doses of the antagonist, or combinations thereof.

Evaluation

Data are treated with either an analysis of variance (ANOVA) followed by Newman–Keuls a posteriori *t*-test, if groups come from a population with homogeneous standard deviations, or with a Kruskal–Wallis nonparametric ANOVA test followed by Mann–Whitney tests if groups come from a population with heterogeneous standard deviations.

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Melanophore Stimulating Hormone

The early investigations of the pigmentary effects of corticotropin, melanotropin, and related peptides derived from the pro-opiomelanocortin family were focused on the control of pigmentary and adrenocortical function (Slominski and Wortsman 2000; Millington 2006). With increasing knowledge and more sophisticated methodology, this role has now been considerably extended (Boehm et al. 2006), including actions on energy balance (Fehm et al. 2004; Butler 2006; Nargund et al. 2006), actions on the cardiovascular system and kidney function (Humphreys 2007), erectile dysfunction (Hadley and Dorr 2006), and even antimicrobial properties (Catania et al. 2006). The pigmentary action of MSH analogs is being commercialized (Hadley and Dorr 2006).

The early studies on synthetic MSH-derived peptide analogs have been considerably extended, with therapeutic applications in view for the control of appetite, and antiobesity compounds of the future.

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Skin Darkening in Whole Amphibia

Purpose and Rationale

MSH (melanocyte-stimulating hormone or melanotropin) is also called melanophore-expanding hormone, based on the microscopical observation of expansion of melanocytes in amphibia (Landgrebe and Waring 1950, 1962; Sandow et al. 1977; Inouye and Otsuka 1987). Several amphibia species can be used, such as *Xenopus laevis*, *Rana temporaria* or *R. esculenta*, or *Hyla arborea*. Hypophysectomized animals are more sensitive than intact ones. Numerous corticotropin analogs have MSH activity based on a common peptide core shared with MSH. The structure of two melanocyte-stimulating hormones has been elucidated: α-MSH containing 13 amino acids and β-MSH containing 18 amino acids.

Hunt (1995) reviewed the role of MSH as a regulator of human melanocyte physiology.

Procedure

The pretest conditions require adaptation for blanching of the skin, e.g., by keeping the animals on a white background prior to the assay. *Xenopus laevis* or *Rana temporaria* are kept in single cages at 16 °C under humid conditions. Injections of various doses of the test preparation or the standard are given into the dorsal lymph sac. After 1 h, the webs between the digits of the hind limbs are investigated under a stereomicroscope, the eyepiece of which is fitted with a photoelectric cell, the other being available for microscopic examination. The state of melanophore expansion can be assessed by direct visual examination and recorded in terms of an arbitrary melanophore index or by modern methods of morphometry.

Evaluation

For test preparation and standard, dose–response curves can be established and potency ratios calculated.

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Assay in Isolated Amphibian Skin

Purpose and Rationale

Melanophores in isolated pieces of pale amphibian skin (from background-adapted animals or those maintained in MSH-free medium) expand within a short period of time after immersion in a MSH-containing buffer. Subsequent immersion of the skin in fresh saline results in contraction of the melanophores (Trendelenburg 1926; Jores 1933; Landgrebe and Waring 1950). Several methods using isolated amphibian skin have been described using either light absorption or light reflection (Landgrebe and Waring 1962). These methods are historical and have been replaced by using cell cultures of melanocytes.

Procedure

For studies on light absorption, the skin of *Rana temporaria* or *R. esculenta* is used. Before sacrificing the donor animal and dissecting the isolated skin, it is important to submit the living animal to varying white and black backgrounds under overhead illumination of a light source of 100 W, so that the melanophores can expand and contract before operation. The areas most suitable for use in this technique are from the thighs. Before using the skin for an actual assay, each piece should be immersed in a sufficiently concentrated MSH solution to expand the melanophores almost but not quite fully. The skin is

then washed with saline until the melanophores are fully contracted again and in a suitable condition for the test. This usually takes about 45 min. A piece of the skin is mounted in a cell which holds the skin stretched and enables it to be immersed in saline or test solution. It is placed in position on the stage of a binocular microscope. An eyepiece is used for visual observation and a photoelectric cell is attached to the other connected directly to a sensitive galvanometer. Various concentrations of the test preparation or the standard are added. At least six skin pieces are used for each concentration of test preparation or standard. Galvanometer readings and readings of the melanophore index are taken simultaneously every 10–15 min until the maximum response is elicited, usually in 30–45 min.

Evaluation

Assessment of potency is made from the readings of the melanophore index and from the light absorbed, which is recorded as a percentage of the light originally passing through the skin, and is plotted against the log concentration.

Critical Assessment of the Method

The methods were originally developed to standardize extracts with (pigmentary) MSH activity by the use of a bioassay. They are still necessary for evaluating compounds for biological MSH activity since not only natural ACTH but also synthetic peptides, such as β^{1-24}-corticotropin (Schuler et al. 1963) or β^{1-23}-corticotropin-23-amide (Vogel 1965, 1969), possess MSH activity.

Modifications of the Method

Measurement of light reflection by skin pieces, instead of light absorption, was used by Shizume et al. (1954).

In **cell-based assays**, Siegrist and Eberle (1986), Bagutti and Eberle (1993), and Sahm et al. (1993, 1996) used cultured mouse B16 melanoma cells in a sensitive in situ melanoma assay to study the structure–activity relationship of melanocyte-stimulating hormone peptides. B16 cells were seeded at a density of 2500 cells per well in 96-well microtest culture plates. After 24 h the cells were incubated in the presence of serial dilutions of MSH peptides for 3–5 days. The melanin released into the medium of each well was then determined spectrophotometrically at a wavelength of 405 nm using an automatic microplate reader calibrated against synthetic melanin.

Sahm et al. (1994, 1996) measured the release of ^3H_2O into the medium from [3′,5′-^{3}H]L-tyrosine by tyrosinase in B16 mouse melanoma cells after incubation with α-MSH analogs.

MSH assays have also been used to study the factors regulating the release of MSH (**MRF**) or inhibiting the release of MSH (**MIF**) (Kastin et al. 1969; Celis et al. 1971).

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Binding to the Melanocortin Receptor

Purpose and Rationale

Much of the initial work on the biological effects of hormones has been expanded by using cloned subclasses of receptors for identification of more specific ligand compounds. A family of five MSH receptor subclasses for the melanocortin peptides has been identified (Chhajlani and Wikberg 1992; Chhajlani et al. 1993; Gantz et al. 1993b; Schiöth et al. 1997a, 1988a; Strand 1999). The melanocortin MC_1 receptor is expressed in melanocytes and melanoma cells and binds α-MSH with high affinity. The MC_1 receptor plays an important role in skin and fur pigmentation in a variety of vertebrates (Cone et al. 1996). The melanocortin MC_2 receptor (i.e., the ACTH receptor) has a well-defined function in steroid production in the adrenal gland (Schiöth et al. 1996a). The melanocortin MC_3 receptor is found in the hypothalamus, the brain, the placenta, gut tissues, and the heart (Gantz et al. 1993a; Desarnaud et al. 1994; Sahm et al. 1994). The melanocortin MC_4 receptor (Schiöth et al. 1996b, 1998b) has been found to affect feeding in rodents and may be important for weight homeostasis (Fan et al. 1997; Huszar et al. 1997). The melanocortin MC_5 receptor is primarily located in various peripheral tissues but has also been found in the brain (Labbé et al. 1994; Fathi et al. 1995).

Schiöth et al. (1997b, 1998a) described binding of synthetic MSH analogs to the human melanocortin receptor subtypes.

Melanocortin MC_4 receptor antagonists increase food uptake in rats (Kask et al. 1998a, b; Skuladottir et al. 1999).

Procedure

Expression of Receptor Clones

The human melanocortin MC_1 and human melanocortin MC_5 receptors are cloned into the expression vector pRc/CMV (Invitrogen). The human melanocortin MC_3 and human melanocortin MC_4 receptors were cloned into the expression vector pCMV/neo. For receptor expression COS-1 cells are grown in Dulbecco's modified Eagle's medium with 10 % fetal calf serum. Eighty percent confluent cultures are transfected with the DNA mixed with liposomes in serum-free medium. After transfection, the serum-free medium is replaced by serum-containing medium and the cells are cultivated for 48 h. Cells are then scraped off, centrifuged, and used for radioligand binding.

Binding Studies

The transfected cells are washed with binding buffer (Schiöth et al. 1995) and distributed into 96-well nonculture-coated plates, which are centrifuged, and the binding buffer is removed. The cells are then immediately incubated in the well plates for 2 h at 37 °C with 0.05 ml binding buffer in each well containing a constant concentration of [125I]NDP (= [Nle^4-D-Phe^7]α-MSH) and appropriate concentrations of the competing

unlabeled ligand. After incubation the cells are washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity is counted by a gamma-counter.

Evaluation

Data are analyzed by fitting to formulas derived from the law of mass action. The method is generally referred to as computer modeling. K_d values for [125I]NDP are calculated (Schiöth et al. 1995, 1996a). The binding assays are performed in duplicate and repeated three times.

Modifications of the Method

Adan et al. (1994) compared the behavioral effects of melanocortins with binding data on MC_3 and MC_4 receptors.

Sahm et al. (1996) studied receptor binding affinities and biological activities of linear and cyclic melanocortins in B16 murine melanoma cells expressing the native MC_1 receptor.

Quillan and Sadée (1996) searched for peptide ligands that cross-react with melanocortin receptors and found several peptides with previously unrecognized agonistic or antagonistic activity on amphibian and human melanocortin receptors.

Peng et al. (1997) compared the actions of C-terminally modified melanocortin peptides at rodent MC_1 and MC_3 receptors.

Bagutti et al. (1993) recommended [^{111}In]DTPA-labeled (where DTPA is diethylenetriaminepentaacetic acid) analogs of α-MSH as ligands for the detection of MSH receptors in vitro and in vivo.

Erskine-Grout et al. (1996) described functional photoaffinity-labeled, biotinylated, and fluorescent probes for the melanoma MC_1 receptor.

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Melanocortin Peptides

The melanocortin system refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by components that

include the five G-protein-coupled melanocortin receptors, peptide agonists derived from the pro-opiomelanocortin preprohormone precursor, and the endogenous antagonists, agouti and agouti-related protein (Gantz and Fong 2003; Cone 2006). This group of regulatory peptides was explored in the context of understanding regulation of body weight development and finding specific antiobesity compounds (Bjorbaek and Hollenberg 2002; Della-Fera and Baile 2005; Padwal and Majumdar 2007). As with the leptin research, there are also indications for the central regulation of thermogenesis by melanotropin peptides (Myers 2004; Fan et al. 2005). The subclasses of melanocortin receptors involved in these several functions have been characterized, and the MC4 receptor is of particular interest (Garcia-Borron et al. 2005; Adan and van Dijk 2006; Adan et al. 2006; Getting 2006). Structure–activity studies on the melanocortin agonists and antagonists have provided further tools for detailed investigation (Cai et al. 2005) by modification of the natural ligands and by the search for small-molecule ligands (Todorovic and Haskell-Luevano 2005). Not surprisingly, the research on body weight regulation has also provided indications for the pathophysiology of cachexia (DeBoer and Marks 2006a, b).

Interactions of the hypothalamic–pituitary–thyroid axis with the central melanocortin system have been investigated and reviewed (Martin et al. 2006).

There has also been considerable interest in the regulation of immune responses and inflammation by melanocortin peptides (Maaser et al. 2006), based on the established relationship of α-MSH and inflammation (Eves et al. 2006; Lin and Fisher 2007).

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Relaxin

General Considerations

Relaxin is a peptide hormone originally prepared from sow ovaries. The hormone acts on collagen and induces increased flexibility of the pelvic girdle of guinea pigs. In mice, the length of the interpubic ligament is increased. Moreover, relaxin inhibits spontaneous uterine motility. In addition to its effects on the reproductive tract, relaxin exerts hemodynamic effects (Coulson et al. 1996). The actions of relaxin as a pleiotropic hormone were reviewed by Bani (1997).

The therapeutic relevance of relaxin in humans is still open to debate (Schwabe and Büllesbach 1994; Goldsmith et al. 1995).

The primary structure of relaxin is highly homologous to insulin. Unlike insulin, the structure of which is remarkably well conserved among the vertebrates, relaxin sequences can vary by more than 50 % between different species (Evans et al. 1993; Layden and Tregear 1996). Despite these large variations, most relaxins have very similar biological activities in animal test systems, probably because the receptor binding region of the B chain, in contrast to the rest of the molecule, is highly conserved between species.

Relaxin of various species has been synthesized (Büllesbach and Schwabe 1993; Wade et al. 1994).

Synthetic human relaxin has been characterized by high-performance liquid chromatography (Canova-Davis et al. 1990).

Klonisch et al. (1999) determined the nucleic acid sequence of canine **preprorelaxin** using reverse transcription-polymerase chain reaction (PCR) and rapid amplification of cDNA ends-PCR. Canine preprorelaxin consists of 534 base pairs encoding a protein of 177 amino acids with a signal peptide of 25 amino acids, a B domain of 35 amino acids, a C domain of 93 amino acids, and an A domain of 24 amino acids.

The **relaxin-like factor (RLF)** is described as a member of the insulin/relaxin/insulin-like growth factor family that is expressed predominantly in the reproductive system, with greatest expression in the Leydig cells of the testis (Pusch et al. 1996; Zarreh-Hoshyari-Khah et al. 1999).

Several **bioassays** have been used for relaxin, such as the pubic symphysis method in guinea pigs and mice, inhibition of uterine motility in rats, and stimulation of interstitial collagenase activity in cultured uterine cervical cells from guinea pigs (see below).

Taylor and Clark (1989, 1992a, b) developed a **reverse hemolytic plaque assay** which allows the quantitative analysis of relaxin secreted by single porcine luteal cells.

Radioimmunoassays for relaxin were described by Sherwood (1979), Jockenhövel et al. (1991), and Steinetz et al. (1996). Lucas et al. (1989) developed an enzyme-linked immunoassay to study human relaxin in human pregnancy and in pregnant rhesus monkeys.

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Relaxin Bioassay by Pubic Symphysis Method in Guinea Pigs

Purpose and Rationale

Experimental relaxation of the pubic symphysis of the spayed guinea pig was described as early as 1929 by Hisaw. The relaxation of the symphysis after relaxin administration is determined by manual palpation.

Procedure

Virgin female guinea pigs of mixed strains weighing 300–400 g are used. Prior to the assay, the animals have to be primed with estrogen and relaxin. Estrogen priming consists of one subcutaneous injection per week of 5 μg of estradiol cyclopentylpropionate in 0.1 ml of sesame oil. Relaxin priming is accomplished by administering 20 μg of relaxin standard in 1 ml saline subcutaneously once a week on day 5 after the estrogen injection.

Six hours after relaxin administration, the symphysis of the animal is palpated. The animal is held head down, ventral side away, between the thighs of the seated observer. The sciatic crests and symphysis pubis are firmly grasped between

the thumbs and forefingers so that the two halves of the pelvis may be moved back and forth alternately. If the pubic symphysis is rigid at this time, the estrogen and relaxin priming are continued weekly until marked mobility of the symphysis is observed. The increased flexibility is transient: the peak response occurs at 6 h and subsides 12–24 h after injection. Mobility responses are estimated subjectively and scored on an arbitrary scale of 0–6. "Zero" indicates no detectable flexibility of the pubic symphysis, whereas "6" represents extreme softening. Scoring should be performed by the same investigator throughout a study.

One week after an animal has responded positively to 20 μg of relaxin standard, it is added to the assay colony. Before assay time, all eligible animals are mixed and divided into groups of 10–20 each. On the day of the experiment, all animals are palpated before injection. Only those with no symphyseal movement are used. Two doses of test preparation and two doses of standard are injected subcutaneously to different groups. Six hours later, two operators palpate and score each animal.

Evaluation

The scores are averaged and a median score calculated. The activity of an unknown preparation is determined by comparison with the dose–response curve of concomitantly administered relaxin standard.

Modifications of the Method

Steinetz and Lust (1994) reported that the relaxin-induced pubic symphyseal relaxation in guinea pigs is inhibited by treatment with glycosaminoglycan polysulfates or pentosan polysulfate. The authors recommended the guinea pig symphysis assay for relaxin as a novel rapid screening test for compounds with potential chondroprotective activity.

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Relaxin Bioassay in Mice

Purpose and Rationale

An increase in length of the interpubic ligament in mice can be used as a bioassay for relaxin. Other methods used include X-ray measurement of pubic separation (Hall 1948; Dorfman et al. 1953) and direct measurement of interpubic ligament (Kroc et al. 1959; Steinetz et al. 1960, 1969a, b).

Procedure

Virgin female mice (e.g., NMRI strain) weighing 18–20 g are used for the assay. Twenty mice are employed for each of three dose levels of standard and test preparation. On day zero each mouse is primed with a single subcutaneous injection of 5 μg estradiol cyclopentylpropionate in 0.1 ml sesame oil. On day 7, the test preparation and the standard are injected subcutaneously. At 18–24 h later, the mice are sacrificed, the abdominal cavities are opened, and the uteri are examined for evidence of estrogen priming. Mice exhibiting threadlike uteri are discarded. The anal and vulval areas are then cut away with scissors, and the upper half of the body is cut off to prevent subsequent bleeding at the pubic symphysis. The bony birth canal is freed of the skin, vagina, and rectum, and the fascia is cleaned off

the symphysis pubis. The pelvis is placed on a binocular microscope fitted with a calibrated ocular micrometer. A transilluminating device consisting of a U-shaped Lucite rod is affixed to the microscope. The tip of the rod is beveled to direct light vertically through the exposed pubic ligament. The feet of the carcass are grasped between the thumb and index finger, applying a slight lateral traction.

The shortest distance between the edges of the pubes is measured, using the ocular micrometer. Micrometer readings are then converted to millimeters.

Evaluation

Mean values of the length of interpubic ligament, expressed in millimeters, are plotted versus logarithm of dose. From dose–response curves activity ratios with confidence limits versus the standard are calculated.

Modifications of the Method

Bullesbach and Schwabe (1996) tested rat relaxin and synthetic analogs in the mouse symphysis pubis assay for structure–activity relationships.

Samuel et al. (1998) studied the effects of relaxin, pregnancy, and parturition on collagen metabolism in the **rat** pubic symphysis. During pregnancy and particularly during birth, there was a significant reduction of tissue wet and dry weight, which coincided with an increase in water content and a significant reduction of overall collagen content.

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Inhibition of Uterine Motility

Purpose and Rationale

This is one of the historical assays based on a nonspecific biological response. Its application and reliability depend on the state of purification of relaxin preparations. Relaxin specifically inhibits the spontaneous motility of estrogen-dominated uterus in vivo and in vitro (Downing and Hollingsworth 1993). Wiqvist and Paul (1958) proposed a relaxin assay based on inhibition of motility of the rat uterus in vitro (Steinetz et al. 1969).

Procedure

The donors (female Sprague–Dawley or Wistar rats weighing 150–180 g) are ovariectomized and primed with 2 μg estradiol daily for 3 days. On day 4, uterine horns are removed, bisected, and suspended in Locke's solution gassed with 1 % CO_2 in O_2 at a temperature of 37.5 ± 0.5 °C. The contractions are recorded using a Statham transducer and a polygraph. A symmetrical 4-point assay is adopted with twofold dose increments of standard and test preparation. Responses consisting of slowing down the contraction frequency up to total suppression of the contractions

are classified visually and assigned score values of 1–3. All four test doses (two doses each of standard and test preparation) are run simultaneously on the four uterine segments obtained from each rat.

Evaluation

Mean values of scores are calculated for each dose. Potency ratios with confidence limits are calculated from the 2 + 2-point assay.

Modifications of the Method

Felton et al. (1953) described a test for relaxin activity using the inhibition of uterine contraction in anesthetized guinea pigs in vivo.

Inhibition of spontaneous and prostaglandin-driven myometrial activity by relaxin in anesthetized rats was reported by Porter et al. (1979).

Downing and Sherwood (1985) studied the influence of relaxin on uterus contractility and on cervical distensibility in different stages of pregnancy in the rat.

Del Angel Meza et al. (1991) measured the effect of relaxin on uterine and ileum tissue of rats in vivo and in vitro.

Vu et al. (1993) tested the activity of a recombinant prorelaxin in an in vitro bioassay in CHO cells. Human uterine endometrial cells were treated with various dilutions of conditioned medium, and the amounts of intracellular cAMP produced were determined by radioimmunoassay.

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Relaxin Assay by Interstitial Collagenase Activity in Cultured Uterine Cervical Cells

Purpose and Rationale

As an example of the changing approach in assay methods, Mushayandebvu and Rajabi (1995) provided evidence for a biological response suitable for in vitro assay. Relaxin is involved in cervical dilatation by stimulating interstitial collagenase, a key enzyme involved in this process. Human recombinant relaxin induces a dose-dependent increase of collagenase activity in cultured guinea pig cervical cells.

Procedure

Abdominal hysterectomy is performed in anesthetized female Hartley guinea pigs under aseptic conditions. The cervix is excised and washed three times in Hank's balanced salt solution (HBSS) containing penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (1.25 µg/ml). The cervix is cut into 2- to 4-mm pieces and digested in Dulbecco's modified Eagle's medium (DMEM) containing bacterial collagenase type A1 (0.5 mg/ml) and DNase type I (0.05 mg/ml) at 37 °C. The digested mixture is filtered once every 15–30 min through a nylon monofilament with a pore size of 400 µm. Following each filtration, separated cells are collected by centrifugation at 365 *g* for 10 min at room temperature followed by

a resuspension in DMEM containing 10 % heat-inactivated fetal calf serum (FCS). Cells are washed twice with HBSS; this is followed by resuspension in DMEM containing 10 % penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (1.25 µg/ml). The unfiltered material is redigested and the process is repeated until the cervical tissue is completely digested.

Cells are plated in 24-well plates at 1×10^5 cells per well in 500 µl of medium containing 10 % FCS and antibiotics and then are incubated in a humidified atmosphere of 5 % CO_2 in air at 37 °C. After 4 days all viable cells are adhered. Culture medium and unattached cells are removed and fresh medium is added. At confluency (5–7 days), culture medium is removed, cells are washed once with HBSS, and serum-free DMEM containing antibiotics is added. The cells are treated with recombinant human relaxin (1–1000 ng/ml) at 0, 24, and 48 h. Culture media are collected at 96 h from initial treatment with the appropriate controls and frozen immediately at −20 °C until assayed for collagenase activity (Dean and Woessner 1985; Rajabi et al. 1988, 1991).

Enzyme samples [up to 100 µl in assay buffer: 50 mM Tris–HCl (pH 7.5), 0.2 M NaCl, 10 mM $CaCl_2$, 0.02 % sodium azide, 0.035 % Brij-35] are incubated with 10 µl of [^{3}H]telopeptide-free collagen substrate [specific activity 3.1×10^6 cpm/mg collagen at 2.24 mg/ml, in 50 mM Tris–HCl (pH 7.6), 0.3 M NaCl] in 1.5-ml microfuge tubes. Aminophenylmercuric acetate (0.5 mM) is used to activate procollagenase; 1,10-phenanthroline (1 mM) is added to inhibit collagenase and serves as blank control for nonspecific collagenolysis. After 18–48 h of incubation at 29 °C, the reaction is terminated by addition of EDTA to a final concentration of 40 mM. Collagenase cleavage products are further digested by trypsin and chymotrypsin in the presence of BSA (10 mg/ml assay buffer) for 2 h at 29 °C. The undigested [^{3}H]telopeptide-free substrate is precipitated in 10 % trichloroacetic acid at 0 °C for 30 min followed by centrifugation at 18,000 *g* at 4 °C for 30 min. A 100-µl aliquot of the supernatant is mixed in 5 ml Aquasol scintillation fluid and counted in a liquid scintillation counter.

Evaluation

The percentage digestion is calculated as the total cpm in the supernatant minus cpm in the 1,10-phenanthroline blanks divided by original counts in the [^{3}H]telopeptide-free substrate (in 100 µl of 10 % trichloroacetic acid in assay buffer) × 100. One milliunit of collagenase is defined as the amount of collagenase that digests 1 ng of collagen in 1 min at 29 °C.

All results are presented as the mean ± SEM of three separate experiments. Statistical analysis is performed by Student's *t*-test for paired observations and by one-way ANOVA for multiple observations.

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Relaxin Receptor Binding

Purpose and Rationale

Using a monocomponent, high specific activity, carrier-free porcine relaxin tracer [^{125}I], Yang et al. (1992) demonstrated relaxin receptors in the symphysis pubis, uterus, and ovary of mice.

A linear Scatchard plot suggested the presence of only one kind of receptor and a dissociation constant of 5×10^{-10} M.

Procedure

Crude membranes are prepared from uterine horns of estrogen-primed mice. The tissues are homogenized three times for 10 s with a Polytron homogenizer. The homogenate is centrifuged at 700 *g* for 10 min, and the supernatants are recovered and recentrifuged twice at the same speed. Thereafter, the supernatants are centrifuged at 10,000 *g* for 30 min. The crude membrane pellets are washed with HEPES buffer, centrifuged again, and then resuspended in 200–500 µl water, and aliquots are removed for Lowry protein estimates.

The crude membrane suspension is diluted to 3 mg/ml protein with binding buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1.6 mM $CaCl_2$, 0.025 mM $MgCl_2$, 1.5 mM $MnCl_2$, 1 % BSA, 2.8 mM glucose, 0.2 mM phenylmethylsulfonylfluoride, 80 mg/l soybean trypsin inhibitor, pH 7.5). The assay is initiated by adding this to 120 µl crude membranes (300 µg protein) with and without 0.2 µg unlabeled relaxin. At the end of the incubation period, 1 ml HEPES buffer, containing 1 % BSA, is added, and bound and free relaxin are separated by three successive washings and centrifugations.

Evaluation

The tubes are analyzed for radioactivity in a Minigamma 400 (LKB, Rockville, Md., USA). Duplicates are run for each experimental point, and experiments are repeated several times.

Modifications of the Method

The control of relaxin secretion and relaxin receptors by relaxin was studied by Bryant-Greenwood et al. (1982).

Relaxin receptors in the myometrium of rats and pigs were studied by Mercado-Simmen et al. (1982a, b).

Fluoresceinylthiocarbamyl relaxin was prepared by Segaloff and Gabbard (1982) for the demonstration of relaxin receptors.

Experiments by Büllesbach and Schwabe (1988) suggested a unique site for the interaction of relaxin with its uterine and symphyseal receptors.

Using a ^{32}P-labeled human relaxin, Osheroff and Phillips (1991) localized relaxin binding sites in rat uterus, cervix, and brain.

The receptor binding site of human relaxin II was studied by Büllesbach et al. (1992).

Min and Sherwood (1996) identified specific cell types that contain relaxin receptors in various organs of pregnant pigs.

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Calcitonin-Gene-Related Peptide

General Considerations

In relation to the established traditional hormone definition, many new polypeptides have been identified by molecular biology and characterized by receptor binding and in vitro systems, while their definitive function is not yet assigned. Calcitonin-gene-related peptide (CGRP) is an example of this rapid development and change in approach by applying new methods. CGRP is a 37-amino-acid neuropeptide which was first identified as a product of the calcitonin gene by alternative splicing (Amara et al. 1982). This original peptide is referred to as α-CGRP; a second gene, unrelated to calcitonin, produces β-CGRP (Amara et al. 1985). These two forms are closely related in both rat and humans and are widely distributed throughout most parts of the nervous system. CGRP shares about 50 % identity with another 37-amino-acid peptide, amylin (Rink et al. 1993). CGRP shows weak but significant homology with adrenomedullin, a 52-amino-acid peptide (Kitamura et al. 1993). Several forms of CGRP have been sequenced: α- and β-CGRP from rat and humans and single variants from sheep, pig, chick, salmon, and the laughing frog, *Rana ridibunda* (Poyner 1997). The calcitonin/CGRP gene is expressed in specific cell types of both the endocrine and nervous systems. The gene is alternatively spliced to yield mRNA encoding calcitonin in thyroid C-cells or the neuropeptide CGRP in a subset of central and peripheral neurons (Amara et al. 1982; Morris et al. 1984; Born and Fischer 1993). The rat as well as the human calcitonin/CGRP gene consists of six exons. The calcitonin mRNA contains exons 1–4 with a poly (A) tail at exon 4, whereas α-CGRP includes exons 1, 2, 3, 5, and 6 with a poly(A) tail at exon 6 (Van Rossum et al. 1997).

α- and β-CGRP display several biological activities, including peripheral and cerebral vasodilatation, a blood-pressure-lowering effect, cardiac acceleration, regulation of calcium metabolism, reduction of intestinal motility, regulation of glucose metabolism, stimulation of pancreatic enzyme secretion, diminution of appetite, reduction of growth hormone release, influence on inflammation and nociception, and inhibition of interleukin-2 production (Poyner 1992, 1997; Wang et al. 1992; Wimalawansa 1996).

Nuki et al. (1994) compared the vasodilatory activity of chicken calcitonin-gene-related peptide with human α-CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats (see chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus").

Wisskirchen et al. (1998) tested agonists of calcitonin-gene-related peptide, homologues, and antagonists in rat isolated pulmonary artery and rat deferens (see chapter "▶ Cardiovascular Analysis In Vivo").

Tomobe et al. (1998) found that the vasodilatation in isolated superior mesenteric arteries by calcitonin-gene-related peptide was significantly larger in spontaneously hypertensive rats than in normal Wistar–Kyoto rats.

In order to elucidate the mechanism of endogenous CGRP release in peripheral vasodilatation, Brain et al. (1993) used a multiple site ^{133}Xe clearance technique.

Raddino et al. (1997) studied the mechanism of action of human calcitonin-gene-related peptide in rabbit heart and human mammary arteries.

Champion et al. (1997) analyzed the responses of human synthetic adrenomedullin and calcitonin-gene-related peptides in the hindlimb vascular bed of the cat (see chapter "▶ Effects on Blood Supply and on Arterial and Venous Tonus").

Castellucci et al. (1993) investigated the vasodilator activity of CGRP in the rat isolated and perfused kidney.

McMurdo et al. (1997) investigated the effect of the calcitonin-gene-related peptide receptor antagonist CGRP$^{8–37}$ on blood flow in the knee joint of anesthetized rats. Synovial blood flow was measured in both exposed and intact skin-covered knees by laser Doppler perfusion imaging.

Sakai et al. (1998) found a synergism of calcitonin-gene-related peptide with the blood-pressure-lowering effect of adenosine.

Preibisz (1993) reported the beneficial effects of CGRP infusions in patients with congestive heart failure and in subjects with neurological deficits after surgical treatment of subarachnoid hemorrhage.

Cadieux et al. (1999) described bronchoprotector properties of calcitonin-gene-related peptide in guinea pig and human airways. Calcitonin-gene-related peptide inhibited substance-P-induced bronchoconstriction in vivo and in vitro.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin-gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini and to relax precontracted mesenteric arteries (see chapter "► Cardiovascular Analysis In Vivo").

Meini et al. (1995) investigated the propagation of impulses in the guinea pig ureter and its blockade by calcitonin-gene-related peptide. Furthermore, Maggi et al. (1995) studied the mechanisms of the inhibitory effect exerted by calcitonin-gene-related peptide on the spontaneous activity of the guinea pig isolated renal pelvis.

Protective effects of calcitonin-gene-related peptide in different experimental models of gastric ulcers (reserpine-induced gastric lesions, ethanol-induced gastric lesions, gastric damage and acid secretion in pylorus-ligated rats) were reported by Clementi et al. (1993).

The role of nitric oxide in the antiulcer activity of calcitonin-gene-related peptide was investigated by Clementi et al. (1994a).

Evangelista and Renzi (1997) investigated the protective role of endogenous and exogenous calcitonin-gene-related peptide in water immersion stress-induced gastric ulcers in rats.

Li et al. (1997) determined the ability of analogs of human α-calcitonin-gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini (see chapter "► Pancreatic Function") and to relax isolated porcine coronary arteries precontracted with 20 mM KCl (see chapter "► Coronary Drugs").

Calcitonin-gene-related peptide acutely augments the contractile response of skeletal muscle to both direct and indirect stimulations as studied in the isolated rat diaphragm by Fleming et al. (1993).

Dumont et al. (1997) used the isolated guinea pig heart (see chapter "► Coronary Drugs") and the isolated rat vas deferens (see chapter "► Cardiovascular Analysis In Vivo") for in vitro bioassays of CGRP agonists and antagonists.

Poyner et al. (1999) found a concentration-dependent inhibition of the electrically stimulated twitch response of guinea pig vas deferens by calcitonin-gene-rated peptide, amylin, and adrenomedullin (see chapter "► Cardiovascular Analysis In Vivo").

The role of calcitonin-gene-related peptide in the protection of capsaicin-induced gastric mucosal hyperemia in rats was studied by Merchant et al. (1994).

Clementi et al. (1994b) studied the anti-inflammatory activity of calcitonin-gene-related peptide in cutaneous inflammation induced by croton oil, arachidonic acid, tetradecanoylphorbol acetate, or cantharidin.

Schaible (1996) investigated the role of tachykinins and calcitonin-gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons.

The development of tolerance to spinal morphine analgesia in rats was prevented by a calcitonin-gene-related peptide receptor antagonist (Menard et al. 1996).

Lutz et al. (1997) investigated the anorectic effects of CGRP and amylin in rats chronically cannulated in the lateral brain ventricle.

Specific calcitonin-gene-related peptide receptors were characterized in hamster pancreatic cells (Barakat et al. 1993).

In doses of 25–200 μg/kg i.p., CGRP decreased food intake in mice, suggesting a role for CGRP as a satiety factor (Morley et al. 1996).

CGRP inhibits insulin-stimulated glycogen synthesis in rat skeletal muscle (Leighton and Cooper 1988).

Chatzipantelli et al. (1996) described the lipolytic actions of calcitonin-gene-related peptide.

Howitt and Poyner (1997) determined the effects of a series of agonists and antagonists on the calcitonin-gene-related peptide receptor of cultured rat L6 skeletal myocytes.

Kurz et al. (1995) studied the receptors of calcitonin-gene-related peptide in the rat thymus and suggested that CGRP is a paracrine thymic mediator that may influence the differentiation, maturation, and proliferation of thymocytes.

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Receptor Binding of CGRP

Purpose and Rationale

Calcitonin-gene-related peptide receptors are localized in many tissues, such as the brain, heart, spleen, blood vessels, liver, lung, and kidney (Born and Fischer 1993; Muff et al. 1995). They are abundant in the brain, and the pattern is similar in the rat, pig, cow, sheep, and humans (Wimalawansa and El-Kholy 1993, Wimalawansa 1996).

Multiple CGRP receptors (subclasses) have been observed: based on pharmacological properties, they are divided into at least two subtypes and denoted as $CGRP_1$ and $CGRP_2$ (Dennis et al. 1989, 1991; Quirion et al. 1992; Poyner 1997). CGRP-(8–37), which lacks seven terminal amino acid residues, is a selective antagonist of $CGRP_1$ receptors, whereas the linear analog of CGRP, diacetamidomethyl cysteine CGRP (Cys [ACM2,7]CGRP), is a selective agonist of $CGRP_2$ receptors.

An atypical CGRP subtype has been reported (Dennis et al. 1991; Van Rossum et al. 1997).

A sensitive and specific radioreceptor assay for calcitonin-gene-related peptide was described by Wimalawansa (1989).

Procedure

For membrane preparation, tissue samples, e.g., dissected brain areas from rats, are placed in 10 vols of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose, 0.5 mM dithiothreitol, and 5 mM EDTA. After centrifugation of the homogenate at 100 *g* for 10 min, the resultant pellet is rehomogenized with 5 vols of fresh buffer and recentrifuged. The pellet is discarded and the supernatants are pooled and recentrifuged at 30,000 *g* for 45 min at 4 °C. The resulting crude membrane pellet is resuspended in 5 vols of assay buffer (50 mM Tris–HCl, 10 mM KCl, 3 mM sodium azide, and 200 IU/ml aprotinin) and recentrifuged at 30,000 *g* for a further 45 min. This procedure is repeated twice with fresh assay buffer. Protein concentrations are determined and adjusted to 1–2 mg/ml. The samples are frozen on dry ice and stored at −70 °C until radioligand binding studies.

For radioligand binding studies, [^{125}I]CGRP (25 pM, ~5 fmol containing ~20,000 cpm/tube) is incubated with various membrane preparations (150–200 µg/ml membrane protein in an incubation medium of 200 µl) in the presence of 200 IU/ml aprotinin and 0.2 % heat-inactivated BSA at 4 °C for 120 min in polypropylene microcentrifuge tubes in a shaking water bath. At the end of the incubation period, 700 µl of chilled assay buffer is added and immediately centrifuged at 11,000 *g* for 2 min in a refrigerated microcentrifuge. The supernatant is discarded and the resulting membrane pellet is rewashed, and the radioactivity remaining in the pellet is counted.

Evaluation

Specific binding is calculated by subtracting the [^{125}I]CGRP binding in the presence of unlabeled CGRP. Results are expressed as fmol of [^{125}I] CGRP bound/mg membrane protein.

Modifications of the Method

Van Rossum et al. (1994) described the binding profile of a selective calcitonin-gene-related peptide receptor antagonist ligand, [^{125}I-Tyr] $hCGRP_{8–37}$, in rat brain and peripheral tissues.

Muff et al. (1992) reported that the calcitonin-gene-related peptide receptor in a human neuroblastoma cell line (SK-N-MC) is clearly different from the calcitonin receptor in a human breast carcinoma cell line (T47D).

Aiyar et al. (1996) described a cDNA encoding the calcitonin-gene-related peptide type-I receptor. Stable expression in human embryonic kidney 293 (HEK293) cells produced specific high-affinity binding sites for CGRP that displayed

pharmacological and functional properties very similar to those of the native human $CGRP_1$ receptor.

Poyner et al. (1999) characterized the receptors for calcitonin-gene-related peptide and adrenomedullin on the guinea pig vas deferens.

Juaneda et al. (2000) reviewed the molecular pharmacology of CGRP and related peptide receptor subtypes.

Schindler and Doods (2002) described the binding properties of the nonpeptide CGRP receptor antagonist radioligand, [^{3}H]BIBN4096BS.

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Inhibin

General Considerations

Inhibin is a gonadal dimeric glycoprotein that has an inhibitory effect on the secretion of follicle-stimulating hormone by the pituitary gland.

The existence of a nonsteroidal gonadal hormone was described by McGullagh as early as 1932. The hormone was isolated and purified from human seminal plasma, from ram rete testis fluid, and from bovine follicular fluid (Franchimont et al. 1979, 1989; Robertson et al. 1986; Vale et al. 1986; De Kretser and Robertson 1989). After the determination of the full structure of bovine (Forage et al. 1986) and human (Mason et al. 1986; Stewart et al. 1986; Tierney et al. 1990) inhibin, it was found that inhibin shares structural homology with a family of glycoproteins which includes Mullerian-inhibiting substance, transforming growth factor-β, follistatin, activin, and bone morphogenic proteins (Robertson 1991; Moore et al. 1994). Inhibin is a disulfide-linked dimer of an α-subunit and a structurally related β-subunit, either β_A or β_B (Robertson et al. 1992). Inhibin A and inhibin B are related dimeric protein hormones and endocrine regulators of the reproductive axis which show differing patterns during the period of follicular development (Woodruff et al. 1996).

Clinically, inhibin determination may have uses in reproductive medicine and oncology; the diagnosis of some forms of cancer including granulosa cell tumors, cystadenocarcinoma of the ovary, and hydatidiform mole; and in the physiology and pathology of pregnancy including placental function (Halvorson and DeCherney 1996).

An international standard for porcine inhibin was described by Gaines Das et al. (1992). An international standard for human recombinant inhibin was established by Rose and Gaines Das (1996).

Tio et al. (1994) purified gonadotropin surge-inhibiting factor (GnSIF), a monomeric polypeptide that shares some biological activities with inhibin, from Sertoli cell-enriched medium.

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In Vitro Bioassay for Inhibin

Purpose and Rationale

Several modifications of in vitro bioassays for inhibin have been used to establish the international standard for porcine inhibin (Gaines Das et al. 1992) and for human recombinant inhibin (Rose and Gaines Das 1996). Mason et al. (1996) used rat anterior pituitary cells for characterization and determination of the biological activities of noncleavable high-molecular-weight forms of inhibin A and activin A.

Procedure

Anterior pituitary cells are prepared from adult male rats and added to 48-well cluster plates at an initial density of 75,000 cell per 0.4 ml of medium per well. Cultures are preincubated in DMEM–Ham's F12 (1:1) medium with bicarbonate supplemented with nonessential amino acids, antibiotics (100 U/ml penicillin, 100 pg/ml streptomycin, and 250 ng/ml Fungizone), and 10 % charcoal-stripped FBS. Two days later, the cells are washed, and the medium is replaced with serum-free medium supplemented with antibiotics and recombinant human inhibin A (20–100 pM) as standard. Cultures are incubated with test samples for 65–72 h, after which the samples of medium are collected and stored at −20 °C until assayed for follicle-stimulating hormone (FSH) by radioimmunoassay (RIA) using goat second antibody precipitation with the following reagents (NIDDK): rat FSH-RP-2, rat FSH-I-8 iodinated using Iodogen, and anti-rat FSH-S-11 primary antiserum. Each sample for bioassay is tested at least twice in independent cell cultures to confirm the observations.

Evaluation

Characteristics of the concentration–response curve, including the maximum effect and medium inhibitory concentration (IC_{50}), are computed using the ALLFIT program (Munson and Rodbard 1980).

Modifications of the Method

Robertson et al. (1991) studied in vivo the FSH-suppressing activity of human recombinant inhibin A in the serum of male and female rats.

Wreford et al. (1994) studied the age dependence of gonadotropin-suppressing activity of human recombinant inhibin in the serum of male rats.

Simpson et al. (1992) induced bilateral cryptorchidism in adult male Sprague–Dawley rats under ether anesthesia by cutting the gubernaculum of both testes, translocating the testes to the abdominal cavity, and ligating the inguinal canal to prevent redescent of the testes into the scrotum. After 28 days, Sertoli cell cultures from these rats were prepared, and inhibin secretion in response to follicle-stimulating hormone (FSH) was measured.

Brown et al. (1991) investigated the effects of inhibin-rich porcine follicular fluid administration on serum bioactive and immunoreactive FSH

concentrations and compensatory testosterone secretion in hemicastrated adult rats.

Hertan et al. (1999) used primary cultures of ovine anterior pituitary cells for bioassays of inhibin and identified high-affinity binding sites for inhibin using iodinated recombinant human 31-kDa inhibin.

Jakubowiak et al. (1989) found similar effects of inhibin and cycloheximide on gonadotropin release in superfused rat pituitary cell cultures.

Demura et al. (1996) studied the levels of inhibin α, β_A, and β_B subunit mRNAs by a quantitative reverse transcription-polymerase chain reaction and the changes in their levels caused by adding inhibin α, β_A, and β_B subunit mRNA antisense oligonucleotides and inhibin A, activin A, or gonadotropin-releasing hormone (GnRH) to cultured rat anterior pituitary cells.

Robertson et al. (1996) investigated the specificity of several immunoassay methods in terms of their ability to detect the range of inhibin forms found in the plasma and their relationship to bioactivity.

A commercially available enzyme-linked immunosorbent assay (ELISA) for inhibin A (Serotec, Oxford) was used by several authors (Wenstrom et al. 1997; Blumenfeld et al. 1998; Wallace et al. 1998). Magoffin and Jakimiuk (1998) used specific and sensitive two-site enzyme-linked immunosorbent assays for determination of inhibin A, inhibin B, and activin A.

An in vitro method has been developed by Allenby et al. (1991) for culturing isolated seminiferous tubules from adult rats for 1–3 days and optimized on the basis of the secretion of immunoactive inhibin under basal conditions and after maximal stimulation with rat FSH or dibutyryl cAMP. Inhibin was measured using a double-antibody radioimmunoassay based on an antibody generated in a sheep to the 1–26 sequence (plus glycine^{27} and tyrosine^{28}) of the N-terminus of the α-subunit of porcine 32-kDa inhibin. The effect of three known testicular toxicants (meta-dinitrobenzene, nitrobenzene, and methoxy acetic acid) on these cultures was assessed.

Knight et al. (1991) described the development of a two-site immunoradiometric assay for dimeric inhibin using antibodies against chemically synthesized fragments of the α- and β-subunit. Knight and Muttukrishna (1994) described the measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidized (Met O) inhibin.

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Activin

General Considerations

Activin is a pluripotent growth factor that was originally isolated based on its ability to stimulate follicle-stimulating hormone (FSH) but is now known to have other important roles during development, erythropoiesis, inflammation, and wound healing (De Paolo 1997). Three types of activin, formed by dimerization of two inhibin-β-subunits β_A and β_B, are termed activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$), and activin B ($\beta_B\beta_B$) (Ling et al. 1986; Vale et al. 1986; Mason et al. 1989; Nakamura et al. 1992). In addition, a third β-subunit (β_C) has been identified (Hötten et al. 1995; Loveland et al. 1996). Lee et al. (1989) suggested that, in the testis, the Leydig cells secrete activin and the Sertoli cells produce inhibin or a combination of both. As members of the transforming growth factor-β (TGF-β) family, the activins are involved in a diverse range of physiological processes, including the regulation of FSH biosynthesis and secretion (Mason 1988; MacConnell et al. 1999), steps in embryonic development (Thomsen et al. 1990), spermatogonial mitosis (Mather et al. 1990), and erythroid differentiation (Eto et al. 1987). Activin acts via a family of activin receptor subunits that includes one type-I (Act RI or ALK-2) and two homologous type-II (IIA and IIB) subunits (Dalkin et al. 1996; Hashimoto et al. 1998).

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In Vitro Bioassay for Activin

Purpose and Rationale

In vitro bioassays for activin have been developed using the production of follicle-stimulating hormone (FSH) from cultured pituitary cells (Robertson et al. 1992), the accumulation of hemoglobin in K562 erythroleukemia cells (Schwall and Lai 1991), and the ability to induce mesoderm tissue in animal cap explants of *Xenopus* (De Winter et al. 1992). One of the sites of production of activin A is the bone marrow (Shao et al. 1992; Yamashita et al. 1992; Uchimaru et al. 1995). Brosh et al. (1995) found that the mouse plasmacytoma cell line MPC-11 (Laskov and Scharff 1970) was exquisitely sensitive to inhibition by activin A without being influenced by a variety of other cytokines and growth factors. On this basis, Phillips et al. (1999) evaluated the MPC-11 cell line as the basis for an in vitro bioassay for activin.

Procedure

MPC-11 plasmacytoma cells are cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 24 mM bicarbonate, L-glutamine, nonessential amino acids, and Pen–Strep in a 95 % air/5 % CO_2 atm at 37 °C and passaged at 1×10^6 cells/25-cm^2 flask every second day. For experiments,

100 µl cell suspension is added to 96-well plates at a density of 1000 viable cells/well in culture medium containing 25 µM β-mercaptoethanol. In activin, other test reagents and sera (100 µl/well) are added to the cultures diluted in phosphate buffer (pH 7.4) containing 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 0.01 % BSA, and Pen–Strep. Cells are cultured in the presence of test reagents for 2 days; on the third day 25 µl [^{3}H]thymidine (0.25 µCi/25 µl, 6.7 Ci/mmol) is added to each well, and 24 h later the cells are harvested and thymidine incorporation is assessed. Proliferation of MCP-11 cells, as measured by thymidine incorporation, is inhibited by increasing doses of activin A or activin B, whereas follistatin, inhibin A, LH, and interleukin-1β are ineffective.

Evaluation

All test preparations, including activin standards and sera, are assayed in quadruplicate, and experiments are repeated at least twice. Relative bioactivity and parallelism are assessed using parallel-line bioassay statistics.

Modifications of the Method

Mesoderm induction assays in *Xenopus* were used by Wuytens et al. (1999). *Xenopus* embryos were obtained by in vitro fertilization. They were maintained in 10 % Normal Amphibian Medium and staged according to Nieuwkoop and Faber (1967). Animal pole regions were dissected from mid-blastula (stage 8) embryos and cultured in 75 % Normal Amphibian Medium containing 0.1 % bovine serum albumin and wild-type or mutant activin (2.5 ng/ml). A preliminary assessment of mesoderm induction was based on the elongation of the animal caps. Animal pole regions were then frozen on dry ice, and expression of the mesoderm-specific gene Brachyury (Xbra) was assessed by RNase protection analysis.

LaPolt et al. (1989) examined the effects of purified porcine activin on inhibin secretion and messenger RNA levels in cultured granulosa cells obtained from immature, estrogen-treated rats. Western blot analyses performed with affinity-purified antisera to inhibin α- and β_A-subunits revealed that treatment with either FSH or activin increased the secretion of inhibin $\alpha\beta$ dimer, with a further increase after co-treatment.

Attardi and Miklos (1990) examined the effect of purified recombinant human activin A on steady-state levels of mRNAs for the gonadotropin subunits in pituitary cell cultures prepared from adult male rats.

Carroll et al. (1991) used rat anterior pituitary cells in vitro and determined the apparent half-life of FSHb mRNA in the presence and absence of recombinant human activin A after addition of actinomycin D.

Demura et al. (1993) measured follistatin-free activin and inhibin in the culture medium of porcine granulosa cells by a competitive protein binding assay and N-fragment RIA, respectively. Both activin and inhibin were secreted under the control of FSH and LH.

Miyamoto et al. (1999) investigated the effect of activin A on the secretion of LH, FSH, and prolactin by female cultured rat pituitary cells at the single-cell level by means of the cell immunoblot assay. Anterior pituitary cells were preincubated with or without activin A for 24 h, after which they were monodispersed and immediately used for cell immunoblot assay.

Peng et al. (1999) examined the expression of activin receptor mRNAs in human ovary and placenta. Primers specific for two type-I and two type-II receptors (ActR-I, Act-RI_B, ActR-II, and Act-RII_B) were used in polymerase chain reaction to amplify cDNAs prepared from granulosa–luteal cells, placental tissues, and trophoblast cells.

Liu et al. (1996) measured the release of immunoreactive activin A from cultured rat anterior pituitary cells by a specific radioimmunoassay.

Shintani et al. (1991) developed a radioimmunoassay for the measurement of activin A, which is identical to erythroid differentiation factor.

For measurement of activin in biological fluids by radioimmunoassay, McFarlane et al. (1996) added sodium deoxycholate, Tween 20, and sodium dodecyl sulfate as dissociating agents in order to remove interference by follistatin.

Knight et al. (1996) developed a two-site enzyme immunoassay for the determination of total activin A concentrations in serum and follicular fluid.

Saito et al. (1991) developed an assay method for activin-binding protein, which exploits its high affinity for sulfated polysaccharides, and used this method to investigate the production of activin-binding protein by rat ovarian granulosa cells in vitro.

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Follistatin

General Considerations

Follistatin is a monomeric glycosylated polypeptide chain which was identified from bovine and porcine follicular fluids on the basis of its inhibition of pituitary follicle-stimulating hormone secretion (Robertson et al. 1987; Ueno et al. 1987; Ying et al. 1987; Bohnsack et al. 2000). Follistatin exerts its inhibitory effect on FSH secretion by neutralizing activin activity (Namakura et al. 1990; Shimonaka et al. 1991; De Winter et al. 1996). Follistatin is able to bind and neutralize the actions of many members of the transforming growth factor-β family of proteins and plays a significant role during organogenesis (Patel 1998). There are two main forms of mature mammalian follistatin which occur as a result of alternative modes of precursor mRNA splicing, giving core proteins of 315 amino acids and the carboxy-truncated variant of 288 amino acids. Further variants in the molecular weight of mature follistatin occur as a result of varying degrees of glycosylation (Inouye et al. 1991; Sugino et al. 1993).

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Immunoassay for Follistatin

Purpose and Rationale

Evans et al. (1998) developed an ultrasensitive two-site enzyme immunoassay for human follistatin.

Procedure

Female BALB/c mice are immunized subcutaneously with 20 μg recombinant human follistatin rh-FS288 in an emulsion with complete Freund's adjuvant. The immunization is repeated on two further occasions at monthly intervals in complete Freund's adjuvant, before finally boosting intravenously with rh-FS288 (total 100 μg in saline). The spleen is removed and the splenocytes are fused to Sp2/0 myeloma cells using polyethylene glycol following a standard fusion protocol (Galfre and Milstein 1981). Hybridoma supernatants are screened on a 96-well plate coated with rh-FS288 (0.2 μg/ml in sodium bicarbonate buffer, pH 9.4). Positive clones are expanded and recloned in methyl cellulose. The supernatants from these clones are titrated against rh-FS288 (0.2 μg/ml) under a standard ELISA protocol (Groome et al. 1995). On the basis of these experiments, clones are selected (29/9 and 17/2) and isotyped with a commercial kit. The clones are grown to produce ascitic fluid in pristane-primed BALB/c mice. Purification of IgG is carried out using protein-G affinity chromatography.

Antibody to a selected clone (29/9) diluted in 0.2 M sodium bicarbonate buffer, pH 9.4, is coated by simple absorption onto 96-well ELISA plates overnight at room temperature (10 μg/ml). The following day, the plates are banged to dryness on paper toweling, and then 100 μl of dry coat reagent per well is added to the plates. After a 1-h incubation at room temperature, the plates are banged to dryness and stored in a sealed box.

Both standard (rh-FS288) and samples are diluted in dissociation solution (84 mmol/l sodium deoxycholate, 3.4 % Tween 20, 1 % BSA, 5 % mouse serum in PBS). Standards are prepared by serially diluting the stock rh-FS288 to give a high standard of 2500 pg/ml and a low standard of 19.53 pg/ml.

Duplicate 50-μl amounts of standard or test samples are added to wells on the plate, which is then sealed and incubated overnight stationary at room temperature in a sealed moist box. The following day, the plate is washed and to each well is added 50 μl of approximately 1 μg/ml of Fab fragment of clone 17/2, which has previously been coupled to alkaline phosphatase by heterobifunctional chemistry (Ishikawa et al. 1983). This is diluted in Tris conjugate buffer: 1 % BSA in 25 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl and 0.5 % Tween 20. After a 2-h incubation in a moist chamber at room temperature, the plate is washed thoroughly and banged to dryness on paper toweling. Alkaline phosphate substrate (50 μl) is added to each well and the plate incubated for 2 h stationary at room temperature. Amplifier solution (50 μl) is then added to each well, and the ensuing chromogenic reaction is stopped by adding 0.4 M HCl (50 μl/well) once the color begins to develop in the zero analyte wells, and the top standard has an absorbance of approximately 1.8. The well absorbencies are read at 490 nm with a reference wavelength set at 620 nm using a microplate reader.

Evaluation

To determine whether dose–response relationships of serially diluted standard and test samples are identical (parallel), the slope values (±95 % confidence intervals) of log-transformed data for each response curve are compared by linear regression. The curves are deemed to be parallel if the slopes (±95 % confidence intervals) are found to overlap.

Modifications of the Method

Nakamura et al. (1992) investigated the effect of follistatin on activin-induced granulosa cell

differentiation in freshly harvested granulosa cells from diethylstilbestrol-treated rats. Activin induced a remarkable change in granulosa cellular morphology from elongated fibroblast-like to round cells, which was prevented by follistatin.

Xiao et al. (1992) studied the effects of activin and follistatin on FSH receptors and differentiation of cultured rat granulosa cells in vitro.

DePaolo et al. (1991) determined FSH and LH levels by RIA in the serum of ovariectomized rats and compared in vivo the FSH-suppressing activity of follistatin and inhibin.

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Further Peptide Hormones Discussed in Chapters Related to the Respective Indications

Further peptide hormones are discussed in chapters related to the respective indications such as:

Chapter A	**Cardiovascular Activity**
Renin–Angiotensin System	Inhibition of the Angiotensin-Converting Enzyme in Vitro
Renin–Angiotensin System	Inhibition of Neutral Endopeptidase (Neprilysin)
Renin–Angiotensin System	Angiotensin Receptor Binding
Renin–Angiotensin System	Renin Activity
PAF Binding and Endothelins Activity	Endothelin
PAF Binding and Endothelins Activity	Inhibition of Endothelin-Converting Enzyme
Effect of Different Peptides	Adrenomedullin
Effect of Different Peptides	Urotensin II
Effect of Different Peptides	Apelin
Chapter E	**Psychotropic and Neurotropic Activity**
Neuroleptic Activity	Neurotensin
Chapter F	**Drug Effects on Learning and Memory**
Non-behavioral Methods Used in the Study of Learning and Memory	Secretion of Nerve Growth Factor (Cultured Neurons/Astroglial Cells)
Chapter H	**Analgesic, Anti-Inflammatory, and Anti-Pyretic Activity**
Central Analgesic Activity	Nociceptin
Central Analgesic Activity	Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Peptide (PACAP)
Peripheral Analgesic Activity	Antagonism to Nerve Growth Factor
Anti-Inflammatory Activity	$_3$H-Bradykinin Receptor Binding
Anti-Inflammatory Activity	Substance P and the Tachykinin Family
Anti-Inflammatory Activity	Neurokinin Receptor Binding

(*continued*)

Anti-Inflammatory Activity	Influence of Cytokines
Anti-Inflammatory Activity	Screening for Interleukin-1 Antagonists
Anti-Inflammatory Activity	Inhibition of Interleukin-1β-Converting Enzyme (ICE)
Anti-Inflammatory Activity	Nuclear Factor-κB
Anti-Inflammatory Activity	Binding to Interferon Receptors
Anti-Inflammatory Activity	Influence of Peroxisome Proliferator-Activated Receptors (PPARs) on Inflammation
Chapter J	**Activity on the Gastrointestinal Tract**
Pharmacological Effects on Gastric Function	Gastrin Activity
Pharmacological Effects on Gastric Function	Gastrin-Releasing Peptide/Bombesin/ Neuromedin
Pharmacological Effects on Gastric Function	Bombesin Receptor Binding
Pancreatic Function	Somatostatin Activity
Pancreatic Function	Secretin Activity
Pancreatic Function	Cholecystokinin
Chapter K	**Antidiabetic Activity**
Measurement of Insulin and Other Glucose-Regulating Peptide Hormones	Glucagon-Like Peptide I
Measurement of Insulin and Other Glucose-Regulating Peptide Hormones	Insulin-Like Growth Factors
Measurement of Insulin and Other Glucose-Regulating Peptide Hormones	Amylin
Chapter L	**Antiobesity Activity**
Assays of Anti-Obesity Activity	Uncoupling Protein and GLUT4 in Brown Adipose Tissue
Assays of Obesity-Regulating Peptide Hormones	Agouti-Related Protein
Assays of Obesity-Regulating Peptide Hormones	Melanin Concentrating Hormone (MCH)
Assays of Obesity-Regulating Peptide Hormones	Cocaine- and Amphetamine-Regulated Transcript (CART)
Assays of Obesity-Regulating Peptide Hormones	Resistin
Assays of Obesity-Regulating Peptide Hormones	Leptin
Assays of Obesity-Regulating Peptide Hormones	Neuropeptide Y
Assays of Obesity-Regulating Peptide Hormones	Orexin
Assays of Obesity-Regulating Peptide Hormones	Galanin
Assays of Obesity-Regulating Peptide Hormones	Adipsin
Assays of Obesity-Regulating Peptide Hormones	Ghrelin
Chapter M	**Anti-atherosclerotic Activity**

(*continued*)

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Part XV

Ophthalmologic Activity

Intraocular Pressure

Beat P. Mertz

Contents

Purpose and Rationale

Intraocular pressure (**IOP**) is the fluid pressure inside the eye. Tonometry is the method eye care professionals use to determine this. IOP is an important aspect in the evaluation of patients at risk from glaucoma. Most tonometers are calibrated to measure pressure in millimeters of mercury (mmHg).

Physiology

Intraocular pressure is mainly determined by the coupling of the production of aqueous humor and the drainage of aqueous humor mainly through the trabecular meshwork located in the anterior chamber angle.

An important quantitative relationship is provided below:

$$\mathrm{IOP} = \mathrm{F}/\mathrm{C} + \mathrm{PV}$$

where F = aqueous fluid formation rate, C = outflow rate, and PV = episcleral venous pressure. The above factors are those that drive IOP.

Procedure

Intraocular pressure is measured with a tonometer as part of a comprehensive eye examination. Tonometry is a test to measure the pressure inside your eyes. The test is used to screen for glaucoma.

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People over age 40, especially African Americans, have the highest risk for developing glaucoma. Regular eye exams can help detect glaucoma early. If it is detected early, glaucoma can be treated before too much damage is done.

The test may also be done before and after eye surgery.

The most accurate method measures the force needed to flatten a certain area of the cornea.

- The surface of the eye is numbed with eye drops. A fine strip of paper stained with orange dye is touched to the side of the eye. The dye stains the front of the eye to help with the examination.
- The slit lamp is placed in front of you, and you rest your chin and forehead on a support that keeps your head steady. The lamp is moved forward until the tip of the tonometer just touches the cornea.
- The health-care provider looks through the eyepiece on the lamp and the machine gives a pressure reading. There is no discomfort with the test.

A slightly different method uses a handheld device similar in shape to a pencil. Again, you are given numbing eye drops to prevent any discomfort. The device touches the outside of the eye and instantly records eye pressure.

The last method is the noncontact method (air puff). In this method, your chin rests on a padded stand.

- You stare straight into the examining device. The eye doctor shines a light into your eye to properly line up the instrument and then delivers a brief puff of air at your eye.
- The machine measures eye pressure by looking at how the light reflections change as the air hits the eye.

Measured values of intraocular pressure are influenced by corneal thickness and rigidity (Grieshaber et al. 2007; Tanaka 1998). As a result, some forms of refractive surgery (such as photorefractive keratectomy) can cause traditional intraocular pressure measurements to appear normal when in fact the pressure may be abnormally high. Normal eyes with thick corneas have higher readings and normal eyes with thin corneas have lower readings. A thin cornea with a high reading may be very abnormal (the actual eye pressure will be higher than shown on the tonometer). Currently, a corneal thickness measurement (pachymetry) is needed to get a correct pressure measurement. There is a device now available to asses this aspect, the IOPac™ pachymeter, a powerful, pocket-sized CCT measurement for glaucoma diagnosis made by Starfish Medical (Victoria, BC, Canada). IOPac™ pachymeters provide powerful, pocket-sized corneal measurements for glaucoma diagnosis and are used by optometrists and ophthalmologists.

Evaluation

Current consensus among ophthalmologists and optometrists defines normal intraocular pressure as that between 10 and 20 mmHg (webMD 2012; Noecker 2014). The average value of intraocular pressure is 15.5 mmHg with fluctuations of about 2.75 mmHg (Janunts 2014).

Ocular hypertension (OHT) is defined by intraocular pressure being higher than normal, in the absence of optic nerve damage or visual field loss (Viera et al. 2006; American Optometric Association).

Hypotony, or ocular hypotony, is typically defined as intraocular pressure equal to or less than 5 mmHg (Sanders 2014; Henderer et al. 1999). Such low intraocular pressure could indicate fluid leakage and deflation of the eyeball.

Critical Assessment and Influencing Factors

Daily Variation

Intraocular pressure varies throughout the night and day. The diurnal variation for normal eyes is between 3 and 6 mmHg and the variation may increase in glaucomatous eyes. During the night, intraocular pressure may not decrease (Liu and

Weinreb 2011) despite the slower production of aqueous humor (Brubaker 1991). In the general population, IOP ranges between 10 and 21 mmHg with a mean of about 15 or 16 mmHg (plus or minus 3.5 mmHg during a 24-h cycle).

Fitness and Exercise

There is some inconclusive research that indicates that exercise could possibly affect IOP (some positively and some negatively) (Koçer and Dane 1999; Qureshi 1995). However, some other forms of exercise may raise IOP (Viera et al. 2006).

Musical Instruments

Playing some musical wind instruments has been linked to increases in intraocular pressure. One 2011 study focused on brass and woodwind instruments observed "temporary and sometimes dramatic elevations and fluctuations in IOP" (Schmidtmann et al. 2011). Another study found that the magnitude of increase in intraocular pressure correlates with the intraoral resistance associated with the instrument and linked intermittent elevation of intraocular pressure from playing high-resistance wind instruments to incidence of visual field loss (Schuman et al. 2000). The range of intraoral pressure involved in various classes of ethnic wind instruments, such as Native American flutes, has been shown to be generally lower than Western classical wind instruments (Clinton 2013).

Other Factors

Intraocular pressure also varies with a number of other factors such as heart rate, respiration, fluid intake, systemic medication, and topical drugs. Alcohol consumption leads to a transient decrease in intraocular pressure, and caffeine may increase intraocular pressure (Pardianto et al. 2005a).

Taken orally, glycerol (often mixed with fruit juice to reduce its sweet taste) can cause a rapid, temporary decrease in intraocular pressure. This can be a useful initial emergency treatment of severely elevated pressure (Drance 1964).

Significance

Ocular hypertension is the most important risk factor for glaucoma.

Intraocular pressure has been measured as a secondary outcome in a systematic review comparing the effect of neuroprotective agents in slowing the progression of open-angle glaucoma (Sena and Lindsley 2013).

Differences in pressure between the two eyes are often clinically significant and potentially associated with certain types of glaucoma, as well as iritis or retinal detachment.

Intraocular pressure may become elevated due to anatomical problems, inflammation of the eye, injury to the eye or head, hyphema, genetic factors, and glaucoma or as a side effect from medication. Intraocular pressure usually increases with age and is genetically influenced (Pardianto et al. 2005b).

Risks

If the applanation method is used, there is a small chance the cornea may be scratched (corneal abrasion). This will normally heal itself within a few days.

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Aqueous Humor Flow Rate

Beat P. Mertz

Contents

Purpose and Rationale

According to CB Toris and CB Camras, September 2007, measuring the outflow of aqueous humor has helped elucidate the pathophysiology of glaucoma and the mechanism of action (MoA) of IOP-lowering treatments. However, difficulties are inherent in each method of measurement, and these issues need to be considered when assessing flow rate. Both authors best describe the situation below.

The production, circulation, and drainage of aqueous humor into and out of the anterior chamber of the eye maintain the IOP at a relatively constant level (aqueous humor dynamics). When the pressure is higher than normal, the problem usually resides in the tissues of the drainage pathways, areas targeted by many IOP-lowering drugs, surgical procedures, and drainage devices. Aqueous humor drainage is measured by several methods, each with advantages and inherent weaknesses. Understanding the limitations of each method, as noted above, ensures a proper interpretation of the results of clinical trials and animal studies, an essential aspect of this assessment.

This was primarily based on the work by Carol B. Toris, PhD, Professor, and Director of Glaucoma Research for the Department of Ophthalmology and Visual Sciences at the University of Nebraska Medical Center in Omaha, and Carl B. Camras, MD, Professor, and Director of the Glaucoma Service and is Chairman of the Department of Ophthalmology and Visual Sciences at the University of Nebraska Medical Center in Omaha.

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Procedure

Trabecular Outflow Facility

Overview

The trabecular meshwork offers a certain resistance to the outflow of aqueous humor that is needed to maintain a steady-state IOP. The inverse of this resistance is trabecular outflow facility, a measure of the compliance of the trabecular meshwork.

Techniques for Measuring Outflow Facility

Tonography

Researchers use a Schiotz tonometer or the tonography setting on a pneumotonometer to determine outflow facility in a noninvasive manner. Tonography was developed originally in the 1940s to assess outflow facility in patients and assist in the diagnosis of glaucoma. A value of less than 0.2 μL/min per mmHg generally was considered to be in the glaucomatous range. Tonography shows a wide variation among healthy subjects and in the same patient over the course of several visits. Nevertheless, it continues to be a valuable research tool in studies of aqueous humor dynamics in human and animal eyes.

The tonography procedure involves placing a tonometer probe with a calibrated weight on the anesthetized cornea of the supine subject for 2 or 4 min. The weight causes the IOP to rise initially, but, over time, the pressure slowly decreases, because aqueous humor drains at an increased rate from the anterior chamber into the drainage pathways. The drop in IOP during the measurement is assumed to be caused solely by increased drainage of aqueous humor from the trabecular meshwork, the pressure-dependent pathway. The rate of fluid outflow from the eye during the time of the test is determined from reference tables (Friedenwald 1948). Outflow facility is the ratio of the flow rate (from tables) to the change in pressure (determined by tonometry). If the IOP decreases little during the test, the fluid flow rate would be small and trabecular outflow facility would be calculated to be low. This is expected in the eyes with ocular hypertension with or without glaucoma.

An important factor affecting the tonography measurement is ocular rigidity. This factor is a measure of the resistance that the eye exerts to distending forces. The stiffer the eye, the greater the ocular rigidity, with more force required to indent the cornea. Ocular rigidity increases by 25 % in older versus younger people (Armaly 1959; Gaasterland et al. 1978). Elderly eyes are less compliant than younger eyes; thus, measurements of outflow facility assessed by tonometry are lower in older individuals on the basis of increased ocular rigidity rather than a true reduction in outflow facility. Tonography performed with an indentation tonometer (Schiotz) assumes that the change in pressure, as a function of time, is based on the accuracy of the ocular-rigidity coefficient during the measurement. Indentation tonography makes no compensation for individual variations in ocular rigidity. Tonography assessed with a pneumatic tonography unit is less affected by ocular rigidity than the Schiotz unit, because the probe that is acting on the eye creates a relatively smaller corneal indentation. Both instruments derive a change in flow from standard tables. There is a device now available to asses this aspect, the IOPac Pachymeter, a powerful, pocket-sized CCT measurement for glaucoma diagnosis made by StarFish Medical (Victoria, BC, Canada). IOPac™ Pachymeters provide powerful, pocket-sized corneal measurements for glaucoma diagnosis and are used by optometrists and ophthalmologists.

CB Toris and CB Camras (September 2007) describe outflow facility as measured by tonography (Cton) that includes pseudofacility (Cps) and uveoscleral outflow facility (Cfu), in addition to trabecular outflow facility (Ctrab), as in Eq. 1:

$$\text{Cton} = \text{Ctrab} + \text{Cfu} + \text{Cps}. \qquad (1)$$

Cfu is the facility of fluid flow through the ciliary muscle. This facility is about 10-fold less than trabecular outflow facility. Pseudofacility is the facility of the flow of aqueous humor from the

posterior chamber into the anterior chamber, resulting from the probe-induced increase in IOP. An assumption in tonography is that the rate of aqueous humor's inflow into the anterior chamber during the measurement remains unchanged by the applied pressure (i.e., pseudofacility is zero). If pseudofacility and/or uveoscleral outflow facility are disturbed during the measurement, a change in tonographic outflow facility may not indicate a change in true trabecular outflow facility.

Fluorophotometry

Fluorophotometry provides another way to assess outflow facility (Hayashi et al. 1989). Aqueous flow (F) is determined by measuring the disappearance rate of a tracer from the anterior chamber. Next, an aqueous flow suppressant such as acetazolamide, dorzolamide, or timolol is given to reduce the IOP and aqueous flow. Brimonidine and apraclonidine are not appropriate for this purpose, because these drugs affect outflow, as well as aqueous flow. The drug-induced change in IOP (IOP2−IOP1) is measured by tonometry, and the change in aqueous flow (F2−F1) is measured by fluorophotometry. Outflow facility is calculated by Eq. 2:

$$C = (F2 - F1)/(IOP2 - IOP1). \quad (2)$$

C by fluorophotometry usually is labeled Cfl.

The main advantage of fluorophotometry over tonography is that fluorophotometry directly measures changes in aqueous flow, instead of referencing standard tables. Additionally, ocular rigidity and pseudofacility are not part of the measurement, because a weight is not applied to the eye. Researchers have found different results and come to different conclusions when using tonography versus fluorophotometry to assess outflow facility. For example, 1 week of twice-daily treatment with apraclonidine did not change outflow facility when measured by tonography, but it increased outflow facility when measured by fluorophotometry (Toris et al. 1989). The reason is because apraclonidine was thought to reduce pseudofacility, an effect that hid the increase in trabecular outflow facility when measured by tonometry but not fluorophotometry (see Eq. 1). In another example, there is an age-related decrease in outflow facility when measured by tonography (Gaasterland et al. 1978; Becker 1958) but not fluorophotometry (Toris et al. 1999). This discrepancy may be caused by the increased rigidity in older versus younger subjects. Ocular rigidity is part of the tonography but not the fluorophotometry measurement.

A few problems are associated with the fluorophotometric method. First, it is assumed that uveoscleral outflow facility is very small and affected little by the measurement. If an experimental manipulation were to increase uveoscleral outflow facility, it could be interpreted erroneously as an increase in trabecular outflow facility. This problem is also inherent in the tonography measurement. Second, the method does not work well in normotensive eyes, in which a change in IOP by the aqueous flow suppressant is not effective. Similarly, tonography does not work well in normotensive eyes, in which the IOP changes little by the weight of the probe. Third, fluorophotometry requires several hours for a complete determination versus 4 min for tonography.

Invasive Methods

The flow-to-blood method is arguably the most accurate technique to assess trabecular outflow facility, especially compared to the two-level, constant-pressure perfusion technique (Bárány 1964) used to measure outflow facility in research animals. In the flow-to-blood method, a radioactive isotope is infused into the anterior chamber at a set pressure (IOP1) for a set period of time. One collects a blood sample at a specific time interval and measures it for radioactivity. Any radioactivity in the blood is thought to have drained solely through the trabecular meshwork, and the rate of its accumulation in the blood is assumed to be trabecular outflow (F1). Next, the isotope is infused at a different pressure (IOP2), and the new rate of accumulation of radioactivity in the blood is assumed to be a new trabecular outflow (F2). Equation 2 is used to calculate trabecular outflow facility. If done carefully, this method is

repeatable and can be used to evaluate outflow facility over time.

The major problems with all invasive techniques are the direct and indirect effects of anesthesia on the IOP and the trauma of the needle's insertion into the eye. Additionally, ocular rigidity, pseudofacility, and uveoscleral outflow facility confound the measurement. An important assumption with the flow-to-blood method is that any tracer in the blood enters solely through the trabecular meshwork. In reality, some tracer may enter the blood through the uveoscleral pathway and vortex veins, thus resulting in an overestimate of trabecular outflow.

Uveoscleral Outflow

Overview

Uveoscleral outflow is the drainage of aqueous humor from the anterior chamber into the ciliary muscle, where it seeps out of the eye in several different directions. The route of uveoscleral outflow is anatomically ill-defined, and its flow rate is relatively independent of pressure.

Techniques for Measuring Uveoscleral Outflow

Mathematical Calculation

Currently, the only noninvasive means by which to assess uveoscleral outflow (Fu) is via mathematical calculation using Eq. 3:

$$\mathrm{Fu} = \mathrm{F} - \mathrm{C}(\mathrm{IOP} - \mathrm{Pv}). \qquad (3)$$

Aqueous humor flow (F) is measured by fluorophotometry, outflow facility (C) by one of the methods described earlier, IOP by tonometry, and episcleral venous pressure (Pv) by venomanometry (Zeimer et al. 1983). A commercially available venomanometer (Eyetech Ltd., Morton Grove, IL) attaches to a slit lamp. One places the membrane at the device's tip on the conjunctiva near the limbus. The user identifies the episcleral veins underlying the conjunctiva with the aid of the slit-lamp biomicroscope. One raises the pressure within the membrane until the episcleral veins collapse. The pressure required to cause the vessels' collapse is read off the dial on the side of the device; it is a measure of episcleral venous pressure.

One limitation of the calculation method for uveoscleral outflow is the large standard deviation generated by the inherent variability in each parameter in the equation. Many subjects are needed to achieve sufficient power to detect clinically relevant differences between experimental and control groups. Another limitation is that calculated uveoscleral outflow can vary tremendously, depending on which value of episcleral venous pressure is used in the equation. It is difficult to obtain an accurate measurement of Pv. For this reason, a value of 9 or 10 mmHg is often used in the equation with the assumption that the value is unchanged during the course of a study. If Pv were to change, one might draw erroneous conclusions concerning the cause of a response in IOP.

Despite its limitations, the mathematical calculation of uveoscleral outflow has provided reasonable explanations for differences in IOP with respect to aging, pharmacological drugs, clinical syndromes, and surgical procedures. In the end, it is the relative changes in uveoscleral outflow, not necessarily its absolute value, that are of greater clinical importance. For example, research by T. Johnson (Johnson T at ARVO 2006) has shown exfoliation syndrome to be associated with reduced uveoscleral outflow when compared to age-matched, healthy control subjects (Zeimer et al. 1983). From a physiological perspective, it would be preferable to treat the area of pathology than simply to prescribe the drug with the best effect on IOP. As a class, prostaglandin analogs may be a good treatment for exfoliation syndrome, because uveoscleral outflow is increased in patients treated with these drugs (Weinreb et al. 2002).

Invasive Methods

Two invasive methods are used to measure uveoscleral outflow. They are more direct than the mathematical calculation, but they cannot be used in clinical studies. The "intracameral tracer method" involves infusing a radioactive or

fluorescent tracer into the anterior chamber at a set pressure and for a specific period of time. The total amount of tracer found in the uvea and sclera during the specified time interval is assumed to be uveoscleral outflow. If the time interval is excessive, some tracer can exit the globe and be lost to analysis. Under these circumstances, uveoscleral outflow would be underestimated. Enucleation of the eye makes this method unrepeatable.

The "indirect isotope method" involves infusing a radioactive tracer in the anterior chamber and monitoring the rate of the tracer's appearance in the blood (trabecular outflow) and the rate of the tracer's disappearance from the anterior chamber (aqueous flow). Uveoscleral outflow is the difference between aqueous flow and trabecular outflow. This method is advantageous in that changes in uveoscleral outflow can be assessed over time. Its invasive nature, however, precludes its use in clinical studies.

Evaluation and Critical Assessment

Many methods are available to assess aqueous humor outflow. The noninvasive methods are indirect, highly variable, and fraught with many limitations and assumptions. The invasive methods require anesthesia, may damage the eye, are usually terminal, and are also laden with limitations and assumptions. Nevertheless, these methods are valuable tools in the study of outflow in the healthy and the diseased eye. They have provided clinicians with a better understanding of diseases that affect and treatments that reduce IOP. Such information may be useful in selecting specific treatments or combinations of treatments for glaucoma or ocular hypertension.

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Experimental Glaucoma

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Contents

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Purpose and Rationale

Based on Rachida A. Bouhenni et al. (2012), glaucoma is a heterogeneous group of disorders that progressively leads to blindness due to loss of retinal ganglion cells and damage to the optic nerve. It is a leading cause of blindness and visual impairment worldwide. Although research in the field of glaucoma is substantial, the pathophysiologic mechanisms causing the disease are not completely understood. A wide variety of animal models have been used to study glaucoma. These include monkeys, dogs, cats, rodents, and several other species. Although these models have provided valuable information about the disease, there is still no ideal model for studying glaucoma due to its complexity. In this chapter, based on a very extensive review by RA Bouhenni et al. (2012), I will present a summary of most of the animal models that have been developed and used for the study of the different types of glaucoma and, thanks to the original authors, the strengths and limitations associated with each species use, including some potential criteria to develop a suitable model.

Introduction

Animal models have greatly improved our understanding of the causes and progression of human diseases and have proven to be a useful tool for discovering targets for therapeutic drugs. However, several diseases remain incurable, because not all models used for studying these diseases mimicked the human disorders completely.

In glaucoma, a wide variety of animal models of different species have been used to study the disease (Gelatt 1977; Vecino 2008). These included large animals such as monkeys (Rasmussen and Kaufman 2005), dogs and cats (Brooks 1990; Dietrich 2005), and pigs (Ruiz-Ederra et al. 2005) and small animals such as rodents (Pang and Clark 2007). Glaucoma in these animals was either spontaneous or induced. Although these models have provided valuable information about the disease, they all had drawbacks and glaucoma remains incurable.

Several types of glaucoma have been described. These have been broadly classified as acute and chronic, secondary and primary. In general, glaucoma in humans is classified into three major types: primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), and primary congenital glaucoma (PCG), with POAG being the most common type in most populations (Quigley 1993). Below I will describe, according to RA Bouhenni et al. (2012), a wide variety of the animal species that have been developed and used to study the different types of glaucoma and outline their features, unique strengths and limitations, as well as some potential criteria to develop a suitable model.

Primary Open-Angle Glaucoma (POAG)

POAG is the most common form of glaucoma in most populations. More than 20 genetic loci have been reported for POAG but only three causative genes have been identified to date (Myocilin, Optineurin, and WDR36) (Harada et al. 2007). POAG is characterized by elevated IOP and acquired loss of RGCs and atrophy of the ON (Quigley 1993). Animal models (spontaneous and induced) that have been used to study POAG and provided valuable information about the disease are described below.

Monkeys

Experimental monkey models have also been developed for the study of POAG. Gaasterland and Kupfer developed an experimental monkey model using argon laser photocoagulation (Gaasterland and Kupfer 1974). They used a modified Koeppe-type goniolens to laser the entire circumference of the trabecular meshwork (TM) which resulted in IOP elevation in 70 % of the animals. The IOP range was between 24 and 50 mmHg after the fourth treatment and remained elevated for 25 days. Histopathologic specimens from the eyes with elevated IOP and ON cupping showed selective loss of RGCs and thinning of the

nerve fiber layer compared with specimens from untreated controls suggesting that glaucoma was achieved. Several studies after that used the monkey model to describe the functional and anatomic changes that occur within the eye and ON in an effort to understand the reasons that lead to elevated IOP (Quigley et al. 1987; Glovinsky et al. 1991, 1993; Quigley and Hohman 1983; Toris et al. 2000). Other experimental monkey models of chronic IOP elevation were developed by Weber and Zelenak using latex microspheres (Weber and Zelenak 2001) and Quigley and Addicks using autologous fixed red blood cells (Quigley and Addicks 1980a, b). Another model that develops acute elevation of IOP was also used to study the mechanism of ON damage (Wax et al. 2008).

Dogs

In 1981, Gelatt et al. described an inherited POAG in the beagles bred in their laboratory (Gelatt et al. 1981). The condition appeared to be autosomal recessive. Elevation of IOP (30–40 mmHg) in this model developed bilaterally at 1–2 years of age, tonographic recordings, and constant pressure perfusions indicated a reduction in the aqueous humor outflow. Gonioscopically, the disease had two phases: open iridocorneal angle during the onset and the first 2–4 years of the disease and closed iridocorneal angles associated with lens subluxation and displacement from the anterior vitreous patellar fossa. The animal also exhibited cupping and atrophy of the optic disk, buphthalmia, cataract formation, vitreous syneresis, and eventually phthisis bulbi. This model was recently used in a genome-wide SNP array study to map the disease genes and led to the identification of the metalloproteinase ADAMTS10 as a candidate gene for POAG (Kuchtey et al. 2011).

The advantage of using this model, and dogs in general in glaucoma research, is the spontaneous inheritance of the disease without congenital anomalies and the availability of the genome sequence. Dogs have relatively large eyes but can be aggressive and difficult to handle in the laboratory. Also, anatomically, dogs have an intrascleral plexus, rather than a Schlemm's canal; this difference may be minor but can be a limitation and their availability may be limited.

Mice

A mouse strain expressing the Tyr423His myocilin point mutation corresponding to the human MYOC Tyr437His mutation was developed to study POAG (Senatorov et al. 2006; Zhou et al. 2008). Myocilin is one of the causative genes of POAG in humans (Joachim et al. 2009) and has been extensively studied. At 18 months of age, the myocilin model demonstrated loss of ~20 % of the RGCs in the peripheral retina, axonal degeneration in the ON, detachment of the endothelial cells of the trabecular meshwork (TM), and moderate and persistent elevation of IOP (2 mmHg higher than normal) (Senatorov et al. 2006).

Another transgenic mouse strain with a targeted mutation in the gene for the α1 subunit of collagen type I has also been developed to study POAG. This model demonstrated open angles, progressive ON axonal loss, and gradual elevation of IOP suggesting an association between IOP regulation and fibrillar collagen turnover (Mabuchi et al. 2004; Aihara et al. 2003a).

There are several advantages of using mice in glaucoma research. These include the high degree of conservation between mice and human genomes, enabling genetic manipulation by altering the mouse genome, and the ability to breed the animals as desired. In addition, they are inexpensive and easy to house and handle, their eyes are easy to obtain, and the sample number for studies can be large. The disadvantages of the mouse model in glaucoma are the absence of the lamina cribrosa in the ON, the very small size of the globe which makes it hard to access clinically, and the availability of specific models may be limited.

Rats

A glaucoma rat model, induced by topical application of dexamethasone, was also developed to

study the expression of myocilin. Although IOP was elevated after 2 weeks of treatment, the protein and mRNA levels of myocilin in the TM and around the Schlemm's canal in the treated eyes were not different from those of the controls suggesting that myocilin may not be directly linked to ocular hypertension (Sawaguchi et al. 2005).

Similar to mice, rats have many advantages. In contrast to other nonprimate models, the rat shares similar anatomical (Quigley et al. 1980; Fingert et al. 2002) and developmental (Fingert et al. 2002; Van Der Zypen 1977) characteristics of the anterior chamber, especially in the aqueous outflow pathway, with the human. Therefore, results obtained from the rat are expected to mimic changes that occur in the human. In addition, there is reasonable IOP elevation as retinal and ON changes are similar to those seen in humans. Also, reduction of IOP in response to glaucoma medications has been described, but the medication effects were not all identical to those observed in humans (Daimon et al. 1997). Furthermore, rats are easier to maintain in the laboratory and, similar to mice, they enable genetic manipulation and can be used in large numbers.

Zebrafish

Transgenic teleost *Danio rerio* (zebrafish) models have been developed for studying glaucoma (Reme et al. 1983; Nucci et al. 1992). The bug eye mutant that was developed by Simon et al. shows RGC death and high IOP (John et al. 2003; Stujenske et al. 2011). The mutant develops buphthalmia shortly after sexual maturation and an average IOP of 32.9 ± 16.2 mmHg compared to that in the wild type (14.7 ± 3.6 mmHg). This model was recently used in a study that led to the identification of a mutation in the low-density lipoprotein receptor-related protein 2 (lrp2) that is important for myopia and other risk factors for glaucoma (Veth et al. 2011). The lrp2 mutant exhibited a phenotype that included high IOP, enlarged eyes, decreased retinal neurons, activation of RGC stress genes, and ON pathology. Another zebrafish glaucoma model, the wdr36 mutant that was developed by Skarie and Link, was used to characterize the wdr36 function (Skarie and Link 2008). This model, however, was only developed to study the function of wdr36, as it did not show a typical glaucoma phenotype (Skarie and Link 2008).

The zebrafish model has received attention for its usefulness in studying glaucoma and other human diseases (Link et al. 2004; Barut and Zon 2000) because of its short generation times and a well-supported genomic infrastructure. It allows the combination of forward and reverse genetic approaches in order to identify critical genetic interactions required for normal and pathological events. This model would be ideal for studying developmental changes in glaucoma such as those occurring in PCG. It is easily adapted to laboratory settings and can be maintained in a relatively small space. The fish typically reaches sexual maturity in 3–4 months, and a breeding pair can produce more than 200 fertilized eggs per mating. Fertilization is external, and the egg and embryo are transparent, which makes it easy to visualize the changes with a regular dissecting microscope. The fish develops quickly, and all major organ systems are formed by 24 h after fertilization. Mutagenesis in zebrafish is performed by gamma ray and chemical approaches. The fish also enables haploid screens and diploidization, transgenesis, and forward and reverse genetic approaches which make it an attractive model for genetic manipulations of the visual system.

Other POAG Models

Administration of glucocorticosteroids can lead to the development of ocular hypertension and POAG through a reduction in aqueous humor outflow (Goldsmith et al. 2003; Jones and Rhee 2006). Models using steroid-induced ocular hypertension have been developed in many animals such as rabbits, bovine, and sheep (Bonomi et al. 1978; Ticho et al. 1979; Wood et al. 1967; Candia et al. 2010; Gerometta et al. 2010). The consistency and robustness of the IOP response and the low cost of maintaining the animals

developed using steroids (rats, rabbit, sheep, and cows) compared to primates are all advantages of this model. However, the prolonged topical corticosteroid treatment required to achieve glaucoma can cause significant adverse effects such as cataracts and corneal ulcers.

Avians

Light-induced avian models of POAG have also been described (Lauber 1987; Kinnear et al. 1974). IOP in these models appeared to be responsive to several antiglaucoma drugs (Lauber et al. 1985). Birds may be easy to handle in the laboratory and are not expensive. This model could be potentially valuable for studying the effect of glaucoma medications on IOP.

Primary Angle-Closure Glaucoma (PACG)

Similar to POAG, PACG is characterized by elevated IOP, damage to the ON, and visual field loss. The iris in PACG obstructs the TM, whereas in POAG the TM is open and unobstructed (Kersey and Broadway 2006). There are several animal models that have been developed for the study of PACG, some of these are congenital, such as dogs and turkeys, and some are induced such as mice and rats.

Dogs

Since the disease is rare in dogs and the genotype and phenotype of glaucoma have not been well characterized, this model has not been used to study angle-closure or congenital glaucoma.

Turkeys

An inherited eye disease leading to secondary angle-closure glaucoma is good for studying angle-closure glaucoma; however, its availability may be limited.

Mice

Genetically manipulated Vav2/Vav3-deficient mice were also described and found to have elevated IOP, which eventually manifests as buphthalmos (Fujikawa et al. 2010). Loss of Vav2 and Vav3 expression in these mice is associated with changes in the iridocorneal angle, which leads to chronic angle closure. The characteristics that make this model useful for glaucoma research are as follows: (1) the elevated IOP occurs spontaneously in these mice and does not require the ocular manipulation necessary in induced models, (2) the frequency of the ocular phenotype is high and onset occurs at a relatively young age, and (3) ocular hypotensives commonly used to treat human glaucoma show efficacy in lowering IOP in this model. The most significant advantage of this mouse glaucoma model is that the deleted genes, Vav2 and Vav3, are well-focused targets that have been studied for over 20 years, providing a useful starting point for further investigation of the potential molecular mechanisms underlying this phenotype.

Additional PACG Models

A wide variety of rat and mouse models have been developed to study the effect of elevated IOP on the ON and RGC degeneration. Though these models were primarily developed to study retinal IOP-related posterior segment damage, the histopathological examination showed varying degrees of angle closure. IOP elevation has been induced by a number of techniques that include the use of hypertonic saline injection into the episcleral veins, cauterization or ligation of the episcleral veins, or laser photocoagulation of the perilimbal region.

Rats

Episcleral vein saline injections of Brown Norway rats resulted in sustained IOP elevations after 4 weeks in 45 % of rats with 35 % developing sustained elevations after subsequent injections (Morrison et al. 1997). Additional models using fluorescent polystyrene microbeads and

hyaluronic acid injections have also been developed (Sappington et al. 2009; Moreno et al. 2005). These models showed a significant IOP elevation and glaucomatous damage in the retina. Wistar rats injected with a solution of microbeads demonstrated an IOP of 29.7 mmHg that remained stable for 13 days and resulted in an axon density that was 16 % lower than that in the control groups (Sappington et al. 2009). Wistar rats receiving weekly injections of hyaluronic acid had elevated IOP in the low 20 s for the duration of the 10 weeks.

As mentioned previously, rats are easy and more economical to maintain, and a large number can be treated in one day by one person reducing the cost associated with additional personnel. However, similar to other induced animal models, the technique may need multiple sessions to achieve IOP elevation. Although IOP elevation is achieved, the response to induction of glaucoma may be inconsistent. The hypertonic saline model is likely to be the most consistent model but is technically difficult to perform and has mainly been used in Brown Norway rats. The IOP elevation in all these models is sustained for a period of 2–6 weeks.

Mice

Other mouse models that have been developed to study PACG included those developed by photocoagulation of the episcleral vessels (Fu and Sretavan 2010; Gross et al. 2003; Coleman 1999) and episcleral vein cauterization (Ruiz-Ederra and Verkman 2006). These models exhibited elevated IOP for up to 4 weeks, loss of RGCs, and damage of RGC axons. Translimbal-photocoagulation-treated eyes of Black Swiss mice reached a maximum IOP of 39.6 mmHg with IOP elevation being statistically significant compared with controls for up to 6 weeks (Aihara et al. 2003b), whereas in photocoagulation of episcleral- and limbal-vein-treated albino CD1 mice, the eyes had doubling of their IOP within 4 h (Fu and Sretavan 2010). Using fluorescent polystyrene microbeads injection in C57BL/6 mice as well, a consistent 30 % elevation in IOP that persisted for more than 3 weeks was achieved using one single injection (Sappington et al. 2009).

Rabbits

Rabbit models for angle-closure glaucoma were also created by either water loading (McDonald et al. 1969; Thorpe and Kolker 1967) or argon laser energy applied to the TM (Gherezghiher et al. 1986; Johnson et al. 1999; Seidehamel and Dungan 1974). Both pigmented and albino rabbits were used in these studies. Although elevated IOP and buphthalmia were achieved in these animals, these models all had drawbacks. For example, in the water loading models, the damage produced included the whole eye and the IOP rise was of insufficient duration (1 h) and caused selective loss of RGCs. In the laser-induced glaucoma models, the IOP elevation lasted for a few weeks, but it was hard to achieve a successful model because of the structure of the iridocorneal angle, which is different from that of humans. The longest IOP elevation was reported in the α-chymotrypsin-injected models.

Primary Congenital Glaucoma

Primary Congenital Glaucoma (PCG) is an autosomal recessive disease caused by an abnormal development of the anterior chamber angle. PCG has been linked to several genetic loci. CYP1B1 and LTBP2 are the only genes in which mutations are currently known. However, the role that these genes play in the pathophysiology of PCG and development of the anterior chamber is not known. An assortment of spontaneous glaucoma models has been described in different animal species. These included rabbits, dogs, monkeys, mice, rats, cats, and albino quails. The study of these models has provided valuable information on the pathophysiology of glaucoma as it relates to changes in the anterior chamber angle, the ON, and the retina but the mechanisms leading to these changes are still elusive.

Rabbits

Many studies have indicated that glaucoma in rabbits is most likely autosomal recessive with incomplete penetrance (semilethal) (Hanna et al. 1962). It typically manifests in the first 6 months and is associated with variable IOP elevation, enlarged

cloudy corneas, and elongated globes. The outflow facility is decreased, suggesting a defect in the outflow pathway, which correlates with the reported histological findings. RGC loss and cupping of the optic nerve were also observed in these rabbits. The phenotypic similarities between rabbits and human patients with congenital glaucoma include the age of onset, IOP elevation, and buphthalmia. In addition, the rabbit eye is also relatively large, which makes it a good model for eye research. However, there are some limitations that make this animal unsuitable for glaucoma research. These include differences in the structure of the trabecular meshwork and aqueous outflow pathways between the human and the rabbit making it difficult to make direct correlations between the developmental changes in the anterior chamber angle in both species. In addition, IOP levels in the buphthalmic rabbit were found to decrease with age. IOP was found to be comparable to normal (18–20 mmHg) until about 5 months of age, followed by intermittent elevation into the 30 mmHg range. A decrease in outflow facility precedes the elevation of IOP. The IOP elevation among animals is variable (20–30 mmHg) up to about 18 months of age; then it decreases to near the normal range between 24 and 48 months. The cause of IOP reduction to normal levels despite decreased outflow facility is unclear. The genome sequence of the rabbit was recently made available at http://www.ncbi.nlm.nih.gov/projects/genome/guide/rabbit/. This will help identify the genetic defects that cause glaucoma.

The biggest disadvantage of this model is its limited availability from commercial vendors. This model has recently been used to study the protein changes in the aqueous humor and has provided valuable information about proteomics and histopathological changes seen in the anterior chamber of this rabbit, although a CYP1B1 mutation could not be identified in this model (Edward and Bouhenni 2011).

Rats

Recently, an RCS-rdy^{-} rat model that develops glaucoma spontaneously was also described (Heywood 1975; Naskar and Thanos 2006). The mutant animals had either a unilateral or bilateral enlargement of the globes with an IOP that ranged from 25 to 45 mmHg, as compared to control values of 12–16 mmHg. The IOP increased significantly with age to reach a value of 35 $\pm$ 7.3 at 12–18 months of age. The animals also had decreased number of RGCs with age as well as atrophic ONHs. The anterior chamber was narrow and the iridocorneal angle was open. These rats were used in other studies in glaucoma research and yielded valuable information about RGC loss (Gatzioufas et al. 2008).

Cats

Feline glaucoma is a rare condition. It has been described in Burmese cats (Hampson et al. 2002), domestic cats (Brooks 1990), and Siamese cats (Gelatt et al. 1981; McLellan et al. 1995). Examination of the Siamese cats revealed bilateral mild-to-moderate buphthalmos and moderate elevation in IOP, which was as high as 31.6 mmHg. Clinical features identified in these cats were similar to those seen in human PCG, though details such as IOP levels and clinical course were not described in these reports. Similar to rabbits, cat eyes are relatively larger, making them attractive for use in glaucoma research. However, no further reports were published describing clinical, pathological, and genetic characterization of the disease in cats.

Mice

A knockout model with Cyp1b1 has also been developed to simulate PCG where CYP1B1 mutations are the predominant cause of PCG in humans in some populations (Bejjani et al. 1998). Similar to the buphthalmic rabbit described previously, Cyp1b1 mutation could be specific to the human PCG only which makes this model and other PCG animal models not suitable for studying the genetics in this disease.

Albino Quails

The albino quail model of glaucoma (al mutant) was described in 1986 by Takatsuji et al. (Takatsuji et al. 1986). The al mutation is sex-linked semilethal recessive of known penetrance. The gene mutation has not been described. The mutant bird would be a good model to study glaucoma, as it is easy to maintain and to handle in a laboratory. However, the cornea of these birds is very small and IOP measurement may be challenging, although a tonopen can be used. In addition, availability of the albino quail is also limited.

Other Types of Glaucoma

Normal-Tension Glaucoma

Normal pressure or normal-tension glaucoma (NTG) is a condition where the clinical features are largely identical to those seen in POAG except the IOP, which, in affected patients, is below the statistically normal upper limit (21 mmHg). To explore the possible pathways of RGC degeneration, genetically modified mice with normal IOP have been utilized as models of NTG as described below.

Because glutamate excitotoxicity and oxidative stress have been implicated in RGC death, mice deficient in the glutamate transporter genes Glast and Eaac1 have been developed as models for normal-tension glaucoma. These mice demonstrate RGC and ON degeneration without IOP elevation (Harada et al. 2007) suggesting that these transporters play important roles in preventing RGC degeneration by keeping the extracellular glutamate concentration below the neurotoxic level and maintaining the glutathione levels in Müller cells by synthesizing and transporting glutamate into the cells.

Autoimmune Glaucoma

Some studies have examined serum samples from glaucoma patients to look for autoantibodies and have found increased levels of heat shock protein 27 (HSP27) and heat shock protein 60 (HSP60). HSP27 and HSP60 immunization in the Lewis rat induced RGC degeneration and axonal loss 1–4 months later in a pattern similar to human glaucoma (Wax et al. 2008), suggesting the role of these proteins in the development of glaucoma. The models also showed IOP-independent RGC loss and changes in serum antibody patterns (Joachim et al. 2009). Experimental autoimmune glaucoma offers a valuable tool to examine the diverse roles of the immune system in glaucoma. It may also facilitate the identification of treatment strategies to prevent pressure-independent RGC degeneration as it may occur in select patients with glaucoma. However, depending on the animal used, limitations can be encountered such as the size of the eye, the cost of the animal, and the anatomical similarities of the animal's eye to that of the human.

Critical Assessment

This paper describes most of the animal models utilized in glaucoma research to date, according to an extensive review by RA Bouhenni et al. (2012). These animal models have provided valuable information about certain aspects of the disease process, but the search for appropriate models continues. The validity of each of these models depends upon the degree of similarity to the human condition, as well as considerations of the model being economical and practical. Since the mechanisms of glaucoma differ among animal models, data obtained from a particular model cannot be generalized and should be interpreted within the context of that model. The animal model used should be selected based on the experimental needs and the hypothesis being tested. Experimentally induced models have the advantage of studying some of the changes in glaucoma over a short period of time. However, sophisticated equipment and trained personnel to induce glaucoma are often needed. In addition, glaucoma induction can be somewhat unpredictable. These models may be useful in testing responses to medications. It is likely that genetic models developed to address specific hypotheses will provide

valuable information on the pathophysiology of the various types and aspects of glaucoma.

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Local Anesthesia of the Cornea

Beat P. Mertz

Contents

Purpose

Local anesthetics act by blocking the conduction of impulses along the neuronal membrane. Many types of chemical structures do so to some degree, including ethanol and barbiturates. However, those agents that are clinically useful as local anesthetics are more potent in this regard and are less toxic.

Rationale

Mechanism of Action and Efficacy

Local anesthetics block the formation or conduction of action potentials. They stabilize the neuronal membrane and prevent the generation of impulses; the standing potential remains intact.

The action potential begins with extracellular sodium flowing into the neuron, followed by intracellular potassium flowing out of the neuron. At low concentrations, local anesthetics primarily affect sodium conductance. At higher concentrations, potassium conductance may also be affected. The clinically used local anesthetics act primarily by reversibly blocking the sodium ion channel at its internal neuronal membrane.

Neuronal factors that affect local anesthetic activity are axon diameter, myelinization, and rate of firing. The greater the diameter of the axon, the more internal membrane surface area and number of sodium channels there are to

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block and, therefore, the lower the intensity and duration of anesthetic action (Staiman and Seeman 1977). The greater the amount of myelin, the less readily the anesthetic penetrates the axon. Frequency dependency describes the observed phenomenon that the more rapidly a neuron discharges, the more rapidly the anesthetic enters the axon. Anesthetic agents whose molecules alternate between positively charged and neutral form at physiologic pH are most likely to show frequency dependency. Benzocaine, which is uncharged at physiologic pH, does not show frequency dependency.

The principal manufacturing factors that affect activity are the drug concentration, pH of the solution, and presence of a vasoconstrictor. Cocaine contains intrinsic vasoconstrictor properties. It blocks the reuptake of norepinephrine released by sympathetic nerve endings. All other local anesthetics block sympathetic neuron release of norepinephrine, resulting in vasodilation. The addition of a vasoconstrictor reduces drug absorption by the vasculature, thereby increasing the local effect of the anesthetic, reducing in theory its systemic toxicity, and reducing surgical bleeding. Norepinephrine is less stable than epinephrine, the vasoconstrictor that is usually added. Concentrations of epinephrine of approximately 1/200,000 (i.e., 0.0005 %) are maximally effective.

Termination of Activity

Most anesthetic molecules reaching the systemic circulation are metabolized to inactive forms before elimination from the body. Hydrolysis of the ester and amine groups and oxidation and dealkylation of amino groups can occur (Dubbels and Schloot 1983). For the amide anesthetic, lidocaine, dealkylation is the major, but not only, metabolic pathway (Nelson et al. 1977). However, the role of local (e.g., orbital) tissue enzymes in terminating the anesthetic effect is limited (Zoglio et al. 1971). Passive diffusion of an anesthetic away from its site of action seems much more important in terminating action. Because motor axons tend to be of larger diameter than sensory axons, the former require a larger quantity of drug to maintain anesthesia. Therefore, as the tissue level decreases, motor activity recovers before sensory (Winnie et al. 1977).

Procedure

Anesthesia of the Eye and Orbit

Topical, corneal, and injected orbital anesthesia are relatively safe ways to perform surgery. In 195 patients with known previous myocardial infarctions who underwent 288 ophthalmologic procedures under local anesthesia, there were no postoperative myocardial infarctions or deaths during, or in the week after, surgery. In 255 patients with previous angina pectoris, but no documented myocardial infarctions, who underwent 373 ophthalmologic procedures under local anesthesia, four patients had myocardial infarctions, two of whom died. In 9,617 ophthalmologic procedures performed on patients with a history of neither myocardial infarction nor angina, there was one myocardial infarction (Backer et al. 1980).

Topical Anesthesia

The cornea and conjunctiva can be anesthetized by drops of any of the local anesthetics.

Bupivacaine, for example, is effective as an eyedrop (Carruthers et al. 1995). It is perplexing to see the surgeon stand over the patient with a syringe full of local anesthetic and call impatiently for a vial of topical anesthetic. Generally, those agents chosen to be topical anesthetics differ only in penetrating the sensory nerve endings more rapidly, thereby having a faster onset of action and a shorter period of producing ocular irritation.

The sympathetic β-receptor antagonist, propranolol, stabilizes neuronal membranes and produces anesthesia. The local anesthetic potency of propranolol is approximately equal to that of lidocaine. Several other β-blockers also have local anesthetic properties. For example, oxprenolol, acebutolol, and alprenolol have approximately half the anesthetic potency of propranolol.

Timolol has no significant anesthetic properties (McDevitt 1977).

The depth and duration of topical anesthesia are dose dependent (Polse et al. 1978; Matsumoto et al. 1981). Return to full recovery after one drop of proparacaine 0.5 % may take more than 1 h and the maximal effect may last 5–20 min. Pathologic tissues (e.g., the hypesthetic corneas associated with previous herpes simplex or herpes zoster infections) may take longer to recover (Weiss and Goren 1991).

In general, the ester topical agents, because of their relatively rapid tissue penetration, have a lower margin of safety and are often not marketed in an injectable form. Tetracaine is such an example. Procaine is an exception; it is an ester-containing local anesthetic, available as an injectable agent. When used as a topical agent, procaine has the dual advantages of a rapid onset and a shorter duration of action.

Specific Uses

Diagnostic Tests

Topical anesthetics are routinely used when performing applanation tonometry. However, small but statistically significant reductions in intraocular pressure have been reported, lasting for 1 to at least 15 min, after their application. In healthy patients measured 1, 5, 10, and 15 min after one drop of 0.4 % benoxinate, the intraocular pressure decreased from a baseline of 14.80 ± 3.28 mmHg to 14.01 ± 3.33 mmHg, 13.56 ± 3.12 mmHg, 13.17 ± 1.12 mmHg, and 13.98 ± 3.45 mmHg, respectively (Baudouin and Gastaud 1994). One drop of tetracaine 2 % produced a small but significant reduction in intraocular pressure from baseline at 10 min (10.67 ± 2.55 mmHg to 9.94 ± 2.17 mmHg) (Carel et al. 1979). The mechanism of this reduction may be an increased aqueous humor flow rate leaving the eye, but the evidence is not strong (Bloom et al. 1976).

Postoperative Analgesia

Postoperative pain after strabismus surgery performed on children, 1–12 years old, was found to be reduced for up to 8 h by applying one drop of a topical anesthetic after intubation and a second drop immediately before tubation (Watson 1991). Those receiving placebo drops required more analgesic drugs during the postoperative period. The pain after laser (photorefractive) keratectomy was reduced during the first 24 h after surgery by using local anesthetic drops every 30 min; there was no impairment of corneal epithelial healing or visual acuity as measured 1, 3, and 6 months after the procedure (Verma et al. 1995).

Therapeutic Use

Most ophthalmologists reject the thought of using long-term topical corneal anesthetic drops, because of their potential side effects and toxicities. However, as treatment for trigeminal neuralgia, a small amount of literature indicates that topical anesthetics are efficacious and advocates their use (Zavon and Fichte 1991; Spaziante et al. 1992).

Toxicity

Topical anesthetic agents can cause a decrease in corneal epithelial cell glycolysis, respiration, and healing. Penetration of other topically applied agents, simultaneously or subsequently applied, is enhanced. The intraocular penetration of topical fluorescein is increased, aiding fluorophotometry. Mydriasis and cycloplegia are more rapid and profound when a topical anesthetic precedes the parasympatholytic eyedrop. However, at least one study has found evidence that the preservatives chlorhexidine and, especially, benzalkonium chloride, which are used in commercial preparations of local anesthetics, are primarily the cause of this increased corneal epithelial permeability (Ramselaar et al. 1988). It is, therefore, difficult to evaluate the role of the anesthetic agent itself when commercial preparations are reported to produce increased corneal thickness (Bjornstrom et al. 1994) or epithelial permeability (Stolwijk et al. 1990).

Topical anesthetics retard healing of corneal abrasions by interfering with epithelial cell mitosis and migration (Marr and Wood 1957). However, it is accepted practice for the physician to administer a topical anesthetic to assess the acutely traumatized eye. The pain,

blepharospasm, photophobia, and epiphora after a corneal abrasion can prevent an accurate assessment of visual acuity and the structural integrity of the anterior segment. A drop or two of topical anesthetic will produce a dramatic, if only temporary, improvement in these symptoms.

Prolonged administration of topical anesthetics can produce a toxic keratopathy consisting of corneal epithelial defects, stromal edema and opacification, and anterior chamber inflammation. Corneal scarring and endothelial damage may require a penetrating keratopathy for visual rehabilitation (Risco and Miller 1992). A sterile corneal ring infiltrate may mimic an infectious process, such as *Acanthamoeba* keratitis (Rosenwasser et al. 1990). Healthcare workers, especially physicians (Henkes and Waubke 1978), and the psychiatrically disturbed are the two groups most likely to abuse corneal anesthetics. If the cause of the keratopathy is not recognized, corticosteroid and antibiotic eyedrops may be given, increasing the chances of secondary fungal superinfections and resistant bacterial infections (e.g., *Candida* species, staphylococci, streptococci, and *Proteus* species) (Chern et al. 1996).

Topical anesthetics are routinely used when obtaining scrapings from a presumably infected cornea. A portion of the scrapings can be used to culture the causative microorganism. However, the anesthetic preparation may be toxic to the organism and impair its growth. The preservative chlorobutanol 0.2 % or 0.4 % was much less effective than benzalkonium 0.01 % or chlorhexidine 0.001 % in inhibiting growth; benzalkonium 0.004 % had little inhibitory effect, as did unpreserved anesthetic agents (Kleinfeld and Ellis 1966). The local anesthetics tetracaine, benoxinate, and cocaine and the preservatives chlorobutanol and butyl parahydroxybenzoate could each alone inhibit growth. This effect depended on the drug concentration and the organism. Unpreserved proparacaine in concentrations of up to 0.5 % did not show significant growth inhibition of the strains of staphylococci, *Pseudomonas*, and *Candida* that were tested.

pH

Anesthetic solutions usually are commercially formulated to be in a pH range of 5.0–6.0. This favors formation of the ionic form of the molecule, which is more stable and increases the shelf life of the product (Robinson et al. 2000).

Anesthetic blocks have been given with solutions after their pH has been increased. When an anesthetic solution with a pH of 5.4 was compared with bicarbonate-adjusted solutions with a pH between 6.7 and 6.9, the peribulbar blocks using the higher pH solutions had a more rapid onset of action but no shorter time to complete akinesia (Lewis et al. 1992). Furthermore, the higher pH solutions required more supplemental injections for an effective block.

Another theoretical justification for increasing the pH has been patient comfort. Lower pH solutions may be more irritating to tissues. Subcutaneous eyelid injections of buffered anesthetic solutions at pH 7.4 were less painful to receive than those at pH 4.6 (Eccarius et al. 1990). However, subcutaneously injected procaine at pH 4.3 produced less pain than lidocaine, pH 6.3 (McKay et al. 1987). When the pain caused by the peribulbar injection of combination bupivacaine-lidocaine solutions at pH 4.87 or pH 7.44 were compared, there was no significant difference (Minasian et al. 2000). These findings tend to undermine any theories attributing tissue irritation to lower pH or comfort to increasing pH.

Vasoconstrictors

The addition of a vasoconstrictor, usually epinephrine, at concentrations of 0.0005–0.001 %, results in an anesthetic solution that retards its own absorption into the circulation. This prolongs the effect of the shorter-acting anesthetics (e.g., lidocaine) but may provide little or no benefit for the longer-acting ones (e.g., bupivacaine) (Krohn et al. 1995; Bjornstrom et al. 1994; Chin and Almquist 1983). The elevations in serum epinephrine caused by commercial preparations of local anesthetics containing epinephrine are probably no more than those caused by stress-induced adrenal gland release (Dimsdale and Moss 1980).

Evaluation and Critical Assessment

Side Effects, Toxicities, and Allergies

Orbital

Bupivacaine, Specifically

Bupivacaine has a special role among local anesthetics, because it is much more myotoxic to the skeletal muscle of experimental animals than other anesthetics are. The mechanism involved may be reduced protein synthesis and may require the presence of extracellular calcium (Foster and Carlson 1980; Johnson and Jones 1978; Steer and Mastaglia 1986). Multiple clinical papers have theorized that the postoperative diplopia, especially vertical diplopia and ptosis being reported, were caused by myotoxicity from retrobulbar and peribulbar bupivacaine (Mather et al. 1994; Rainin and Carlson 1985; Hamed and Mancuso 1991; Hunter 1996).

Increased Intraocular Pressure

It is usually an advantage to have a low intraocular pressure when an eye is lacerated by trauma or when a penetrating surgical incision is to be made. However, immediately after a retrobulbar or peribulbar injection, the intraocular pressure is increased, presumably because of the mass effect of the solution. By 5 min after the injection, the intraocular pressure tends to have returned to baseline and by 8–10 min after the injection, the intraocular pressure has decreased significantly. This sequence has long been known (Atkinson 1934; Gifford 1949). The presence or absence of epinephrine in the anesthetic solution has little or no influence on the sequence (Gjotterberg and Ingemnsson 1977). The explanations for the reduction in intraocular pressure have varied (e.g., paralysis of the extraocular muscles, a reduced ciliary body secretion, and an indirect ischemic effect caused by compression of the blood vessels) (O'Donoghue et al. 1994; Horven 1978). The mechanisms causing the rare central retinal artery or vein obstructions found postoperatively are not clear, but the injection of a retrobulbar or peribulbar solution may play a role (Klein et al. 1982; Sullivan et al. 1983).

Central Nervous System

Direct injection of the anesthetic solution into the cerebrospinal fluid surrounding the optic nerve has been blamed for producing central nervous system toxicity after retrobulbar blocks. Extraorbital cranial nerve pareses, amaurosis, grand mal seizures, and respiratory arrest have occurred (Meyers et al. 1978; Javitt et al. 1987; Cohen et al. 1992).

It is unlikely that these events were the result of absorption from the orbital tissue producing toxic blood levels. To put this possibility in perspective, lidocaine is usually administered for the treatment of ventricular tachycardias by using an intravenous bolus injection of 50–100 mg, followed by a continuous drip of 2–4 mg per minute (Greenblatt et al. 1976). Five milliliters of lidocaine 2 % anesthetic solution contains only 100 mg of lidocaine. Intravenous lidocaine, 2 mg/kg, before intubation has been recommended to reduce the incidence of postoperative vomiting, from more than 50 % to less than 20 %, in children undergoing strabismus surgery (Warner et al. 1988). Intravenous lidocaine, 1.5 mg/kg in children and 2 mg/kg in adults, has been advocated for prevention of the intraocular pressure increase that occurs during intubation for general anesthesia (Lerman and Kiskis 1985).

An important factor in determining whether a given plasma level of local anesthetic may be toxic is the quantity of α-1 acid glycoprotein. This protein binds basic drugs, such as lidocaine. Neonates and cirrhotic patients have lower levels of α-1 acid glycoprotein, and patients with acute inflammatory responses have higher levels (Lerman et al. 1989; Barry et al. 1990).

Methemoglobinemia

Local anesthetics are a potential cause of surgery-related methemoglobinemia, and prilocaine is the most potent in this regard (Olson and McEvoy

1981). This condition can be caused by the inability of NADH-dependent methemoglobin reductase to reduce the hemoglobin iron atom from Fe^{+3} to Fe^{+2}. Prilocaine has less central nervous system and cardiovascular toxicity than lidocaine; thus, complications other than a clinically insignificant methemoglobinemia are rare. However, a peribulbar injection of prilocaine has been implicated as a contributory factor in the development of acute methemoglobinemia with respiratory distress. Improvement occurred rapidly with the injection of the antidote, methylene blue, 1.5 mg/kg (Eltzschig et al. 2000).

Allergies

The most commonly reported anesthetic allergies are contact allergies from topically administered drugs. Anaphylaxis is rare, and if it were to occur, it would most likely be from sensitivity to an additive in the solution rather than to the anesthetic agent itself (Barker and McAllen 1982). However, at least one death has been reported from a rechallenge to a local anesthetic after instilling a drop in the conjunctival sac (Adriani 1972).

Summary

Table 1 below includes a list of local anesthetic agents using essential ester bond structures, as well as others. Structurally, their syntheses require a secondary (−NH) or tertiary ($-NH_2$) amino group in the hydrophilic portion of the molecule. With few exceptions, local anesthetics also contain a lipid-soluble group (e.g., benzoic acid), separated from the water-soluble group (containing the amide) by an intermediate chain. The intermediate chain contains an oxygen, nitrogen, or sulfur atom. This general structure has been called the anesthesiophore group. It is important that the amino group in the hydrophilic portion of the molecule be a proton receptor and, thus, allowing it to become positively charged (e.g., a tertiary amide, $-NH_2$, becomes quaternized, $-NH^{3+}$, and thereby ionized) at physiologic pH (7.3–7.4).

Table 1 Local anesthetic agents

Ester bond	Amide bond	Neither
Para-aminobenzoic acid containing	Bupivacaine	No sympathetic activity
Benoxinate	Dibucaine	Dimethisoquin
Benzocaine	Diperodon	Dyclonine
Butacaine	Etidocaine	Pramoxine
Butamben	Lidocaine	Adrenergic β-blocking
Butethamine	Mepivacaine	Acebutolol
Butyl aminobenzoate	Oxethazaine	Alprenolol
Chloroprocaine	Phenacaine	Oxprenolol
Dimethocaine	Prilocaine	Propranolol
Naepaine	Pyrrocaine	
Procaine	Ropivacaine	
Tetracaine		
Meta-aminobenzoic acid containing		
Metabutethamine		
Primacaine		
Proparacaine		
Non-aminobenzoic acid containing		
Cocaine		
Cyclomethycaine		
Hexylcaine		
Piperocaine		

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Experimental Cataract Formation

Beat P. Mertz

Contents

Purpose and Rationale

A **cataract** is a clouding of the lens inside the eye which leads to a decrease in vision. It is the most common cause of blindness and is conventionally treated with surgery. Visual loss occurs because opacification of the lens obstructs light from passing and being focused on the retina at the back of the eye.

It is most commonly due to aging, but there are many other causes. Over time, a yellow-brown pigment is deposited in the lens, and this, together with disruption of the lens fibers, reduces the transmission of light and leads to visual problems. Those with cataracts often experience difficulty in appreciating colors and changes in contrast, driving, reading, recognizing faces, and coping with glare from bright lights.

Nuclear sclerosis is the most common type of cataract and involves the central or "nuclear" part of the lens. Over time, this becomes hard or "sclerotic" due to condensation of the lens nucleus and deposition of brown pigment within the lens. In advanced stages, it is called brunescent cataract. Cortical cataracts are due to the lens cortex (outer layer) becoming opaque. They occur when changes in the water content of the periphery of the lens cause fissuring. When these cataracts are viewed through an ophthalmoscope or other magnification systems, the appearance is similar to white spokes of a wheel pointing inwards. Posterior subcapsular cataracts are cloudy at the back of

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the lens adjacent to the capsule in which the lens sits.

Procedure

Different Experimental Approaches in Cataractogenic Research

The different experimental approaches in cataractogenic research are listed below according to Kyselova (2010) and other refs.

Especially in rodents, eye lens proteome-related changes have been studied preferentially on the aging model of cataract (Cenedella 1998; Lampi et al. 2002; Ueda et al. 2002; Descamps et al. 2005) or on the hereditary cataract model (Fujii et al. 2004). A few research articles were found to be related to a diabetic cataractous state in particular (Satake et al. 2003; Kumar et al. 2005a, b). Although dogs (Kador et al. 2007; Gift et al. 2009) and rabbits (Cheng 2002; Babizhayev et al. 2009) might be commonly used, rodents still remain as the most common experimental animals used to study the mechanisms of cataract formation. Several experimental treatments aimed at inducing cataracts in rats include streptozotocin-induced diabetes (Kyselova et al. 2005a, b), galactose feeding (Huang et al. 2000), ionizing radiation (Worgul et al. 1996), inhibition of cholesterol synthesis and steroid treatment (Dickerson et al. 1997), overdose of selenite (Shearer et al. 1997), and finally culture with oxidants or calcium ionophore (Chandrasekher and Cenedella 1993; Fukiage et al. 1997; Nakamura et al. 1999; Mitton et al. 1999).

In most of these models, covalent modification of crystallins, followed by phase separation of the lens cytosol and formation of water-insoluble aggregates, may play important roles in opacification. Some of the modifications detected in rat crystallins that could contribute to insolubilization are mixed disulfide formation (Lou et al. 1995; Kyselova et al. 2005c), glycation (Swamy-Mruthinti et al. 1996), cross-linking by UV light (Dillon et al. 1989), transglutaminase (Groenen et al. 1994), or disulfides (Ozaki et al. 1987), phosphorylation (Ito et al. 1999), and proteolysis (David et al. 1993). The ocular lens would appear to be an ideal organ for maintaining culture conditions, because it lacks blood vessels and nerves. The lens in vivo obtains its nutrients and eliminates waste products via diffusion with the surrounding fluids.

Lens opacification observed in vivo can be mimicked in vitro by the addition of a cataractogenic agent to the culture medium – e.g., galactose (Saxena et al. 1996) or high glucose (Padival and Nagaraj 2006; Dickerson et al. 1995; Olofsson et al. 2007; Son et al. 2007).

Incidentally, the lenses from various species have been incubated successfully since the middle of the last century (Kuck 1970). Different research groups utilized rat lenses in organ culture as a model system for studying the effects of various stresses on the lens and mechanisms of cataract formation and for screening potential anti-cataract agents (Kinoshita 1974; Spector et al. 1998; Zigler et al. 2003; Ghosh and Zigler 2005). In certain instances, lens opacification induced in vivo by administration of a particular cataractogenic agent can be mimicked in vitro by addition of the same agent to the culture medium – e.g., naphthalene (Xu et al. 1992; Lee and Chung 1998), selenite (Biju et al. 2007a), transforming growth factor-β (Hales et al. 1995), methylglyoxal (Shamsi et al. 2000), or high glucose (Padival and Nagaraj 2006; Dickerson et al. 1995; Olofsson et al. 2007; Son et al. 2007; Devamanoharan and Varma 1995). Further, agents which prevent such cataracts in vivo may also be effective in culture (Son et al. 2007; Zigler et al. 2003; Chandra et al. 2002). Thus, there is good evidence to support the idea that the lenses in culture can be an effective model for the lens in vivo.

Two-Faced Biological Function of Selenium

Selenium (Se) is an essential trace element for humans, animals, and some bacteria. It is pharmacologically active and at supranutritional dietary levels can prevent the development of many cancers, thus demonstrating chemoprevention and/or

carcinostatic activities (Rayman 2005). Selenium has however been shown to induce widespread oxidative stress in biological systems (Manikandan et al. 2009). Ironically, it forms an important part of biological defense, being the key component of selenoproteins, such as GPx, selenoprotein P, and thioredoxin reductases (Stadtman 1991).

Selenite Model of Cataractogenesis

The selenite cataract model is the most commonly used as it partially mimics senile nuclear cataract in humans, according to Z. Kyselova at SETOX 2010. The model has been used by several investigators to screen a variety of agents having anti-cataract potential; their representatives will be outlined.

Experimental Approaches In Vivo

Selenite-overdose cataract is an extremely rapid and convenient model of nuclear cataracts in rats in vivo. Sodium selenite is a cataractogenic agent commonly used in experimental studies since 1978 (Ostadalova et al. 1978). Selenite cataract is usually produced by a single subcutaneous injection of 19–30 μM/kg body weight of sodium selenite (Na_2SeO_3) into suckling rats 10–14 days of age, definitely before the completion of the critical maturation period of the lens at approximately 16 days of age (Shearer et al. 1997). Repeated injections of smaller doses of selenite (Huang et al. 1992) and oral administration (Shearer et al. 1983) are also cataractogenic. Severe, bilateral nuclear cataracts are produced within 4–6 days. Precursor stages include: posterior subcapsular cataract (day 1), swollen fibers (day 2–3), and perinuclear refractile ring (day 3). Although the model has been used extensively as a model for nuclear cataract, a transient cortical cataract also forms 15–30 days after injection (Shearer et al. 1992). The cortical cataract then clears after several months, but the nuclear cataract is permanent. Similarly, Anderson et al. (1988) observed that after a single injection of an overdose of sodium selenite at 30 μM/kg b. w., the nuclear cataract appeared rapidly within 3–5 days after injection and was permanent, while cortical cataract developed 15–30 days after injection and cleared within a few months. The selenite cortical cataract appeared to arise from early epithelial damage that interrupted normal fibrogenesis and interfered with normal ion control, resulting in an influx of water, cellular destruction, and opacity.

Experimental Approaches In Vitro

Usually Wistar rats of either sex in the weight range of 100–200 g can be used for the study. When these rats are killed, the eyes are enucleated without delay. The lenses are carefully dissected at posterior to avoid damage. Next, according to Biju et al. (2007b), the lenses are cultured as organs in an M-199 medium with a HEPES buffer, supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin under 5 % CO_2 at 37 °C in a CO_2 incubator.

The selenite medium is prepared by adding sodium selenite to the medium to give a final concentration of 0.1 mM. The lenses are maintained in a 24-well culture plate with 2 ml medium/well and one lens/well for 5 days. The lenses developing opacification in the first 24 h are discarded because of high suspicion for ruptures during preparation. Then, as early as 24 h of incubation in the presence of sodium selenite, the lenses result in a dense cortical vacuolization and opacification.

Mechanism of Cataract Formation Induced by Sodium Selenite

As indicated above, in both experimental approaches, either in vivo or in vitro, sodium selenite manifests its effect on the lens by inducing primarily oxidative stress in the lens tissue. However, its exact mode of action is still open to debate. Fris et al. (2006) hypothesized that the formation of selenite-induced nuclear cataract is a result of GSH loss from the lens. Thereafter, the capacity of GSH to buffer the oxidation/reduction status of lens metabolism is diminished, and the sensitivity of rat lenses to oxidative stress is enhanced. The selenite-induced nuclear cataract formation is caused by various contributing mechanisms as summarized, including calpain-induced hydrolysis and precipitation of lenticular proteins.

Calpains (EC 3.4.22.17) are a family of non-lysosomal cysteine proteases with a neutral pH optimum and a requirement of calcium for activation. Studies on experimental cataract have demonstrated calpain-induced proteolysis of β-crystallin as a major mechanism in lens maturation, as well as cataractogenesis (David et al. 1994). Lp82 is the dominant isoform of calpain in rodent lens, suggesting that it may be responsible for the proteolysis attributed to calpains in experimental cataract.

Evaluation and Critical Assessment

Concluding Remarks on the Relevance of the Selenite Model

According to Doganay et al. (2002), selenite cataract has many general similarities to human cataract, e.g., lipid membrane vesicle formation, increased level of calcium, elevated amount of insoluble proteins, enhanced proteolysis, decreased amount of water-soluble proteins, and declined level of GSH. Major dissimilarities are also present when compared to human cataract: no high molecular weight covalent aggregates or increased disulfide formation. Selenite cataract appears to be dominated by rapid calpain-induced proteolytic precipitation (Shearer et al. 1997), while human senile cataract may be caused by oxidative stress over a long period. Currently, the authors' best conclusion about the relevance of the selenite cataract model to human cataract may be that the selenite cataract model is a useful biological model for initial drug testing. However, important differences between human and selenite cataracts have to be taken into consideration.

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Models on Eye Inflammation

Beat P. Mertz

Contents

Purpose and Rationale

Ocular inflammation encompasses a variety of disorders affecting the eye, including keratitis, uveitis, and CMV-induced retinitis. Many of these disorders are associated with the use of contact lens in human patients.

Keratitis can be caused by bacterial (staph), fungal, and immune-mediated response but is most commonly associated with herpes simplex 1 viral infections.

Mouse, rat, rabbit, and primate keratitis models based on these types of infectious agents all are available.

Uveitis is defined by inflammation of the uvea or middle layer of the eye, responsible for blood supply. Uveitis is an inflammation inside the eye, specifically affecting one or more of the three parts of the eye that make up the uvea: the iris (the colored part of the eye), the ciliary body (behind the iris, responsible for manufacturing the fluid inside the eye), and the choroid (the vascular lining tissue underneath the retina). Problems associated with uveitis are relatively underappreciated by the general population and ophthalmologists alike. Few people realize, for example, that the third leading cause of blindness in the United States is uveitis.

The disorder is most often associated with autoimmune issues, such as rheumatoid arthritis or ankylosing spondylitis. Common animal models involve the use of S-antigen to induce uveitis.

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CMV retinitis often occurs in immunocompromised patients in reaction to *Cytomegalovirus* infection, an otherwise harmless human parasite.

Animal models of CMV retinitis include SCID-humanized mice and immunocompromised guinea pigs and monkeys.

In vitro eye models can aid in prioritizing active compounds, evaluating lead compounds, and assessing drug-to-drug interactions while minimizing animal use. As the first step in the preclinical drug development process, in vitro eye models offer effective systems to screen preclinical candidates without the costs and time associated with in vivo models. In vitro models for indications including dry eye, eye infection, and pain, wound healing, retinal diseases, and ocular cancer (neoplasm) have been developed, including cells and cell lines, such as corneal cultures, human retinal capillary endothelial cells *(HRCECs)* and human retinal pigment epithelial cells *(ARPE-19)*, and bovine retinal capillary endothelial cells *(BRCECs)* and pericytes.

Models such as the human corneal epithelium *(HCE)* eye irritation assay, nuclear translocation model of hypoxia-induced factor-1alpha *(HIF-1α)* and nuclear factor kappa B *(NFκB)*, and human embryonic stem cells *(hESCs)* can provide efficient, predictive models during preclinical drug development.

Procedure

Experimental Autoimmune Uveitis (EAU) Model (Developed by Iris Pharma)

Experimental autoimmune uveitis (EAU) is an organ-specific autoimmune disease and serves as a model of certain ocular inflammatory pathologies in human. In this model, rats develop EAU in up to 75 % of induced eyes. Treatment with oral cyclosporine A administration totally reduced the inflammation in this model with 100 % of the eyes protected; rat eyes treated with oral cyclosporine A administration do not show any incidence of EAU.

The most common form of uveitis is anterior uveitis, which involves inflammation in the front part of the eye. Pars planitis is the inflammation of the pars plana, a narrow area between the iris and the choroid. Posterior uveitis affects the back portion of the uveal tract and involves primarily the choroid. The inflammation causes spotty areas of scarring on the choroid and retina that result in areas of permanent vision loss.

Destructive inflammatory diseases of the eye such as uveitis are the third leading cause of blindness worldwide.

Uveitis in Spondyloarthritis

Environmental factors such as bacillary dysenteries are strongly implicated in some forms of SpA, such as reactive arthritis (Rosenbaum and Rosenzweig 2012). Components of bacterial cells have been detected in the inflamed synovium (van der Heijden et al. 2000 and might activate the innate immune system and enable ingress of leukocytes, including T cells, into the joint tissue. Whether a similar phenomenon occurs in the uvea remains unknown. Nevertheless, adhesion molecules do vary between different tissue beds (Ruoslahti and Rajotte 2000), and these "addresses" could conceivably be shared between the uvea and the synovium, directing inflammatory cells to both locations.

Animal models could help clarify the mechanism underlying the dual vulnerability of the eyes and joints in SpA, where most patients have ankylosing spondylitis (AS) and about a third have uveitis involvement. However, JT Rosenbaum et al. are aware of only two such models describing this combination of organ involvement, perhaps because the majority of investigators do not meticulously examine the eye for evidence of inflammatory disease. Adjuvant arthritis can be induced in specific strains of rats by an injection of Freund's adjuvant, which consists of killed mycobacteria in mineral oil. The affected rodents develop many features characteristic of SpA including spondylitis, new bone formation, and nongonococcal urethritis (Petty et al. 1989), while uveitis is also well described in this model

(Petty et al. 1989). In adjuvant arthritis, granulomas can be detected in the uveal tract and the inflammation is often bilateral (Petty et al. 1989). These features differ from uveitis associated with AS, as eye inflammation is typically unilateral and is clinically described as nongranulomatous in this disease.

JT Rosenbaum et al. have also detected uveitis in an established model of arthritis, in which BALB/c mice are immunized with aggrecan (Kezic et al. 2012). Aggrecan is the main proteoglycan in entheses, the tendinous insertions in the bone, which are a major target for inflammation in SpA. This model of eye disease is more consistent in transgenic mice whose T-cell receptors specifically recognize the G1 epitope of aggrecan (Kezic et al. 2012). Aggrecan is detectable in synovial and peripheral joints along with the uveal tract, but expression of this proteoglycan is not limited to these sites. Moreover, although the uvea and synovium are both affected in this model, the cytokine dependence of inflammation in each of these organs is substantially different; the absence of IFN-γ ameliorates joint disease while markedly exacerbating eye disease (Kezic et al. 2012). Nevertheless, this model could be useful in determining whether T cells that migrate into the eye use the same adhesion molecules as those that infiltrate the joints and will enable further investigation into the link between joint and uveal inflammation.

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Part XVI

Acoustic Activity

Pharmacologic Intervention for Acquired Hearing Loss: Assays of Drug-Induced Inner Ear Damage

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Contents

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Pharmacologic Intervention for Acquired Hearing Loss: Assays of Drug-Induced Inner Ear Damage

Hearing can be permanently impaired by therapeutic drugs, most notably the aminoglycoside class of antibiotics and the platinum-based chemotherapeutic drugs. The prevalence of this hearing loss in patients (ototoxicity) has prompted great interest in better understanding the mechanism of injury and prevention. Aminoglycoside ototoxicity usually affects hearing in the high-frequency range before progression to lower frequencies. For in-vitro studies, the organotypic organ of Corti model provides an inner ear (cochlear) tissue preparation for investigating the effects of drugs on the mammalian ear, including testing of drug toxicity, protective effects, and delineation of cellular and molecular mechanisms. Gentamicin is a prototype aminoglycoside, and a gentamicin injury model is presented here as an established preparation for testing potential protective drugs. Similar injury models can be developed using other ototoxic agents such as the chemotherapeutic drug cisplatin. For in-vivo investigation, the guinea pig is a preferred model. Two advantages of the guinea pig in hearing research include ease of access to the cochlea and a pattern of aminoglycoside-induced injury similar to humans.

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Organotypic Organ of Corti Explant Model

Purpose and Rationale

- This aminoglycoside-treated preparation allows to perform an in-vitro assay of drugs that may prevent injury to cochlear hair cells and thus serve as potential protective agents.
- A classic preparation involves exposure of the explant to gentamicin, creating a preferential outer hair cell loss, with a gradient of injury from the cochlear base to apex. This pattern of injury mirrors that observed in the human ear.
- An important feature of the model is its efficiency, in that it allows relatively rapid evaluation prior to investing the time and expense of in-vivo studies.
- The organotypic culture is derived from mammalian tissue and hence is physiologically relevant to the human cochlea.

Procedure

Animal Selection

- CBA/J mice postnatal day 2–3 pups
  - Other strains and species, including transgenic mice, may be used depending on the experimental questions.
  - Guinea pig models are precocious in development and more closely resemble adult cochlea, making it challenging to maintain in culture compared to mice neonatal models.

Procedure

- Prepare culture dish
  - Prepare collagen gel: combine 180 μL collagen I rat tail (BD Biosciences), 20 μL of 10× Basal Medium Eagle (Sigma), and 20 uL of 2 % sodium carbonate.
    - Store on ice for 15 min after preparation.
  - For each cochlear explant, place one 15 μL drop of collagen gel on 35-mm culture dishes.
    - Allow to gel at room temperature for 10–15 min.
  - Prepare 200 mL of serum-free medium as below and add 1 mL of solution to each culture dish.
    - 2 g bovine serum albumin (Sigma)
    - 2 mL Insulin-Transferrin-Selenium 100x supplement, (Invitrogen)
    - 4.8 mL of 20 % glucose (5 mg/mL) (Sigma)
    - 0.5 mL of penicillin G (8.8 U/mL) (Sigma)
    - 190.8 mL 1× Basal Medium Eagle (Sigma)
    - 0.44 g sodium bicarbonate (2.2 g/L)
- Dissection
  - Mice pups are anesthetized and euthanized.
  - Inner ears and cochlea are extracted and placed in cold Hank's Balanced Salt Solution (HBSS).
  - Dissect away auditory nerve bundle and lateral wall tissues, including removal of stria vascularis while avoiding injury to the basilar membrane.
    - Avoiding trauma to the cochlear hair cells (located at periphery of the basilar membrane) will ensure quality of the preparation.
  - Meticulous care is required when placing the organ of Corti on previously prepared culture dish to ensure proper orientation.
  - The tectorial membrane should face upward. Take care to prevent it from being folded along the undersurface of the specimen, as this will obscure visualization. The explant should not be twisted or rotated but rather be allowed to have a gentle turn.
  - Incubate explants for 4 h at 37 °C, 5 % CO_2, then add additional 1 mL of culture medium.
  - Continue incubation for 2 days to allow recovery from dissection stress.
  - Treat with drug of choice for 3 days.
- Videos describing the technique are available at:
  - http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/video-tutorial-for-cochlear-dissection/

- http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology-resources/cochlear-dissection-summary/
- Treatment
  - Incubate in the presence of the vehicle used in experimental group.
    - Care should be taken to control for the effect of solvents or vehicles.
      - DMSO, which is often used to dissolve putative protectants, has intrinsic antioxidant properties.
      - Similarly, phenol red frequently used in media also has antioxidant effect.
    - Replace medium with new media containing a concentration of gentamicin that creates a known gradient of injury.
      - *Note*: Commercial gentamicin is a mixture of three related compounds. Each batch therefore must be titrated to induce 50 % outer hair cell loss. The optimal concentration of gentamicin varies with lot but often falls in the 2–5 μM range.
  - Fixation and staining
    - Rinse explants 3× with phosphate-buffered saline (PBS).
    - Fix with 4 % paraformaldehyde overnight at 4 °C.
    - Permeabilize for 30 min with 3 % Triton X-100 in PBS at 22–24 °C.
    - Rinse explants 3× with PBS for 10 min.
    - Incubate with fluorescent phalloidin stain or Myosin VIIA antibody stain for 30 min.
    - Rinse explants 3× with PBS for 10 min.
    - Mount on slide.

Evaluation

- **Morphology**
  - Hair cells
    - Hair cells are examined under light or confocal microscopy to examine for hair cell death and structural changes from the apex to the base.
    - The hair cells are evaluated for integrity of the hair cell outline, stereocilia, and ultrastructural evaluation of organelles (mitochondria, nucleus, endoplasmic reticulum).
  - Ribbon synapse
    - The presynaptic terminal can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
    - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
  - Spiral ganglia
    - Examination of the spiral ganglia cells requires different dissection and sectioning techniques, described by Szabo et al. 2014.
- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from base to apex and entered into programs designed for cytocochleograms (KHRI Cytocochleogram is one such program available).
  - This provides comparison of cell counts to normative data established from control specimens.
  - Number of preserved hair cells is calculated as a percent and plotted as function of distance from apex to basal turn of explant.
- **Immunohistochemistry**
  - The explant has distinctive geometry, which must be preserved. The tissues are not thin sections but rather a complete tissue. Microscopy should consider vertical stacking and need to evaluate in two planes for nucleus and organelles.
  - Triton X-100 is the most commonly used permeability agent.
  - Immunohistochemistry is useful to examine specific molecules that may be up- or down-regulated.
- **Other approaches**
  - The sensory hair cells represent a small percentage of the cellular tissue within the explant, with most of the tissue mass contributed by supporting cells and neural elements. Therefore, sensitivity is limited for discerning effects on hair cells.

- **Western blotting** reflects this cellular mix. One protocol involves the following: use of glass/micro tissue grinder to pool and homogenize explants in lysis buffer. Follow general procedures for western blotting.
- **Gene expression**: RT-PCR, microarray, and in-situ hybridization have been described for evaluation of gene expression. A variety of other techniques such as electroporation have also been described.
- http://www.jove.com/video/1685/primary-culture-plasmid-electroporation-murine-organ
- This video describes isolation and culture of the murine organ of Corti with or without the spiral limbus and spiral ganglion neurons. It also shows expression of an exogenous reporter gene in the organ of Corti explant by electroporation.

Critical Assessment of the Method

- The organotypic organ of Corti model has a proven track record for predicting toxicity and potential protection for in-vivo experimentation.
  - Allows study of hair cell death and protection in a living cellular model
  - Highly sensitive assay for injury to outer hair cells or organ of Corti
- Assay is a starting point for screening and requires follow-up in-vivo experimentation for the assessment of likely clinical implications.
  - Some protective agents may overcome aminoglycoside toxicity by preventing drug entry or inactivating the parent compound; assessment for such effects using antimicrobial interference assays is a necessary step.
- Considerations
  - By virtue of removal of explant from intact organism, this model eliminates the first-pass effect and may not reflect normal metabolism/elimination kinetics of systemic applications.
    - The assay does not allow one to predict the toxicity of prodrugs or toxic metabolites. The impact of P450 activation/inactivation of drugs is not represented in the model.
  - The endolymphatic potential is not preserved due to loss of boundaries between endolymph and perilymph.
  - Removal of stria vascularis and bypass of the blood labyrinth barrier increase sensitivity. The assay is thus highly sensitive to toxicities that might arise in the setting of a compromised blood–brain barrier, which occurs in a variety of disease states and with exposure to certain pharmacological stresses, such as chemotherapy.
  - Cochlear dissections may cause stress to the sensory hair cells (via temperatures, dissection method, etc.) and hence may cause expression of proteins that may lead to data modification (Casado and Cutillas 2011).

Modifications of the Method

- Dissection model variations
  - A variety of animal alternatives to the mouse are available, most commonly including the rat or Mongolian gerbil.
  - Preparations can be made with or without the spiral limbus and spiral ganglion neurons.
  - Immortomouse cell lines
    - Cell lines derived from the organ of Corti (immortomouse) have previously been explored as potential screening tools to evaluate toxic or protective effects on the inner ear.
    - These immortalized cells have been criticized for showing results that do not correlate with those observed in explant hair cells and in vivo.
    - These cells lack mechanoelectrical transduction channels, possess molecular machinery that resists cell death from aminoglycosides, and typically require 100-fold increased dosing versus chronic explant model to elicit effects.

- Zebra fish models
  - These have been studied as screening tools for ototoxic agents and otoprotectants based on the premise that the mechanosensory cells found in neuromasts have similarities to sensory hair cells of the inner ear.
  - These neuromasts exhibit sensitivity to aminoglycoside and cisplatin ototoxicity that is analogous to that observed in the inner ear, although their use in screening has led to false positives and negatives. Results from zebra fish screens thus require verification in other systems.
- Pharmacologic variations: replacing the drug of interest
  - Other aminoglycosides
    - Streptomycin, kanamycin, tobramycin, neomycin, amikacin
  - Cisplatin
    - Platinum-containing chemotherapy agent commonly used for solid malignancies
    - Common toxicities include oto- and nephrotoxicity
- Chronic versus acute model
  - Chronic model example: 2.5–5 μM aminoglycoside exposure for 72 h.
  - In contrast to the chronic treatment model, a 24 h acute treatment regimen typically involves gentamicin doses 10–100-fold higher.
    - Acute model may be useful for monitoring short-term changes in oxidative stress, protein expression, or covalent modifications.
- Toxicology screen: In assessing the inherent toxicity profile of a candidate otoprotectant, it is often helpful to establish the toxicity curve and use this data as a reference for defining target dosing for the putative drug.

Examination of Electrode Insertion Trauma (EIT)

- Cochlear implant surgeries that involve surgical placement of an electrode array into the inner ear cause hair cell trauma, leading to reactive oxygen species formation, inflammation, apoptosis, and fibrosis in the traumatized area (Bas et al. 2012).
  - These reactive measures diminish the effectiveness of the cochlear implant.
  - The molecular mechanisms underlying hair cell apoptosis and inner ear fibrosis in a cochlear injury setting are not well understood.

Procedure

- **Animals**: Postnatal day 3 rats (Wistar strain)
  - Animal is anesthetized and cochlea is dissected out.
- **Groups**
  - Control group: Cochleostomy only
  - Cochleostomy with 0.28 mm monofilament line insertion (EIT).
  - Cochleostomy with 0.28 mm monofilament line insertion and dexamethasone (DXM 20 μg/mL).
  - Dexamethasone has been shown to be protective.
- **Cochleostomy**
  - *Procedure video link*
  - A 0.35-mm diameter hole is created in the cochlea with sharpened No.5 Dumont forceps next to round window.
  - Insert monofilament through hole three times at angles 110–150° to achieve appropriate insertion trauma into scala tympani.
  - Incubate cochlea for 10 min in phosphate-saline buffer.
  - Excise outer hair cell explants.
  - Culture in serum-free media (Dulbecco's Modified Eagle's Medium).
  - Supplement with glucose (6 g/L), 1 % of N-1 supplement.
  - Image using Wild-Heerbrugg M400 stereomicroscope and color camera.

Evaluation of Electrode Insertion Trauma

- Reactive oxygen species detection (cellROX)
- Gene expression studies
  - Pro-inflammatory enzymes (iNOS, COX-2)
  - Pro-inflammatory cytokines (TNF03B1, IL-1β)
  - Wound-healing growth factors
- **Histology and morphology**
  - Morphology (see Evaluation section for more details)

- Cytocochleogram (see Evaluation section for more details)
- Immunohistochemistry: *Cleaved caspase-3, apoptosis-induced factor (AIF), and endonuclease G (ENDO G)* (see Evaluation section for more details)

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Aminoglycoside In-Vivo Model

Purpose and Rationale

- In-vivo aminoglycoside models allow investigators to examine the effect of aminoglycoside mechanisms on the inner ear and test possible therapies.
- Although animal models mirror human disease imperfectly, the intact organism preserves many of the physiologic features that are absent with in-vitro screens.
- The guinea pig model allows easy access to the cochlea and a pattern of aminoglycoside-induced injury that parallels the toxicity seen in humans.

Procedure

Animal Selection: *Guinea Pig Model*

- Pigmented adult guinea pigs weighing 200–400 g.

  Albino guinea pigs carry mutations that affect auditory processing.
  - Control: saline administration
  - Test group: aminoglycoside administration
    - Gentamicin, aminoglycoside ototoxicity model, in the guinea pig in-vivo model is well established.
    - Generally aminoglycosides are well tolerated but body weight should be monitored.

Procedure

- **Treatment**
  - Aminoglycoside group
    - Inject 100 mg gentamicin base/kg body weight intramuscularly (subcutaneous and intraperitoneal are also available options).
      - Monitor body weight daily and adjust dose accordingly.
      - *Note*: Commercial gentamicin is a mixture of three related compounds. Each batch therefore must be titrated to induce 50 % outer hair cell loss.
    - Treat daily for 14 days.
  - Control group
    - Inject equivalent volume of saline in control group guinea pigs.
    - Treat daily for 14 days.
  - Animals are monitored and evaluated at 12 weeks after beginning of treatment.

- **Testing**
  - **Auditory brainstem response (ABR) testing**
    - ABR is measured at scheduled intervals after ototoxic effects have stabilized.
      - ABR is used to determine auditory thresholds and also can yield latency and amplitude functions for more detailed analysis.
      - Animals are anesthetized by intramuscular injection of 40 mg/kg ketamine +10 mg/kg xylazine or equivalent drug combinations.
      - Needle electrode placement
        - The active electrode is placed at ipsilateral vertex.
        - The reference electrode is placed subcutaneous below ipsilateral right pinna.
        - Ground electrode placed either in opposite ear or right thigh.
      - Insert transducer speculum into either ear external auditory meatus, creating a closed acoustic system.
        - Drug-induced hearing loss is generally bilateral and thus it is only necessary to test one ear.
      - Present tone burst stimuli at low, mid, and high frequencies (for example, 4, 16, 32–48 kHz).
      - Output is fed to an amplifier, viewed on oscilloscope and stored for later evaluation.
      - Threshold shifts are calculated in comparison to individual prestudy established thresholds.
        - Threshold is defined as the intensity that produces a detectable change from the non-stimulus condition.
        - Thresholds are verified twice.
  - **Evaluation of vestibular function**
    - Place animals inside restraining box on rotation table.
    - Insert ground needle electrode to contralateral pinna and active electrode into periocular region.
      - In the dark, induce nystagmus by abruptly starting or stopping rotational table.
      - Measure duration of post-rotary nystagmus and number of beats.
      - For a control, measure optokinetic nystagmus in the light.
  - **Endocochlear potential (EP)**
    - The EP is a positive voltage of 80–100 mV in the cochlear endolymphatic space.
    - EP is highest in the cochlear basal turn, decreased toward the apex.
    - It can be measured by passing double-barrel K-selective microelectrodes into the endolymph through the stria vascularis.
  - **Distortion product OAE (DPOAE)**
    - *Most commonly used method in rodents*.
    - DPOAE is evoked using a pair of primary tones, f_1 and f_2.
    - Evoked frequency (f_{dp}) is mathematically related to the primary frequencies.
    - Place spectrum analyzer microphone in the ear canal and record the amplitude of f and f_1 and calculate $2f_1–f_2$.
  - **Dissection to examine cochlear morphology or immunocytochemistry**
    - Animals are anesthetized and sacrificed and cochlea dissected out.
    - For surface preparation and light microscopy
      - A small opening is made in the apical portion of the cochlea.
        - (Process analogous to the in-vitro explant prep; continue dissection and stain)
      - Fix with 4 % paraformaldehyde overnight at 4 °C.
        - Rinse 3× with phosphate-buffered saline (PBS) for 10 min.
        - Decalcify cochleae in 3 % EDTA at 4 °C for 2–3 days if necessary. In contrast to rodents, guinea pig cochleae can be dissected for surface preparations without decalcification.

- Rinse explants 3× with PBS for 10 min.
- 0.1 % Triton x-100 in PBS for no longer than 1 h.
- Rinse explants 3× with PBS for 10 min.
- Incubate with antibodies and/or stain.
- Rinse explants 3× with PBS for 10 min.
- Mount on slide.

- For electron microscopy
  - A small opening is made in the apical portion of the cochlea.
  - Perfuse cochlea through opening with
    - 0.1 M sodium cacodylate
    - 2 % glutaraldehyde
    - 2 mM calcium chloride
  - Incubate overnight at 4 °C.
  - Next, dehydrate cochleae with alcohol and embed in EMbed 812 resin.
  - Slice 6 mm para-modiolar planar sections and mount on slide with Richardson stain.
  - Examine under bright-field and differential interference contrast optics.

Evaluation

- **Daily or weekly evaluation**
  - Body weight: Measure daily and adjust dose accordingly.
  - Evaluate blood urea nitrogen (BUN) and creatinine (Cr) for assessment of renal function, which is commonly impaired by aminoglycosides.
  - Serum albumin provides assessment of protein and nutrition.
- **Assaying of serum drug levels**
  - Obtain from control and treatment groups at desired times.
  - Only include animals who survived entire study period in analysis.
    - Retrieve blood via toenail clipping.
    - Centrifuge sample and store at −20 °C until analysis.
    - For analysis, thaw and dilute 1:100 with normal saline.
    - Analyze by conventional procedures.

Histology and Morphology

- **Morphology**
  - Hair cells
    - Hair cells are examined under light or confocal microscopy to examine for hair cell death and structural changes from the apex to the base.
    - The hair cells are evaluated for integrity of the hair cell outline, stereocilia, and ultrastructural evaluation of organelles (mitochondria, nucleus, endoplasmic reticulum).
  - Ribbon synapse
    - The presynaptic terminal can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
    - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
  - Spiral ganglia
    - Examination of the spiral ganglia cells requires different dissection and sectioning techniques, described by Szabo et al. (2014).
- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from base to apex and entered into programs designed for cytocochleograms (KHRI Cytocochleogram is one such program).
  - This provides comparison of cell counts to normative data established from control specimens.
  - The number of preserved hair cells is calculated as a percent of total and plotted as function of distance from apex to basal turn of explant.
- **Immunohistochemistry**
  - The explant has distinctive geometry, which must be preserved. The tissues are not thin sections but rather a complete tissue. Microscopy should consider vertical

stacking and need to evaluate in two planes for nucleus and organelles.
- Triton X-100 is the most commonly used permeability agent.
- Immunohistochemistry is useful to examine specific molecules that may be up- or downregulated.

Critical Assessment of the Method

- Other species successfully used in vivo include the chinchilla, gerbil, cat, as well as rodents.
  - Considerations in mice:
    - The drug of choice is kanamycin (Wu et al. 2001).
    - High dosage of aminoglycoside is necessary to achieve comparable ear injury state. However animal may develop renal insufficiency before achieving the desired gradient of hair cell injury.
  - Validity of the model is compelling, especially when protection observed from one in-vitro animal study produces similar results in a different in-vivo species model.
    - Effects of metabolism, absorption, excretion, and development of toxic metabolites (or inactivation) are likely to be more similar to the clinical setting than in the explant model.
- Allows for study of physiologic mechanisms for drugs that are promising in explant studies and protective mechanisms
  - In-vivo model allows examining the pathophysiology of clinical and extreme aminoglycoside dosages.
  - Aminoglycoside dosage necessary to induce extreme hearing loss (greater than 60 dB loss) may cause high morbidity rates.
  - Does conserve endocochlear potential (and fluid spaces of endo-/perilymph) which is lost in the explant model.
  - Effects on stria vascularis can be evaluated.
  - Effects on hair cell ribbon synapse and spiral ganglia can be studied.

Modifications of the Method

- In-vivo models can vary in the species used, the aminoglycoside used, or the drug dosage examined.
  - Common drug variation is the chemotherapeutic agent, cisplatin
- Experimental models to eliminate all hair cells include:
  - Diuretic (ethacrynic acid or furosemide) + aminoglycoside
  - This model is not clinically relevant as it does not mimic a normal disease state.
- Procedure may be modified to examine aminoglycosides against a potential mitigator or aggravator.
  - Example of potential mitigators: antioxidants, modulators of cell death and survival pathways
- Combination models that examine dual insults (such as those that examine noise + aminoglycoside injury) may cause additive or synergistic injurious effects.

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Neurobiology and Pharmacology of Sleep Disorders in Otolaryngology: Head and Neck Surgery

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Contents

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Introduction

The Centers for Disease Control reports that over 40 million Americans suffer from chronic sleep disturbances (Li et al. 2011), and the American Academy of Sleep Medicine identifies over 80 distinct sleep disorders (American Academy of Sleep Medicine 2005a). This number may actually be higher, as sleep disturbances are often underreported or mistaken as a consequence of other pathological or pharmacologic processes. Symptoms associated with sleep disorders, such as decline in memory, concentration, fatigue, or irritability, tend to be nonspecific, making it easy to overlook the contributing role that poor sleep quality plays. Progress in sleep research has revealed that sleep loss and drowsiness play a significant role in cognitive performance, in quality of life, and in causing catastrophic accidents. Growing awareness of sleep quality and habits has allowed us to pay closer attention to the many factors that influence sleep, including diet, mood, technology, and environmental factors.

This chapter will provide an overview of the technical tools commonly utilized in both clinical and animal models of sleep studies with a small discussion on circadian sleep disorders. We will examine translational animal research models used to study four of the most common human sleep disorders: insomnia, narcolepsy, sleep apnea, and parasomnias, such as restless leg syndrome. Finally, we will discuss new developments in the field of optogenetics and their applicability in animal models for the advancement of sleep medicine. Please refer to the Appendix 1 for an overview of sleep medicine history. Information related to sleep/wake states, and the neurotransmitters involved, are in Appendix 2, followed by the pharmacologic drug classes implicated in sleep interference in Appendix 3.

Key Diagnostic Tools in Sleep Medicine

A variety of techniques have been utilized in clinical medicine. Although these have been primarily used to study human patterns of sleep, many have also proven useful in animal models. Our understanding of sleep neurobiology, pharmacology, and circadian rhythms largely derives from what we have learned from animal models. The following are a few of the technical tools commonly utilized in sleep medicine studies of clinical and animal models.

Polysomnography

Purpose and Rationale

- Polysomnography (also known as a sleep study) provides various metrics of the respiratory, cardiac, endocrine, and neural electrical activity in a holistic context to examine sleep and arousal states.
- Sleep and brain activity show unique wave patterns associated with each behavioral state.

Procedure

- Human studies are usually monitored through an entire sleep cycle overnight in a controlled setting (lab, hospital, etc.).
- It simultaneously measures multiple metrics including:
  - Electroencephalography (EEG) – can also be performed independently
  - Eye movements (EOG)
  - Muscle activity (EMG)
  - Cardiac activity/heart rhythms (ECG/EKG)
  - Respiratory activity and flow
  - CSF and blood chemistry, temperature
  - Pulse oximetry

Evaluation

- Comparison of EEG activity recorded in the brains of various animals and humans has resulted in distinct EEG profiles that reflect different states of brain function and arousal (see Appendix 2 for more information).
- Studies have defined specific EEG patterns for sleep and wake behavioral states in most mammals and birds (Toth and Bhargava 2013).

  - Includes fish, flies, nematodes, and birds (Corner and van der Togt 2012)
  - Although EEG-defined sleep architecture varies among species, many similarities exist within circadian rhythms, homeostatic regulation, and neurotransmitter roles.
- Understanding the electrical activity of the brain has proved clinically useful, especially in instances of epilepsy monitoring, seizure characterization, brain death identification, or to monitor behavior/anesthetic states (Paterson et al. 2011).

Critical Assessment of the Method

- Home screening tools are more expensive and not as comprehensive.

Modifications of the Method

- These methods may be used to further investigate the relationship of subjective neural processes (such as attention, learning, and memory) by their relationship to quantifiable metrics as measured through EEG and sleep studies.

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Multiple Sleep Latency Test (MSLT)

Purpose and Rationale

- Used to measure sleep latency – (the time it takes to fall asleep and SOREMPs)
  - SOREMPs (sleep-onset REMS period) – REM sleep that occurs within a sleep latency less than 15 min

Procedure

- The patient is placed in a setting conducive for sleeping (must be done in controlled atmosphere).
- Present four or five 20 min nap opportunities that are 2 h apart.
- MSLT Measures most of the same features as polysomnography.

Evaluation

- Results are interpreted in the clinical context.
  - It is used to test for narcolepsy to distinguish between true daytime sleepiness and tiredness.
- Key features measured include sleep latency and SOREMPs (sleep-onset rapid eye movement period).
  - Normal: sleep latency is greater than 10 min and does occur at onset of sleep.
  - Narcolepsy: sleep latency is less than 5 min and with immediate REM sleep (SOREMP).

Critical Assessment of the Method

- Test is not reliable for narcolepsy diagnosis unless at least two sleep-onset periods have occurred.
- MSLT is usually conducted after an overnight polysomnography.

Modifications of the Method

- Models are mainly for human studies as there is no accurate animal model.

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Actigraphy

Purpose and Rationale

- Used in scenarios where MSLT is not appropriate
- Actigraphy is a noninvasive method of monitoring daytime sleepiness through round-the-clock usage of a sensor.

Procedure

- In humans, this is usually worn on the wrist of the nondominant arm.
- It measures gross motor activities, sleep patterns, and circadian rhythms.

Evaluation

- Unlike polysomnography, actigraphy measures these variables in a user's natural sleep and active environment, rather than requiring laboratory monitoring.

Critical Assessment of the Method

- Harvesting information from natural activity patterns provides more accurate and applicable information for both diagnosis and treatment.
- It has a lower cost than polysomnography, which is conducted in formal setting.
- Its accuracy is similar to that of polysomnography (>90 %) (Jean-Louis et al. 1997).

Modifications of the Method

- Recent technology, such as Fitbit, has brought actigraphy technology and personal habit/pattern monitoring for mass consumerism; many of these technologies record and upload data to servers in real time that can be analyzed by both individuals and healthcare providers.
- It has been increasingly used in drug clinical trials for insight into sleep quality.

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Human Versus Animal Models of Sleep Medicine

Sleep disorders in humans are dependent on many factors, including duration, quality, and continuity of sleep. Endogenous systems mediated by hormones and neurotransmitters play a significant regulatory role, as do external influences such as environment, light, and stress. Studying sleep in the context of these variables is further complicated by the subjective experiences of the affected individual, in which personal circumstance and habits play a strong role (Harvey and Tang 2012; Zhang et al. 2007). Animal models provide an invaluable opportunity to examine the physiology of sleep mechanisms without the personal circumstantial components that affect human sleep. Studying these animals in a controlled laboratory setting has allowed us to understand the similarities and differences that exist between human and animal sleep characteristics. However animal models of sleep do not always correlate with human sleep patterns. Sleep duration and patterns have been examined for over 150 animal species, illustrating a wide variability in how sleep and wake are distributed in a 24-h day cycle (Campbell 1984). Sleep quantity, continuity, and ratio of REM versus NREM may significantly vary, and environmental cues such as photoperiod and temperature can affect sleep, as often seen in hibernation and sleep patterns of various animals (Campbell 1984). Strains of animals that exhibit specific behavioral traits (such as insomnia or sleep apnea) have been inbred to create models for sleep studies. The wide variability in animal behavior and sleep patterns means that studies that examine sleep and associated variables may be interpreted differently based on how the model is manipulated and examined (Siegel 2009). Regardless of these differences, many overarching similarities in sleep pathophysiology have been elucidated that span across species.

Insomnia and Circadian Sleep Disorders

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus plays the role of the "master clock" in sleep regulation. Specifically, this location regulates the body's circadian rhythm, an oscillatory biological process that regulates sleep and wake cycles and associated systems. The markers commonly used to study the circadian rhythm include melatonin levels (which increase significantly during sleep), cortisol, and changes in body temperature. The rhythm is regulated

endogenously by a complex interaction of neural, humeral, and cell cues. Additionally, the circadian rhythm is influenced by environmental factors that help regulate our body's response to both day and season, with light exposure as the primary driving factor through which humans have evolved to trigger these pathways (Dickmeis et al. 2013). Disruptions in the suprachiasmatic nucleus or downstream pathways can result in desynchronization from natural sleep patterns. Although there is great variety in how circadian rhythms may be affected, insomnia is one of the most common sleep-associated problems. Insomnia affects a significant portion of the population (Morin 2012) and is defined as either difficulty in falling asleep, poor sleep quality despite adequate opportunity, frequent awakenings, early waking, or trouble falling back asleep. Along with poor sleep quality and quantity, insomnia may cause excessive daytime sleepiness as well as diminished performance and cognition.

Insomnia Model

Purpose and Rationale

- Insomnia is characterized as:
  - Difficulty falling asleep or poor sleep quality despite adequate opportunity
  - Frequent nighttime awakenings
  - Trouble falling asleep
  - Early awakenings
- Manipulating internal and environmental cues in animal models provide an opportunity to examine the mechanisms that cause insomnia.

Procedure

- Animals are exposed to a stressful environment for a defined period of time. Sleep and behavior disturbances are then monitored for several hours afterward.
  - Pre- and post-stress exposure sleep and behavior patterns are compared.
- Animal models help manipulate sleep latency, duration, and awakenings to cause fragmented sleep.

Animal Selection: Rodent and *Drosophila* Flies

- Rodent models – DBA/2J mice (Wisor et al. 2009)
  - Created through selective breeding – these animals are ideal to study insomnia.
  - Mice have a diurnal distribution of sleep that resembles the fragmented sleep that occurs in insomnia (Franken et al. 1999).
  - They exhibit a high number of brief awakenings and slow wave sleep episodes (Daszuta et al. 1983) and spend more time awake during a 24-h period.
- *Drosophila* flies: ins-1 flies (Seugnet et al. 2009)
  - Ins-1 flies (insomnia like) – sleep less than normal flies, have difficulty with sleep latency and duration, and have cognitive impairment
  - Also created through selective breeding – ideal to study genetic markers involved in drive for sleep and insomnia

Intervention Type: Physical, Pharmacological, or Lesion Intervention

- Physical – animals undergo acute sleep deprivation through use of mild stressors/disturbances (Nair et al. 2011; Ramesh et al. 2012).
  - Animals are continually monitored in light and dark environments.
  - To prevent sleep, animals are touched gently when they exhibit physical (behavioral) cues or neurologic (EEG waves) cues.
    - May also be physically roused with tapping or novel objects
  - Automated method – used for examining chronic sleep deprivation (see Modifications)
- Pharmacological – stimulants provide a simple and effective model for sleep-onset insomnia treatment (Bonnet and Arand 1992; Paterson et al. 2007; Richardson 2007).
  - Examples: caffeine, amphetamines, modafinil
  - See drug class "CNS stimulants" in Appendix 3 for further details.
- Lesion models
  - Specific area lesions can be used to physically disrupt cortical sleep pathways.

- See Appendix 1 for more about lesion models.

Evaluation

- Detailed examination of pre- and post-stress behavior including:
- Food intake: EEG/polysomnography
- Immunohistochemistry
- Serum corticosteroid levels
- Blood chemistries
- Glucose tolerance
- Brain temperature
- Spatial learning and/or memory deficits (Ward et al. 2009a)

Critical Assessment of the Method

- An ideal animal model for insomnia should closely mimic human insomnia patterns – thus applicable animal models require manipulation of sleep cycles so that:
  - The animal initiates sleep at times offset from its natural circadian rhythm.
  - Sleep is fragmented.
  - A sleep debt is accrued.
  - Sleep quality suffers despite adequate opportunity to sleep.
- Few animal models have been able to successfully encompass these characteristics.
- It is difficult to determine if manipulations of animal sleep models are translatable to a human model due to variations in baseline sleep characteristics.
  - There is intrinsic variation even within inbred animal strains (Franken et al. 1999).
  - Natural animal rhythms may differ greatly from human sleep.
    - Example: many commonly studied rodent species are active during the night and sleep during the day.
    - Human sleep is significantly consolidated (hours) compared to the fragmented sleep of rodents.
  - There is limited applicability to human studies.
    - In rodents, light and dark may have different effects on the circadian rhythm.
    - Sleep fragmentation may have lesser influence on sleep quality, function, and cognition for rodents compared to humans who have a different baseline for sleep fragmentation.
- Animal insomnia models provide a valuable opportunity to explore and understand the underlying pathophysiological processes that contribute to sleep.

Modifications of the Method

- Experiments done in light and dark environments help identify and compare effects of light on natural sleep patterns.
- Chronic sleep deprivation can be achieved with 2 techniques:
  - "Disk over water" approach (Rechtschaffen and Bergmann 1995)
    - Causes total and/or REM sleep deprivation
  - Platform technique (Machado et al. 2006)
    - Affects REM and slow wave sleep
- Genetic manipulation has revealed clock gene point mutations.
  - Per2 – causes advanced sleep-phase syndrome
  - Per3 – is associated with delayed sleep-phase syndrome (Hamet and Tremblay 2006)
  - Higher incidence of insomnia in individuals homozygous for polymorphism of clock gene (Serretti et al. 2003)

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Narcolepsy

Narcolepsy is characterized by four key symptoms: (1) excessive daytime sleepiness, (2) cataplexy, (3) sleep paralysis, and (4) hypnagogic hallucinations (Akintomide and Rickards 2011). Of these four features, cataplexy is a feature that has been widely studied. Cataplexy is characterized as a sudden loss of muscle tone during wakefulness and is usually triggered by some strong emotion. Loss of muscle tone may be localized or affect the whole body and usually lasts between a few seconds up to 10 min. Narcoleptic patients also report disturbed and fragmented sleep, limb movements during sleep, and increased time spent in REM and NREM sleep. Patients also admit to sleep attacks, where they are overwhelmed by the urge to sleep. Clinical diagnosis of narcolepsy usually requires an MSLT and polysomnography study (American Academy of Sleep Medicine 2005b). Animals have played a significant role in the study of narcolepsy. Lin et al.'s landmark discovery illustrated the role of the orexin receptor mutation to cause narcolepsy in Doberman Pinscher dogs (Lin et al. 1999; Riehl et al. 1998; Nishino and Mignot 1997). Subsequent development of knockout mouse (mice lacking the orexin gene) have provided an animal model through which to study narcolepsy and pharmacologic interventions (Chemelli et al. 1999).

Purpose and Rationale

- To study cataplexy and the role of orexin/hypocretin neurotransmitter in narcolepsy

Procedure

Animal Selection: Canine or Rodent Models

- Canine model – Doberman pinschers (Stanford canine narcolepsy colony)
  - Familial model – has hereditary loss of orexin/hypocretin type II receptor (Ox2R)
    - Cataplexy can be induced via FECT and PECT.
  - Sporadic model – unknown etiology of orexin neuron loss
  - The canine model has been instrumental in the discovery of the orexin gene mutation and its role in narcolepsy.
- Rodent – knockout models (KO)
  - Two types: orexin KO and OxR2 KO mice
  - Both closely mimic human narcolepsy symptoms of cataplexy and EDS (excessive daytime sleepiness or sleep attacks).
  - Both have attacks during their dark period.

Intervention Type/Triggers

- Canines (Riehl et al. 1998; Nishino and Mignot 1997)
- Food-elicited cataplexy test (FECT)
  - Food is placed at regular intervals in a semicircular fashion around the dog. The dog is trained to eat the food in a particular order.
  - Narcoleptic dog – will have multiple partial or complete cataplexy attacks before completing all food
  - Normal dog – will be able to finish all food without pause
- Play-elicited cataplexy test (PECT)
  - Better for younger canines
  - Two or more dogs are brought together in a playroom and allowed to freely interact with one another. Various stimuli (toys, play fighting) induce cataplexy attacks.
- Rodents
  - There appears to be multiple triggers for rodent cataplexy.
    - Group housing (increased social interaction) (Chemelli et al. 1999)
    - Tickling causes ultrasonic vibrations resulting in cataplexy; this is thought to be analogous to laughter-induced cataplexy in humans (Panksepp 2007).
    - Highly palatable food (Clark et al. 2009)
    - Increased locomotor activity (Espana et al. 2007; Overeem et al. 1999)

Evaluation

- FECT and PECT – number and duration of attacks
- Duration required to complete the entire task
- EEG recordings, general rest, and activity patterns

Modifications of the Method

- Usage of different triggers to induce cataplexy (noted in procedure)
- Pharmacologic blockade
  - Blockade of both orexin receptors increased REM sleep but did not induce cataplexy (Brisbare-Roch et al. 2007).
  - Selective Ox1R antagonists – did not induce cataplexy (Smith et al. 2003)
- Neuron ablation
  - Use of cytotoxic transgene-induced apoptosis in orexin neurons causes similar behavior and sleep patterns as orexin KO mice (Hara et al. 2001).
  - Both orexin KO mice and orexin-ataxin3 transgenic mice (non-pure C57BL/6J background) were found to have higher weights and serum leptin levels (Zhang et al. 2007; Hara 2005).
- Models in Rats
  - Neurotoxin (saporin) injection caused nonselective loss of orexin and melatonin neurons in rats (Gerashchenko et al. 2003).

- Orexin knockout via RNA interference technology (RNAi) (Chen et al. 2006)
  - Microinjection of siRNA (short interfering RNA) into rat hypothalamus targets prepro-orexin mRNA causing suppression of prepro-orexin.
  - In vivo method: fast, reversible, and highly selective

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Sleep Apnea

Sleep apnea is one of the most common issues encountered by otolaryngologists, affecting over 18 million Americans (Dempsey et al. 2010). Sleep apnea is defined as intermittent or cyclic disruption of airflow and breathing during sleep. In sleep apnea, there are periods of airflow cessation. Sleep apnea can be divided into central or peripheral causes; central sleep apnea is due to dysfunctions in neural circuitry that result in diminished neural input to the respiratory drive. Obstructive sleep apnea (OSA), in comparison, is

due to airway obstruction in the naso- and oropharynx. This airway obstruction may occur due to airway collapse, as a result of relaxed or excessive pharyngeal musculature. From this point forward, our discussion will focus on peripheral causes of sleep apnea.

Risk factors for OSA include obesity, large neck, and physical abnormality of the upper airway. Individuals may report poor sleep quality and frequent awakenings, which can cause daytime symptoms of fatigue, inability to concentrate, and increased likelihood of motor vehicle accidents. OSA also negatively impacts multiple comorbid conditions including cardiopulmonary disease (pulmonary hypertension), cardiovascular events, diabetes, and neuropsychological consequences on cognition and memory (Veasey 2009). Although sleep apnea can be diagnosed from a clinical history of apneic events, symptoms, and physical exam, polysomnography can be helpful as a confirmatory study.

Treatment of sleep apnea is primary done by addressing the contributing risk factors. Weight loss and surgical reduction of redundant tissue can help minimize airway obstruction to improve airflow. Pharmacological interventions are not the first-line or even second-line treatment; further exploration regarding the pharmacodynamics of sleep in OSA is needed. Rather, physical interventions such as CPAP (continuous positive airway pressure) or BIPAP (bi-level positive airway pressure) continue to be the mainstay treatment options. One challenge to studying sleep apnea in animal models is that few animal species have been observed to have natural sleep-disordered breathing. Although they do present spontaneously in some animals, accurate animal models must provide insight into obesity-driven sleep apnea and/or airflow mechanisms that will still be applicable for humans.

Obstructive Sleep Apnea: Noninvasive Model

Purpose and Rationale

- Intermittent hypoxia experiments can be conducted on anatomic models (English bulldog), obesity models (Yucatan minipigs), or mice to examine the pathophysiological of OSA.
  - This allows evaluation of oxygen desaturation, hypercapnia, hypoxia, and sleep fragmentation (Fletcher and Bao 1996)
- An ideal/successful animal model requires the appropriate physical characteristics necessary as well as consolidated sleep patterns similar to humans.

Procedure

Animal Selection: English Bulldogs, *Yucatan* Minipigs, and Mice

- English bulldog model – anatomic and physiologic model
  - Unlike humans, OSA in bulldogs is unrelated to weight.
  - It naturally displays many clinical features of OSA including: (Hendricks et al. 1987, 1991)
    - Narrow oropharynx
    - Enlarged soft palate
    - Snoring and sleep-disordered breathing
    - Oxyhemoglobin desaturation during sleep
    - Frequent arousals
    - Hypersomnolence with shortened sleep latencies
- Obese Yucatan minipigs model – help examine role of obesity in obstructive sleep apnea (Lonergan et al. 1998).
  - It demonstrates significantly higher apneic states and oxyhemoglobin desaturation rates.
  - New Zealand mice have also been used to study obesity models (Brennick et al. 2009).
- Rodent models: C57BL/6J mice – hypoxia models
  - Used to assess respiratory function along with pharyngeal, mechanical, and neuromuscular control (Foutz et al. 1979)
  - Also have been used for genetic and pharmacologic manipulation studies targeting apneic and sleep-disordered breathing
- **Intervention**: Intermittent hypoxemia (repetitive hypoxia and oxygenation exposure) (Dematteis et al. 2009).

- Animals are either mask ventilated or placed in a ventilation chamber.
- Intermittent hypoxia is conducted in the animal's diurnal sleep phase.
  - Nitrogen-enriched air is used to induce oxygen desaturation.

Evaluation

- Arousal states
- Oxygen desaturation
- Hypercapnia
- Oxyhemoglobin desaturation
- Blood chemistries
- Systemic blood pressure
- Spatial learning and/or memory deficits (Ward et al. 2009b)
- Number of awakenings/sleep fragmentation

Note: evaluation of human sleep apnea is primarily gauged on the extent of oxygen desaturation and number of apneic/hypoapneic events (American Academy of Sleep Medicine 2005c).

Critical Assessment of the Method

- Intermittent hypoxia models enable us to isolate and investigate individual factors that contribute to both acute and chronic effects of OSA in animals.
- However, the translational ability of animal sleep studies is limited by differences in sleep patterns in comparison to human OSA (Dematteis et al. 2009).
  - Example: obesity is a significant factor associated with the development of OSA in humans, but not necessarily so in animals.
  - Example: sleep fragmentation in humans has proportionally larger effects when compared to animal species where sleep fragmentation is routine.
- Human metabolic or cardiovascular effects from OSA, such as hypertension, may not be adequately reflected in animal species.
  - Animals may physiologically respond differently to stressors or stimuli.
  - Example: intermittent hypoxemia OSA models cause hypoxemia as compared to hypercapnia in humans (Kanagy 2009).
- The significant cognitive and quality-of-life effects of OSA are difficult to measure in a non-human study.
- Further investigation and model development are necessary to determine the consequences of behavior, cognition, and chronic health due to long-term sleep apnea.

Modifications of the Method

- Imposing fragmented sleep patterns on these animals may help elucidate mechanisms that may be applicable in humans.

Intermittent Type

- There is significant variety in the intermittent hypoxia model – various hypoxic concentrations and durations have been examined.
- The timing of the intermittent hypoxia model also varies and can be applied during the animal's natural active or somnolent phase.

Acute Versus Chronic

- Intermittent hypoxia models introduce temporary stressors to the animal and allow time for recovery. The nature of this model allows it to be applied for a much longer duration, enabling researchers to study the chronic effects of obstructive sleep apnea (Savransky et al. 2007).
- Long-term intermittent hypoxia model can cause irreversible loss of noradrenergic and dopaminergic (wake-active) neurons through vacuolization and dysfunctional c-fos activation (Zhu 2007).

Surgical Intervention

- Surgical intervention occurs with placement of a surgical tracheostomy tube (Farre et al. 2003; Fewell et al. 1988).
  - Temporary partial occlusion of the endotracheal tubes allows manipulation and examination of the animal's physiological, neural, and cardiovascular parameters (Brooks et al. 1997; Jun and Polotsky 2007).

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Restless Leg Syndrome and Related Motor Disorders

Restless leg syndrome (RLS) and sleep-related motor disorders are relatively common, with prevalence rates up to 15 % (Yeh et al. 2012). Although they can occur at any age, they are more likely to occur in older adults, preferentially affecting women (Ohayon 2012). Although it appears that RLS does have an underlying genetic component (Ekbom and Ulfberg 2009), the

pathophysiology of the disease is not fully understood. Two key molecules, iron and dopamine, have been found to play an impactful role in RLS. Human studies have illustrated correlations of low iron content in serum and CSF with RLS severity (Mizuno et al. 2005). However, other contributing factors must play a role in RLS, as not all iron-deficient patients develop the disorder (Earley et al. 2000). Alternatively, the role of dopamine in degenerative motor disorders such as Parkinson's has led to studies that have identified altered dopaminergic profiles in RLS patients through autopsies and functional MRI (Connor et al. 2009). This dopaminergic role is further substantiated by symptom improvement with the administration of dopaminergic agonists (and exacerbation through antagonists) (Cano et al. 2008). Diagnosis of RLS is based on clinical symptoms and may include: (1) urge to move the legs associated with paresthesias or dysesthesias, (2) symptoms that start or worsen with rest, (3) partial relief of symptoms with movement, and (4) worsening of symptoms in the evening or at night (American Academy of Sleep Medicine 2005c). The subjective nature of RLS makes it difficult to replicate in animal models. However, developed animal models explore and exploit the role of iron and dopamine in sleep-associated movement disorders.

Combined Dopamine and Iron Deficiency Model

Purpose and Rationale

- The purpose of this study is to manipulate the effects of iron deficiency and dopamine deficiency in rats to see if they can be used to reproduce behavioral changes that mimic RLS symptoms.
- Animal models that explore the role of dopamine in RLS have been developed based on the findings that RLS symptoms have responded well to dopaminergic agents.
- There is also significant evidence suggesting that dysfunctional CNS iron homeostasis plays a contributing factor to RLS based on imaging and autopsy studies of the striatum and red nucleus (Mizuno et al. 2005; Earley et al. 2000).

Procedure

Animal Selection

- Sprague–Dawley rats

Intervention/Manipulation

- Iron manipulation:
  - For the first month:
    - Half the rats were fed a regular diet.
    - Half were fed iron-deficient diet.
    - After the first month, each group was further subdivided for dopamine intervention.
- **Dopamine** manipulation:
  - Dopamine neurotoxin of 6-hydroxydopamine (6-OHDA) was stereotaxically injected bilaterally into A11–A14 dopaminergic nuclei (Ondo et al. 2000, 2007).
  - Control animals underwent sham surgeries.
- **Pharmaceutical** manipulation:
  - Conducted 1 month after surgery
  - Challenged with three drugs that affect dopaminergic pathway:
    - Ropinirole – D2/D3 agonist
    - SKF-38393 – D1 agonist
    - Haloperidol – D2 antagonist

Evaluation

- Behavior and iron chemistries were recorded several times:
  - At baseline
  - After dietary intervention
  - After 6-OHDA lesioning
  - After pharmacological manipulation
- Locomotor activity, sleep and sensory responses, and circadian rhythm
  - Serum iron was assessed from tail vein samples.
- Autopsy
  - Tissue iron concentrations were taken from brain and spinal cord samples.
  - Brain sections were examined to see if 6-OHDA injections had affected other

dopaminergic regions (substantia nigra or ventral tegmentum).
- Diencephalon and mesencephalon sections were stained for TH-immunohistochemistry.

Findings
- Manipulations of iron and dopamine nuclei were able to induce behavioral changes consistent with RLS (Qu et al. 2007).
- Pharmacologic treatments showed expectedly appropriate effects on hyperactivity (Lu 2011).
  - Agonists returned hyperactivity to baseline levels.
  - Antagonists nonspecifically worsened locomotor activity in both lesion and sham animals.

Critical Assessment of the Method
- Although behavioral responses are similar to those found in clinical RLS of humans, the subjective aspects of paresthesias or dysesthesias cannot be adequately measured (Ondo et al. 2000, 2007).
  - These preliminary studies have not included measurements of electrophysiological sleep metrics and tested a small sample size.
- The substantia nigra, ventral tegmentum, and suprachiasmatic nucleus may be affected by 6-OHDA, as they all have projections to the A11 nucleus.
  - Thus while this lesion model may reproduce features of RLS, it may only give a partial understanding of the pathophysiology.
- Combining neurotoxin lesioning methods with an iron deficiency diet provides a hyperactive animal model that can be used to study clinical RLS and further be applied for future therapeutic trails.

Modifications of the Method
- The above study has been built on prior studies that individually study the role of dopamine (Ondo et al. 2000, 2007) or iron (Dean et al. 2006) in RLS.
  - Iron deficiency alone
    - Iron-deprived mice also show an increase in wakefulness during their circadian phase that correlates with human RLS (Dean et al. 2006), suggesting their role in being a comparable animal model for human RLS.
- Dopamine deficiency alone
  - Studies the involvement of the A11 dopamine cell cluster (diencephalic spinal tract) in RLS using neurotoxin to induce bilateral depletion of the nuclei (Ondo et al. 2007; Clemens et al. 2006)
- Pharmaceutical modification:
  - Usage of other dopaminergic agonists or antagonists such as pramipexole (Ondo et al. 2000)
- Genetic modification
  - D3 receptor knockout mice (D3KO) naturally display behaviors consistent with RLS, including hyperactivity, increased locomotor activity, and hypertension (Ali et al. 1991; Espinar-Sierra et al. 1997).
  - Iron-deficient D3KO demonstrated higher acute and persistent pain and earlier onset of activity (by 3–4 h) compared to control or only iron-deficient mice (Dowling et al. 2011).
- Fluorescent tracer (Fluoro-Gold) can be stereotaxically injected into spinal cord of C57BL mice to further explore dopaminergic pathways in the brain and spinal cord (Qu et al. 2006).

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Optogenetics: A New Tool for Study of Neurotransmission in Sleep

Although our understanding of the brain's neurobiology and circuitry has grown significantly, it is still difficult to specifically study one cell type in the brain while leaving the rest affected. Electrodes unselectively stimulate all circumferential tissue, triggering both activation and inhibitory pathways. Although pharmaceutical manipulation offers more precision, their actions lag in the real-time nanosecond environment of neural circuitry. In the late 1970s, a microorganism protein called opsin was discovered to regulate electric charge across cell membranes in response to visible light in the microorganism's environment. In 1971, Stoeckenius and Dieter demonstrated that one of these proteins, bacteriorhodopsin, a single-component ion pump, activates upon exposure to green light photons (Lozier et al. 1975). In 1977, halorhodopsins joined the family of proteins. These remarkable light-activated proteins were largely forgotten about until 2002, when another protein, channelrhodopsin (ChR), was discovered. Multiple forms of the channelrhodopsin protein were subsequently identified, some of which are activated by yellow light (VChR1) while others by blue photons (ChR1 and ChR2).

These proteins and their potential applications remained relatively untouched until 2005 when scientists discovered they might hold the key toward unlocking neurocircuitry mechanisms with unprecedented specificity. Much of the groundbreaking work surrounding optogenetics has occurred at Stanford University, specifically under the guidance of Karl Deisseroth, a pioneer in the field.

In 2005, Deisseroth and his team demonstrated successful use of transfection techniques to insert spliced opsin genes with a promoter into specific neuronal cells of interest. Then, using safe pulses of visible light, the team was able to manipulate millisecond precision control over the targeted neuronal cells' firing patterns. This discovery laid the groundwork to utilize microbial opsin genes to effectively turn neurons on and off, safely, in response to light in a real-time manner. Since then, opsin genes have been further modified to respond to variations in the light spectrum, including "fast" and "slow" channelrhodopsin mutants that provide precise control over a neuron's firing, timing, and duration (Zhang et al. 2011; Gunaydin et al. 2010). With optogenetics, scientists are able to manipulate a mixed population of cells, specifically activating some while inhibiting others in order to see subsequent effects and interactions. Tools developed in 2006 and 2007 now enable scientists to direct light for optogenetic control to any superficial or deep component of the brain. Combined with the development of millisecond scale instruments to record electrical activity, scientists are now able to study and target a specific cell, at a specific time, in a freely moveable animal.

Tuning Arousal with Optogenetic Modulation of Locus Coeruleus Neurons

Purpose and Rationale

- The purpose of this study was to examine the role of the locus coeruleus (LC) in noradrenergic firing patterns on an organism's behavior and arousal. This study attempted to selectively manipulate the LC and observe behavior in a real-time setting (in vivo) (Carter et al. 2010).
- This study demonstrates the usage of optogenetics to selectively target and activate neurons.

Procedure

Animal Selection

- Tyrosine hydroxylase (TH)RES–Cre knockin mice were mated with C57BL/6J wild-type mice.
- Only male heterozygous mice 8–10 weeks old at the start of the experiment were used.
- All mice were housed individually with constant temperature, humidity, and 12-h light–dark cycles with food and water provided ad libitum.

Procedure

- Virus preparation
  - Optogenetic transgenes were incorporated into Cre-inducible recombinant AAV vectors with AAV5 coat proteins.
  - Viral concentration of 2×10^{12} genome copies (gc) per ml was achieved and stored at −80 °C before stereotaxic injection.
- Surgery
  - Anesthetized mice were placed on a stereotaxic frame.
  - AAV5 carrying recombinant or control virus was injected adjacent to the LC through an internal cannula at a rate of 0.1 μl min^{-1} for 10 min.
  - Mice received either bilaterally or unilateral injections into LC via an inner cannula.
- Only mice that received bilateral injections also had additional surgical implants of an inner cannula and EEG/EMG implant.
  - After surgical recovery, mice were acclimated to a flexible EEG/EMG connection cable which enabled free movement within cage.
  - For photostimulation/photoinhibition experiments, fiber-optic cables were placed inside surgically implanted cannula.
- Light pulses of various frequencies and durations were generated.

Evaluation

- Polysomnography
- Photostimulation and photoinhibition
- Cardiovascular measurements
  - Heart rate
  - Blood pressure
- Microdialysis and high pressure liquid chromatography
  - Fresh artificial cerebrospinal fluid was evaluated for norepinephrine levels.
- After experiment completion, histology and electrophysiology were performed on acute brainstem coronal slices.

Critical Assessment of the Method

- Traditional methodologies that permanently affect neuronal activity via ablation, pharmacologic, or knockout methods may cause pathway adaption through the recruitment of other circuits involved in sleep/wake states, food intake, or endocrine regulation (Luquet et al. 2005b; Wu et al. 2009b).
- Optogenetics offers the ability to specifically activate or inhibit neural function for predefined periods without distressing the animal in a freely moving environment.
  - Offers rapid, real-time results "relevant to natural sleep/wake events" (Carter et al. 2010) in comparison to pharmacologic or genetic manipulation methodologies
- Enables comparison of tonic versus phasic neural stimulation patterns on arousal and sleep behaviors

Modifications of the Method

- The applications for optogenetics are vast; they can be modified to selectively target and study other locations, neurotransmitters, and circuits of the brain, including pathways involved in sleep, psychiatric, movement, or neural disorders.

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Appendices

Appendix 1: Evolution of Sleep Medicine Research

Studies in the past century have started to unveil intricate mechanisms that govern how the body initiates and regulates sleep and the interactions that sleep (or lack of) plays in multiple body and brain systems. Modern medicine's exploration of sleep started during an encephalitis epidemic in the early 1900s when Baron Constantin von Economo noticed that individuals with encephalitis of the posterior hypothalamus suffered from incessant sleepiness compared to those with injury of the preoptic area, who instead had severe insomnia (Berger and Vilensky 2014). This discovery led to the development of lesion models as a core methodology by which to understand sleep. In the 1930s, Bremer demonstrated that cuts on different parts of the medulla resulted in sleep or wake EEG activity, dependent on the location of the cut (Kerkhofs and Lavie 2000). Moruzzi and Magoun built upon this foundation in the 1940s by illustrating that stimulation of specific brain regions (rostral reticular formation) caused brain electrical activity to transition between sleep and wakefulness patterns (Moruzzi and Magoun 1995). Subsequent researchers have identified additional brain lesions/locations that contribute to sleep and wake, confirming our understanding that sleep is governed by the interaction of multiple locations via neurotransmitters. In the 1950s, Kleitman and Aserinsky discovered REM sleep; (Aserinsky and Kleitman 2003) further exploration has defined three unique behavioral states: wakefulness, non-REM sleep (NREM), and REM sleep (Aserinsky and Kleitman 2003).

Appendix 2

See Table 1.

Appendix 3: Neuropharmacology

Although EEG and polysomnograms provide useful data regarding an individual's sleep state and quality, a thorough history and physical exam can reveal significant causes of sleep disturbances, specifically pharmaceutical causes of excessive sleep or insomnia. In many cases, a patient's medication list may help distinguish between a primary sleep disorder that is causing symptoms and one that is concurrent due to some underlying process or drug. Much of our understanding of sleep pharmacology and neurophysiology has been intertwined. Multiple neurotransmitters are found to play key roles in the body's sleep/wake cycle; the role of these neurotransmitters was often discovered through indirect methods; specifically, pharmacological manipulation for mental, behavioral, and movement disorders commonly revealed interruption of normal sleep patterns. Drug classes, such as mood stabilizers/antidepressants and antipsychotic or cholinergic drugs, cause disruption on the sleep/wake cycle, indicating that the neurotransmitter being manipulated plays some role in the sleep pathway. For example, histamine is a key molecule in allergic reactions; drowsiness is common with 1st generation antihistamine usage, yet missing with 2nd generation antihistamines (which cannot cross the blood–brain barrier). Thus, pharmacological manipulation has (and still) plays a significant role in understanding the neurobiological mechanisms governing sleep as well as drug platform development. A quick summary of the involved neurotransmitters and their role in wake, NREM, and REM sleep can be found above in Table 1. Table 2 provides a brief list of drug classes that have been found to cause sleep disturbances based on the pathways or neurotransmitter influenced.

Table 1 Overview of sleep/wake behavioral states

	Other info	Wakefulness	Non-REM sleep	REM sleep
EEG		Desynchronized	↑ voltage	↓ voltage
		↓ voltage	↓ frequency	↑ frequency
		↑ fast frequency of		
		α (8–12 hz)		
		β (8–12 hz)		
		High β (20–30 hz)		
		θ (>30 hz)		θ waves recorded in hippocampus during REM
Muscle movement	Managed mainly by 5Ht and NE	Increase muscle tone and movement	Little movement	No movement recorded in hippocampus
Ach – acetylcholine	Mostly in basal forebrain and midbrain tegmentum	↑↑	↑	↑↑
		Fire in rhythmic bursts during wakefulness-related cortical α and θ activity		High levels in cortex and thalamus
		High levels in cortex and thalamus		
5Ht – histamine		↑↑↑	↑	No activity
				Inhibits REM sleep
NE – norepinephrine	Locus coeruleus NE may help maintain muscle tone	↑↑↑	↑	No activity
	α1 excitatory			
	α2 inhibitory			
Ha – histamine	Maintains cortical activity	↑↑↑	↑	No activity
		Highest in post hypothalamus		
Orexin	Part of flip model to stabilize transitions	↑↑↑	↑	No activity
		Highest in lateral hypothalamus		Some activity in REM-related muscle twitches
D – dopamine	(Schizophrenia, bipolar) – dopamine neurons in substantia nigra and midbrain are believed to be most influential on sleep/wake pathways	Individuals with low D (Parkinson's) suffer from excessive sleepiness	Fire in bursts	
		High D is associated with insomnia as seen in many mental disorders		

Table 2 Drug classes that interact with sleep neurobiology

Disorder	Drug/effect
Antidepressants (affect HT and NE)	Most cause ↓ REM sleep with ↑ serotonin
	Abruptly stopping antidepressant may cause intense dreams and rebound REM sleep
	TCA – causes drowsiness, disturbs REM, may cause nightmares
	Clomipramine, nortriptyline, amitriptyline, doxepin
	MOA inhibitors – prevent/suppress REM. May ↓ total sleep time and sleep efficiency
	Phenelzine, selegiline
	SSRI/SNRI: SSRI – more likely to cause insomnia
	Fluoxetine, sertraline, paroxetine, citalopram
	Fluoxetine – significantly ↓ sleep efficiency and REM time
Antiemetics (affect D, H1, H2, or Ach)	Most penetrate BBB causing sedation
	5-HT3 antagonists
	Block serotonin in CNS and GI tract
	Ondansetron, mirtazapine
	Dopamine antagonists
	Act in brain, may cause muscle spasm
	Metoclopramide, domperidone, olanzapine
	Antihistamines
	See **above** for additional details
	Scopolamine
	Antipsychotics
	See **above** for additional details
	Most have antiemetic effects mediated by D2 blockade
	Prochlorperazine
Antiepileptics (affect GABA)	May have variable effects on sleep and wakefulness
	Carbamazepine – extensively studied drug, may have differing short- and long-term usage effects on sleep
Antihistamines (affect H1 and Ach)	Commonly used for allergies or as antiemetic
	1st generation – nonselective to Ach and H1 receptors, crosses BBB causing sedation
	Diphenylhydramine
	2nd generation – does not cross BBB, selective to peripheral H1 receptors, nonsedative, daily allergy relief
	Cetirizine, fexofenadine, loratadine
Antiobesity drugs (affect MOA)	Most target monoamine systems
	Bupropion, tesofensine
	Insomnia is a common side effect
Antiparkinsonian (increases D)	Dopaminergic agonists can have dosage-related biphasic influence on sleep (can cause both insomnia and excessive sleepiness)
	Levodopa – low doses improve sleep; high doses may cause insomnia
	Ropinirole or pramipexole – associated with sudden excessive sleepiness
Antipsychotics (decrease D)	Sedative effects
	Also have antiemetic effects mediated by D2 blockade
	Insomnia in mental disorders is associated with increased dopamine levels
	1st generation/typical: blocks dopamine pathways
	Haloperidol, chlorpromazine, thioridazine
	2nd generation/Atypical: blocks dopamine and serotonin 5HT receptors
	Clozapine

(*continued*)

Table 2 (continued)

Disorder	Drug/effect
Beta blockers	Sedative effect dependent on lipid solubility of drug
	Propranolol – most lipid soluble, high sleep disruption
	Atenolol – least lipid soluble, limited sleep disturbance
Calcium channel blockers	Exact sleep interference mechanism of action unknown, thought to interfere with dopaminergic system
	Flunarizine – associated with insomnia
CNS stimulants	Cause general arousal by blocking inhibitory pathways to increase dopamine levels
	May cause insomnia as a side effect
	ADHD treatment
	Methylphenidate – dopamine reuptake inhibitor
	Amphetamine – dopamine reuptake inhibitor and releasing agent
	Narcolepsy treatment
	Modafinil
	Other stimulants
	Caffeine – water and lipid soluble, easily crosses BBB
	Primarily blocks adenosine pathways but can interfere with all other neurotransmitter levels
	Ephedrine and *pseudoephedrine*
	Pseudoephedrine has similar structure to epinephrine
	Nicotine – increased catecholamine release due to stimulation of central nicotinic cholinergic pathways
	Ecstasy/NMDA – primary effects 5HT
	Cocaine – SNDRI, easily crosses BBB, affects mesolimbic reward pathway causing addictiveness
Sedatives/hypnotics (affect GABA and Na+ channel blockers)	Sedatives help relax; hypnotics induce sleep
	There is significant overlap between the two
	Using these drugs in combination may cause significant respiratory depression
	Barbiturates – affect GABA receptors
	Significant overdose side effects
	Phenobarbitol
	Benzodiazepines – affect GABA receptors
	Useful for short-term anesthesia but may cause long-term dependence
	Worsen sleep quality by increasing light sleep and decreasing deep sleep
	Variable half-lives: can be short or long acting
	Short and medium acting: alprazolam, lorazepam
	Long acting: clonazepam, diazepam
	Non-benzodiazepine sedatives
	Similar efficacy as benzodiazepines but with fewer side effects
	Zolpidem (Ambien), eszopiclone (Lunesta)
	Other sedatives/hypnotics
	Methaqualone
	Alcohol
	Cannabis
Steroids	Associated with increased frequency of insomnia
	Cortisol, estrogen, progesterone

Ach acetylcholine, *BBB* blood–brain barrier, *CNS* central nervous system, *D* dopamine, *GABA* gamma-aminobutyric acid, *GI* gastrointestinal tract, *H* histamine, *HT* serotonin, *MOA* monamine oxidase inhibitor, *NE* norepinephrine, *SNDRI* serotonin–norepinephrine–dopamine reuptake inhibitor, *SNRI* selective norepinephrine reuptake inhibitor, *SRI* selective serotonin reuptake inhibitor, *TCA* tricyclic antidepressants

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Experimental Models for Drug Evaluation in Noise-Induced Hearing Loss and Age-Related Hearing Impairment

Amrita Ray, Jochen Schacht, and Michael J. Brenner

Contents

The contributions of noise and aging to hearing loss are distinct, yet interrelated. Noise-induced hearing loss (NIHL) is one of the leading – but preventable – causes of hearing loss worldwide. NIHL is usually characterized by an elevation in the hearing threshold, and the area of damage is most pronounced one-half octave above the frequency of noise exposure. A significant body of evidence suggests that NIHL damage results from noise-induced free radical production (Oishi and Schacht 2011). Age is an independent risk factor for acquired hearing loss, although its effects may be difficult to distinguish from those of noise exposure in the clinical setting. The mechanism underlying age-related hearing loss (presbycusis) is heavily influenced by an individual's genetic susceptibility, and there is thus dramatic variation. Four-way cross animal models allow a longitudinal examination of these factors in relation to age and hearing loss.

Noise-Induced Hearing Loss (NIHL) In Vivo

Purpose and Rationale

- Although most human studies have examined the impact of sound on temporary threshold shifts (TTS), animal models allow the investigator to examine the anatomic and physiologic mechanisms underlying both TTS and NIHL.

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- Animal models allow investigators to examine the limits of hearing threshold, recovery and the physiologic changes caused by noise exposure leading to NIHL.
- Established in vivo models of NIHL and age-related hearing loss provide useful tools to evaluate the efficacy of potential pharmacologic treatments and other potential hearing protection strategies.

Procedure

The following section details considerations that the investigator can apply to customize an animal model for NIHL suitable for their experimental questions:

I. ***Animal selection considerations*** – *A wide variety of species have been used (gerbil, guinea pig, mice, rat, chinchilla)* (Le Prell 2012):
   - Noise-induced sensorineural hearing loss has been studied in a variety of animals:
     - Gerbil, guinea pig, and chinchilla models
       Hearing range has overlap with the mid- to high-frequency human hearing range.
       Easily accessible cochlear anatomy.
       Animals are used to study the relationship of noise exposure to temporary threshold shifts (TTS) and permanent threshold shifts (PTS).
       Note: Chinchilla has increased susceptibility to hearing loss at high frequencies, not analogous to human hearing.
     - Mice
       - Mice have become the preferred model due to availability of genetic manipulations and tools of molecular biology:
         While inbred strains help reduce genetic variability; a single strain cannot encompass the diversity of damage patterns in response to noise insults.
         Commonly used to study tinnitus.
       - Rats offer a more favorable anatomy but less versatility for genetic manipulations.

II. ***Animal groups and noise exposure conditions***:
   - Experimental groups (Salvi and Boettcher 2008)
     - Control group
     - Noise exposure group
       Awake animal is exposed to noise in a ventilated sound exposure chamber:
       Choose a noise exposure type (octave band, broadband, etc.) and intensity appropriate to the animal model.
       Monitor noise levels using a sound level meter at the level of the animal's head.
       Please refer to "Modifications of the Method" section for other noise exposure paradigms.
     - Noise exposure + experimental protective agent
       Potential protective drugs/antioxidants can be given pre- or postexposure PO/IP/IM:
       Recent work has also examined local delivery of protective agents using a variety of techniques, such as trans-tympanic, "cut down" procedure with, direct delivery to the middle ear, placement on round window, etc.
   - **Recovery**
     - Allow animals to recover for 2 weeks:
       After noise exposure, threshold shift may require 2 weeks to stabilize (i.e., acute threshold shifts may recover; permanent threshold shift will reach the end point):
       This is dependent on the noise exposure intensity and duration.
       Smaller TTS demonstrate a shorter recovery time and less likelihood of causing PTS.

Note: There are three phases of recovery (log scale):
Early phase (<3 h) – little recovery in threshold hearing
Rapid recovery phase (3 h–5 days) – majority of threshold recovery
Slow recovery phase – plateau phase with minimal additional threshold recovery

- **Dissection of the cochlea to examine cochlear morphology and immunocytochemistry**
  - Anesthetize and sacrifice animals before dissecting the cochleae.
  - For surface preparation and light microscopy:
    Make a small opening in the apical portion of the cochlea.
    Fix with 4 % paraformaldehyde overnight at 4 ° C.
    Rinse 3× with phosphate-buffered saline (PBS) for 10 min.
    For sectioning for light or electron microscopy, decalcify the cochleae in 3 % EDTA at 4 °C for 2–3 days.
    Rinse explants 3× with PBS for 10 min.
    Add 0.1 % Triton 100 in PBS for no longer than 1 h.
    Rinse explants 3× with PBS for 10 min.
    Incubate with antibodies and/or stain.
    Rinse explants 3× with PBS for 10 min.
    Mount on slide.

Evaluation

Physiologic assessment is used to examine the effect of various noise paradigms on inner ear:

- **Auditory brainstem response (ABR) testing** (Willott 2006)
  - Measure ABR at the start of the study and after effects of exposure have stabilized:
    If TTS, measure 1–3 days after exposure.
    If PTS, measure 7–14 days after exposure.
  - ABR is used to determine auditory thresholds and can yield latency and amplitude functions for a more detailed analysis:
    Generally, animals are anesthetized by intramuscular injections of muscle relaxant, anesthetic, and/or sedative in concentrations depending on animal species and strain.
  - Needle electrode placement:
    The active electrode is placed at ipsilateral vertex.
    The reference electrode is placed subcutaneously below the ipsilateral right pinna.
    Ground electrode is placed either in the opposite ear or in the right thigh.
  - Insert transducer speculum into either ear external auditory meatus, creating a closed acoustic system:
    Unless delivered directly into one ear, noise-induced hearing loss is generally bilateral, and thus, it is only necessary to test one ear.
  - Present tone-burst stimuli at low, mid, and high frequencies (4, 16, 32–48 kHz):
    These frequencies are appropriate for animals with a higher frequency range of hearing but may not directly mirror noise injury for humans.
  - Feed output to an amplifier viewed on oscilloscope:
    Threshold is defined as the intensity that produces a detectable change from the non-stimulus condition.
    Verify thresholds twice.
    Threshold shifts are calculated in comparison to individual pre-study thresholds.
- **Otoacoustic emission (OAE)** (Martin et al. 2006)
  - Measuring OAEs is a simple, noninvasive test used to detect hearing deficit and measure outer hair cell (OHC) health.
  - Otoacoustic emissions (OAEs) are low intensity sounds generated by the inner ear due to an OHC electromotile response. Depolarization and contraction of the outer hair cell due to changes in membrane potential cause a longitudinal electromotile response that is detectable in the ear canal.

- OAEs change or disappear after inner ear damage and can be used to gauge inner ear health.
- Can be evoked using three different methodologies:

  Stimulus frequency OAE (SFOAE) – apply a pure-tone stimulus and record the vector difference between the stimulus and the recorded waveform.

  Transient-evoked OAE (TEOAE) – a broad frequency range click or brief pure-tone stimulus induces a response from the inner ear region with the same frequency as the applied pure-tone stimulus.

  Distortion product OAE (DPOAE)

  Most commonly used method in rodents (Jimenez et al. 1999).

  DPOAE is evoked using a pair of primary tones, f_1 and f_2.

  Evoked frequency (f_{dp}) is mathematically related to the primary frequencies.

  The amplitude of f_1, f_2, and $2f_1$–$2f_2$ is recorded and analyzed by placing a sensitive spectrum analyzer microphone in the ear canal.

- **Gap detection** (Turner and Parrish 2008)
  - Gap detection is used to assess the ability to hear brief silence gaps in continuous noise and may be useful to evaluate peripheral and central damage.
  - The animal is trained to a behavioral response in the presence of silence:

    Place animal in an environment with continuous background noise.
  - Animal responds when there are brief gaps of silence:

    This allows examination of how the extent of noise-induced hearing loss impacts auditory temporal resolution.
  - Can also be used to evaluate tinnitus.
- **Frequency sensitivity**
  - The animal is trained to have a behavioral response when it hears a probe (brief, fixed-frequency tone burst).
  - Next, add a continuous pure-tone mask to background whose frequency is significantly lower than that of the probe.
  - Increase frequency of the pure-tone mask until probe is no longer detectable due to masking (threshold frequency).
  - Measure subsequent psychophysical tuning curve.

Invasive Evaluations

- **Endocochlear potential (EP)** (Salt et al. 1987; Konishi and Salt 1983)
  - The EP is a positive voltage of 80–100 mV in the cochlear endolymphatic space.
  - EP is highest in the cochlear basal turn and decreases toward the apex.
  - It is measured by passing double-barrel K-selective microelectrodes into the endolymph through the stria vascularis.

- **Compound action potential (CAP)** (Abbas and Brown 2014)
  - The CAP helps quantify the discharge activity of the Type I auditory nerve fibers (cranial nerve 8) that synapse on the inner hair cells (IHC):

    CAP recordings are usually done during acute surgical preparations, but chronic recordings can be accomplished by implanting the electrode into the round window:

    Access middle ear via a small opening.

    Place silver ball wire electrode on/near the round window.

    Place ground electrode at a remote location.

    Seal middle ear opening with dental cement.

    Secure and mount electrode connector to skull.

    CAP is elicited by click or brief tone bursts.

    The amplitude of CAP peak recordings (N1 and N2) reflects IHC response to the stimulus intensity of the click or tone burst. As stimulus intensity

increases, N1 or N2 amplitude also increases.
In NIHL animals, N1 and N2 amplitude is diminished, which requires a higher intensity stimulus to generate the same response.

Histology and Morphology

- Hair cells
  - Hair cells are examined under light or confocal microscopy to identify hair cell death and structural changes from the apex to the base of the cochlea.
  - The hair cells are evaluated for the integrity of the hair cell outline, stereocilia, and ultrastructure of organelles (mitochondria, nucleus, endoplasmic reticulum).
- Ribbon synapse
  - The presynaptic terminals can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
  - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
- Spiral ganglia
  - Examination of the spiral ganglia cells requires different dissection and sectioning techniques, described by Szabo et al. (2003).
- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from the base to the apex and entered into programs designed for cytocochleogram (KHRI Cytocochleogram is one such program).
  - This allows investigators to compare cell counts to normative data established from control specimens.
  - The number of preserved hair cells is calculated as a percent and plotted as a function of distance from the apex to the basal turn of explant.
- **Immunohistochemistry**
  - The explant has distinctive geometry, which must be preserved. The tissues are not thin sections, but rather a complete tissue. Confocal microscopy should consider vertical stacking and the need to evaluate in 2 planes for nucleus and organelles.
  - Triton X-100 is the most commonly used permeability agent.
  - Immunohistochemistry is useful to examine specific molecules that may be up- or downregulated.
  - *See drugs* in vivo.

Critical Assessment of the Method

- Animal models allow investigators to examine the effect of various noise intensities and durations in a well-controlled setting which is impossible in human models.
- These studies provide the opportunity to study genetic effects through inbred strains and neuronal networks through invasive methods.
- A significant body of data suggests noise-induced metabolic and oxidative stress as a mechanism for hair cell damage.
  - A variety of antioxidants have been used as a preexposure intervention to attenuate metabolic stress induced by noise exposure and to hasten recovery.
- Special considerations in animal models:
  - Certain animal types are more susceptible to acoustic trauma compared to humans (chinchillas are one example).
  - The guinea pig model can be used to detect delayed hearing loss weeks or months after noise exposure (Kujawa and Liberman 1999).
- Behavioral method:
  - These are often more time consuming in comparison to physiological recordings.
    Animals must have adequate training to establish stimulus control.
  - These methods allow investigators to examine how living animals respond to noise exposure.
- Physiologic assessment:
  - DPOAE

Used to assess OHC function
Does not reflect IHC or auditory nerve function
Cannot differentiate severity of NIHL when exceeding 30–35 dB due to a loss of DPOAE

Modifications of the Method

- Animal models allow development of various noise exposure paradigms by manipulating noise (duration, spectrum, and intensity) and examining the pathophysiologic response in the inner ear:
  - Noise exposure duration – days, weeks, and months
  - Variations in noise spectrum and intensity
  - Types of noise (Le Prell 2012):
    Chronic (long-term factory noise) or impulse noise (gunshot)
    Impulse noise is defined as a single-pulse burst of sound at least 140 dB that lasts 0.001–1 s.
    Preconditioning noise: long-term low-level continuous noise exposure causes a decrease in auditory sensitivity to subsequent noise damage.
    Common conditioning paradigm: 4–6 h/day noise exposure for multiple days
    Gaussian noise: spectrally flat noise with relatively level frequency components
    Allows comparison of anatomical damage in relation to exposure time and level
    Kurtotic noise: Gaussian-like broadband noise with impulse-like delivery.
    Mimics industrial noise exposure (high background noise + brief impulse bursts)
    Octave band noise: noise centered at any frequency to induce hair cell death in animal subjects.
    Used to examine specific types of hearing loss (such as high-frequency hearing loss associated with age)
- Note: Many people with a history of noise exposure report tinnitus (ringing or buzzing in the ears)
  - While tinnitus may be a result of stressful noise exposure and thus play a role in NIHL, it can also be caused by a variety of other situations not related to noise exposure such as:
    Age-related tinnitus
    Disease pathologies (Meniere's disease, acoustic neuromas)
    Medications (certain diuretics, antibiotics, quinines, aspirin)
- Drosophila fly (Johnston's organ) (Christie and Eberl 2014)
  - Allows for genetic and molecular investigations
- Physiological applicability is limited in comparison to the human ear.

Behavioral Methods

- **General considerations**
  - Behavioral methods allow threshold determination through psychophysical methods (Clark et al. 1974).
  - These methods help quantify the relationship between stimuli and sensation in the animal model.
  - However, they require sufficient prior training of the animal to ensure that the animal's behavior accurately reflects the stimulus:
    In response to a true-positive or true-negative response, the animal receives a food pellet (or other rewards).
    - **Method of limits** (ascending method): a sound stimulus is presented at levels too quiet for perception. Intensity is gradually increased until sound stimulus is detected. This is reversed for the descending method. The threshold is estimated as halfway between the first *YES* and last *NO* response.
    - **Possible causes of error**
      Error of habituation – animal continues to report/behave the same way even when beyond threshold stimuli.

Error of anticipation – animal anticipates a stimulus and makes a "premature" response.

- **Method of constant stimuli**: stimuli levels are presented randomly rather than in an ascending or descending order, preventing the animal from predicting the stimulus. Animals are trained to respond on stimulus detection. The threshold is where the stimulus is detected 50 % of the time.
- **Staircase procedure**: stimuli intensity is increased or decreased by a prespecified dB step based on the animal's correct or incorrect responses.
  - Accuracy can be increased by reducing the dB step size after a number of correct responses or threshold reversals.

- **Training and assessment protocol (psychophysics procedure)**
  - Animal is trained to hold down key until stimuli tone is presented. Tones are presented in 50 % of the trials. The animal should release key to receive food pellet in response to the tone (true positive):
    True negative (TN) – trial is scored as a (correct) TN if key is held down during a blank tone trial. Animal is then rewarded with a food pellet.
    False positive (FP) – trial is scored as FP if the animal releases the key during a blank tone trial. Blank tones (no sound) are presented 50 % of the time.
    False negative (FN) – trial is scored as FN if animal does not release key during a tone trial. Negative reinforcement may include turning off enclosure lights.
  - Psychophysics study responses to tone and blank tones are used to determine threshold vs. frequency (audiogram) in animal:
    These help examine acute/temporary threshold shifts (ATS) vs. permanent threshold shifts (PTS):
    Chinchillas have been used extensively to examine the relationship between noise exposure and acute and permanent threshold shifts (Mills et al. 1973).
    Psychophysics studies in primates have also been used to examine sensory receptor function (Stebbins et al. 1988).
  - Comparison of normal vs. NIHL psychophysical tuning curve:
    Normal psychophysical curve has two regions:
    The narrow V-shaped portion of the curve has the nadir centered at the best probe frequency.
    Broad, high-threshold tail region.
    NIHL animals have significantly altered psychophysical curve shape:
    NIHL animals have a blunt W- or U-shaped tuning curve with a broad high-threshold tip connected to high-threshold tail region.

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Age-Related Hearing Loss (ARHL)

Purpose and Rationale

- Aging is an independent risk factor for acquired hearing loss, although its effects are often difficult to separate from those of noise exposure in the clinical setting.
- The effect of aging on hearing is modified by genetic susceptibility characteristics, with individuals differing dramatically in their tendency for presbycusis.
- Environmental influences (drugs, noise, disease) also impact ARHL
- Both animal and human studies that examine the impact of aging are either cross-sectional or longitudinal, but animal models allow further investigation of the physiologic mechanisms underlying age-related hearing loss.
- Aging-induced sensorineural hearing loss is studied primarily in mouse models due to the opportunity for genetic manipulation.

Procedure

Animal Selection and Procedure

- No single animal model is considered a gold standard for the study of presbycusis:
  - Gerbil models provide a model of strial degeneration.
  - Rodents (CBA/J mice) mirror oxidative stress mechanisms of the aging process and enable molecular or genetic manipulation.
  - However, different mice strains demonstrate presbycusis at different rates.

Procedure

- **Four 4-way crosses** – the gold standard for robust modeling and aging research in general (NIA recommendation: citation in Schacht et al. 2012):
  - Breed mice from two different F1 parents to ensure that genome is from 4 distinct inbred grandparental stocks.
  - Example: female mice of (MOLF/EiJ × 129S1/ScImJ) F1 stock crossed with males of (C3H/HeJ × FVB/NJ) F1 stock (Schacht et al. 2012).
  - Each mouse shares half of its alleles with all other mice in the same population.

- Breeding
  - Successful breeding largely involves mitigating non-aging-related sources of hearing loss and allowing the animals to age in a stable, low-stress environment:
    Controlled environment that minimizes ambient noise exposure
    Standard diet with avoidance of potential ototoxins that may confound hearing
    Minimization of stress
  - Due to the long duration of experiments, animal housing is particularly relevant. Female mice can generally be housed together, whereas male mice are prone to fighting. Thus, female mice are more amendable and practical for experimental procedures. However, gender differences exist in the onset and rate of age-related hearing loss so that one sex alone does not provide complete information.

Therapeutic Interventions

- Although presbycusis clearly has genetic determinants, pharmacologic interventions may influence the onset of hearing loss.
- The vast majority of interventions have negative results on age-related hearing loss. Some dietary interventions with antioxidants and vitamins may mitigate stress pathways that induce oxidative stress.
- Use of antioxidants to attenuate presbycusis is not well substantiated.
  A long-term intervention in mice from 10 to 22 months of age did not alter age-related hearing loss (Sha et al. 2012).

- Caloric restriction, a treatment known to attenuate oxidative stress and increase longevity, demonstrates different effects dependent on mice strain (Willott 1995):
  Results ranged from having no apparent effect, various reductions in rate of hearing loss and auditory degeneration, to aggravation of hearing loss.

- **Dissection to examine cochlear morphology or immunocytochemistry**
  - Anesthetize and sacrifice animals before dissecting the cochleae.
  - For surface preparation and light microscopy:
    Make a small opening in the apical portion of the cochlea.
    Fix with 4 % paraformaldehyde overnight at 4 ° C.
    Rinse 3× with phosphate-buffered saline (PBS) for 10 min.
    Decalcify the cochleae in 5 % EDTA at 4 °C for 24 h.
    Remove bony shell of otic capsule.
    Stain with 1 % phalloidin with Alex 305 label for 1 h.

Rinse explants 3× with PBS for 10 min.
Remove segments of cochlear spiral and mount on slides.
Process analogous to the in vitro explant prep.

Evaluation

- **Auditory brainstem response (ABR) testing** (Willott 2006)
  - Measure after 2 months.
  - ABR is used to determine auditory thresholds and also can yield latency and amplitude functions for a more detailed analysis:
    Generally, animals are anesthetized by intramuscular injections of muscle relaxant, anesthetic, and/or sedative in concentrations depending on animal species and strain.
  - Needle electrode placement:
    The active electrode is placed at ipsilateral vertex.
    The reference electrode is placed subcutaneously below the ipsilateral right pinna.
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  - Insert transducer speculum into either ear external auditory meatus, creating a closed acoustic system.
  - Present tone-burst stimuli at low, mid, and high frequencies (4, 16, 32–48 kHz).
  - Feed output to an amplifier and view on oscilloscope:
    Threshold is defined as the intensity that produces a detectable change from the non-stimulus condition.
    Verify thresholds twice.
    Threshold shifts are calculated in comparison to individual pre-study thresholds.
- **Gap detection** (Turner and Parrish 2008)
  - Gap detection is used to assess the ability to hear brief silence gaps in continuous noise and may be useful to evaluate peripheral and central damage.
  - The animal is trained to a behavioral response in the presence of silence:
    Place animal in an environment with continuous background noise.
  - Animal responds when there are brief gaps of silence:
    Allows examination of how the extent of noise-induced hearing loss impacts auditory temporal resolution.
  - Can also be used to evaluate tinnitus.

Histology and Morphology

- Hair cells
  - Hair cells are examined under light or confocal microscopy to identify hair cell death and structural changes from the apex to the base.
  - The hair cells are evaluated for the integrity of the hair cell outline, stereocilia, and ultrastructure of organelles (mitochondria, nucleus, endoplasmic reticulum).
- Ribbon synapse
  - The presynaptic terminals can be identified with immunolabeling of CTBP2 (Kujawa and Liberman 2009).
  - Postsynaptic marker can be identified with immunolabeling using either PSD95 (postsynaptic density 95) or GluR2/3 (glutamate receptor subunits 2/3) (Yuan and Chi 2014).
- Spiral ganglia
  - Examination of the spiral ganglia cells requires different dissection and sectioning techniques described by Szabo et al. (2003).

- **Cytocochleogram**
  - The number of preserved outer hair cells is counted from the base to the apex and entered into programs designed for cytocochleogram (KHRI Cytocochleogram is one such program).
  - This allows investigators to compare cell counts to normative data established from control specimens.
  - The number of preserved hair cells is calculated as a percent and plotted as a function of distance from the apex to the basal turn of explant.

- **Immunohistochemistry**
  - The explant has a distinctive geometry, which must be preserved. The tissues are

not thin sections, but rather a complete tissue. Confocal microscopy should consider vertical stacking and the need to evaluate in 2 planes for nucleus and organelles.
  - Triton X-100 is the most commonly used permeability agent.
  - Immunohistochemistry is useful to examine specific molecules that may be up- or downregulated.

Critical Assessment of the Method

- A cautionary note is warranted for certain animal models with anomalous physiology that limits applicability to human hearing loss:
  - The C57/BL mouse is a workhorse of genetic studies, but this mouse has a cadherin 23 (Cdh23) mutation that accelerates hearing loss.
  - Murine models that exhibit hearing loss due to mitochondrial mutations have catastrophic mutations that cause profound perturbation of physiological function. Such extreme derangements make these models of questionable value for modeling human age-related hearing loss.
  - Mutations in DNA repair enzymes also differ from the physiology underlying human presbycusis.
- An advantage of the mouse model is the relatively short life span of mouse versus human:
  - A more complete understanding requires consideration of sensory hair and non-sensory and supporting cells:
  - Mouse models that carry mutations that mimic age-related hearing loss can potentially provide mechanistic insight into the similarities and differences between age-related hearing loss and noise injury.
- Longitudinal sampling is an important consideration in aging research. End points that cannot be tested serially (e.g., endocochlear potential can only be evaluated as a terminal end point) may be less useful in such research:
  Challenges exist in examining the clinical implications of long-term protective agent administration.
  Considerations must be given to avoid confounding from noise sources.
- Although presbycusis is classically a high-frequency sensorineural hearing loss ("flat" hearing loss), it may also present with elevated threshold shifts across high and low frequencies:
  In Harold Schuknecht's classic typology, such a pattern is characterized as strial hearing loss.

Modifications of the Method

- The effect of noise exposure on aging can also be examined:
  - Example of combined aging + noise protocol:
    Group 1: no noise exposure
    Group 2: exposed to noise at early age
      Expose awake animal to noise in a ventilated sound exposure chamber.
      Expose animal to noise that yields at TTS or low PTS.
      Monitor noise levels using a sound level meter at the level of the animal's head.

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Part XVII

Pharmacological Methods in Dermatology

Skin Sensitization Testing

Hans Gerhard Vogel

Contents

Guinea Pig Maximization Assay

Purpose and Rationale

A variety of methods are available for the prospective identification of skin-sensitizing chemicals. The methods that have been used are the Draize rabbit irritancy test (Draize et al. 1944; Phillips et al. 1972), the occluded guinea pig patch test (Buehler 1965), and the guinea pig maximization test (Magnusson and Kligman 1969; Magnusson 1980). The guinea pig maximization test is an adjuvant sensitization test requiring intradermal injections of the test substance, in combination with Freund's complete adjuvant, which stimulates nonspecifically the immune system of treated animals, enhancing their ability to respond to sensitizing chemicals.

Procedure

Groups of 15–20 guinea pigs (Hartley strain) are used. On day 0 an area of 4 × 6 cm over the shoulder region is clipped short with an electric clipper. Three pairs of intradermal injections are made simultaneously, so that on each side of the midline there are two rows of injections each. The injection sites are just within the boundaries of the 2 × 4 cm patch, which is applied 1 week later. Injections are (1) 0.1 ml Freund's adjuvant alone, (2) 0.1 ml test material, and (3) 0.1 ml test material in Freund's adjuvant. Control animals are given

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the same injections but without the test agent, i.e., Freund's adjuvant and vehicle.

On day 7, the same area over the shoulder region is again clipped and shaved with an electric razor. The test agent in petrolatum is spread over a 2 × 4 cm filter paper in an even, rather thick layer or, if liquid, to saturation. The patch is covered by an overlapping, impermeable plastic adhesive tape. This in turn is firmly secured by an elastic adhesive bandage, which is wound around the torso of the animal. The dressing is left in place for 48 h. Control animals are exposed to the vehicle without the test agent in the same way as the experimental group.

On day 21, in experimental and control animals the flanks are shaved on both sides on an area of 5 × 5 cm each. Filter paper pieces, 2 × 2 cm, are sealed to the flanks for 24 h with the same occlusive bandage as for topical induction: (1) left side: patch with the test agent in the highest nonirritant concentration; the same vehicle as for topical induction is used; (2) right side: path with the vehicle.

On day 23, reading is made 24 h after removing of the patches. By then, skin irritation due to the occlusive dressing has usually faded.

Evaluation

If the challenge reactions in the experimental group clearly outweigh those in the control group, the agent is regarded to be a sensitizer. To grade the substances according to the percentage of animals sensitized, the substances are divided into five classes, ranging from weak (grade I) to extreme (grade V) sensitizers (Kligman 1966).

Modifications of the Method

Improvement of the classical guinea pig maximization test was proposed by several authors: Maurer et al. (1980a), Shillaker et al. (1989), Botham (1992), Kashima et al. (1993), Basketter et al. (1995), Frankild et al. (1996), Vohr et al. (2000), Steiling et al. (2001).

Maurer et al. (1980b) developed a model for the evaluation of the photocontact allergenic potential in the guinea pig. Four days before the induction period, Pirbright white guinea pigs were shaved on the back skin and chemically depilated. During the 3-week initial induction period, the animals were treated epicutaneously four times a week with the test drug. Four days before the challenge at week 6 the skin was again chemically depilated. During the challenge periods at weeks 6 and 9, the animals were treated for 3 days consecutively with the test drug. To stimulate the immunological reactivity, the guinea pigs received four intradermal injections of Freud adjuvant/saline 1:1. After each application, the animals were irradiated for 15 min with an Osram Xenon 6000 W UV lamp. The intensity of erythematous reactions was evaluated according to the Draize scoring scale.

A predictive assay for contact allergens using human skin explant cultures has been described by Pistoor et al. (1996).

Use of **transgenic animals** to investigate drug hypersensitivity has been proposed by Moser et al. (2001).

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Popliteal Lymph Node Hyperplasia Assay

Purpose and Rationale

The popliteal lymph node assay in mice or rats has been recommended as a tool for predicting allergies (Kammuller et al. 1989; Bloksma et al. 1995; Koch et al. 2000; Pieters 2001). Moreover, the inhibition of popliteal lymph node hyperplasia can be measured (Schorlemmer et al. 1998; Mollison et al. 1999). The test can be used to study compounds potentially effective in allergic eczema.

Procedure

Spleens from Brown-Norway rats are harvested aseptically, splenocytes expressed by compression with a hemostat in Dulbecco's phosphate-buffered saline (DPBS), red blood cells lysed with Tris (0.16 M) buffered in ammonium chloride (0.17 M) buffer, washed twice (400 g), irradiated (20 Gy), washed in DPBS, and suspended in DPBS at 5×10^7 cells per ml. On day 0, recipient Lewis rats are injected subcutaneously into the plantar surface of the right hind paw with 0.1 ml of the splenocyte suspension. Compounds are dissolved in an appropriate vehicle and dosed daily, 2 ml/kg, on days 0–3. Recipients are sacrificed on day 4 and popliteal lymph nodes (PLNs) from both hind limbs from vehicle control rats, or the right popliteal lymph node from drug-treated rats, dissected free and weighed individually on a microbalance (Mollison et al. 1993). The average weight of PLN from the left leg of vehicle-treated animals is used as background.

Evaluation

Percent inhibition is calculated using the following formula:

$$100 - \frac{PLN_{\text{exp.}} - PLN_{\text{mean leaf}}}{PLN_{\text{mean right}} - PLN_{\text{mean left}}} \times 100$$

$PLN_{\text{exp.}}$ = experimental PLN weight
$PLN_{\text{mean left}}$ = mean vehicle control left PLN weight
$PLN_{\text{mean right}}$ = mean vehicle control right PLN weight

ED_{50} values are derived by simultaneous least squares regression analysis.

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Local Lymph Node Assay

Purpose and Rationale

More recently, the local lymph node assay in mice has been recommended for measurement of allergenic potency (Kimber and Weisenberger 1989; Kimber et al. 1995; Kimber 2001; Basketter and Scholes 1992; Basketter et al. 2001).

Procedure

Groups of mice (CBA strain), weighing 20–25 g, receive topical applications of the test chemical on the dorsum of both ears, once a day for 3 consecutive days. In standard analyses, three concentrations of the test material are evaluated together with the relevant vehicle control. Five days after the initiation of exposure, all mice receive an intravenous injection of [^{3}H]-labeled thymidine into their tail vein. Five hours later, animals are sacrificed and draining auricular lymph nodes excised. A single cell suspension of lymph node cells is prepared by gentle mechanical disaggregation, and the cells are washed and resuspended in trichloroacetic acid for at least 12 h at 4 °C. Precipitates are resuspended in trichloroacetic acid and transferred to an appropriate scintillation fluid. The incorporation by draining lymph node cells of [^{3}H]-labeled thymidine is measured by ß-scintillation counting and recorded as mean disintegrations per min (dpm).

Evaluation

For each concentration of the test material a stimulation index (*SI*) is derived relative to the concurrent vehicle control. Those chemicals that at one or more test concentrations induce a *SI* of three or greater are classified as skin sensitizers.

Dose–response curves are plotted in order to provide information on the relative potencies of skin sensitizers. The concentration of the test chemical required to produce a stimulation index (*SI*) of three (named *EC3* value) is calculated using the formula

$$EC3 = c + [(3 - d)/(b - d) \times (a - c)]$$

where the data points lying immediately above and below the *SI* value of three on the dose–response plot have the coordinates (*a*,*b*) and (*c*,*d*), respectively.

Modifications of the Method

On the basis of a modified local lymph node assay, Homey et al. (1997) analyzed immunosuppressive effects of topically applied drugs. On 4 consecutive days, NMRI mice were treated on the dorsal surfaces of both ears with increasing concentrations of test compound. During the last 3 days, the mice received in addition the contact sensitizer oxazolone (1 %). On day 5, draining auricular lymph nodes were removed in order to assess lymph node cell counts and perform flow cytometric analysis of lymph cell subpopulations.

Hariya et al. (1998) developed a nonradioactive endpoint in a modified local lymph node assay.

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Experimental Dermatitis

Howard Maibach

Contents

Several methods involving skin reactions are discussed in other chapters, such as

- Ultraviolet erythema in guinea pigs (H.3.2.2.1)
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Spontaneous Dermatitis

NC/Nga Mouse as Model for Atopic Dermatitis

The NC/Nga mouse has been recommended as a model for atopic dermatitis (Matsuda et al. 1997; Tsudzuki et al. 1997; Suto et al. 1999; Vestergaard et al. 1999, 2000; Kotani et al. 2000; Aioi et al. 2001; Kohara et al. 2001). When kept in specific pathogen-free conditions, it remains healthy, but when kept in nonsterile conditions,

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it spontaneously develops a disease resembling atopic dermatitis at the age of 6–7 weeks. The level of IgE in the blood gradually increases to very high levels and peaks at the age of 16–18 weeks (Matsuda et al. 1997). This enhanced IgE production has been attributed to an increased sensitivity of the B cell to the CD40 ligand and to IL-4, which is the result of enhanced phosphorylation of Janus kinase 3, a feature found also in atopic dermatitis (Matsumoto et al. 1999). At 16–18 weeks, the mice develop dry skin and, gradually, nodular lesions, which in turn become crusted wounds. The lesions are pruritic and located on the back, the neck, the ears, and the face. Biophysical parameters show impairment of water retention and barrier function. The amount of ceramide in the skin decreases significantly (Aioi et al. 2001).

Histologically, the skin lesions in the NC/Nga mice are characterized by hyperkeratosis and parakeratosis, which resemble the lichenified lesions observed in atopic dermatitis patients. In the dermis, an infiltration is found, similar to that seen in atopic dermatitis patients, containing lymphocytes, eosinophils, mast cells, and macrophages, in addition to a large population of dendritic cells (Vestergaard et al. 1999).

The lesions in the NC/Nga mouse improve after treatment with tacrolimus hydrate (FK506) ointment (Hiroi et al. 1998) and also with topical steroids (Vestergaard et al. 1999). Both treatments reverse the changes in the skin, block the expression of inflammatory cytokines, and decrease the serum levels of IgE.

Further Dermatitis Models

Iwasaki et al. (2001) recommended atopic NC/Nga mice as a model for allergic asthma: after immunization with ovalbumin, severe allergic responses were elicited by a single intranasal challenge.

Arai et al. (2004) showed that a prostanoid DP_1 receptor agonist inhibits the pruritic activity in NC/Nga mice with atopic dermatitis.

Mihara et al. (2004) described the vital role of the itch-scratch response in development of spontaneous dermatitis in NC/Nga mice. Capsaicin-sensitive sensory nerves of these mice were ablated by neonatal capsaicin treatment. Scratching behavior was almost completely prevented in these mice, and the development of dermatitis and elevation of the serum IgE level were significantly suppressed.

Ohmura et al. (2004) studied the involvement of substance P in scratching behavior in a picrylchloride-induced dermatitis model in NC/Nga mice.

Takano et al. (2004) evaluated the antipruritic effects of several agents on scratching behavior by NC/Nga mice.

Takaoka et al. (2005, 2006) tested the expression of IL-31 gene transcripts in NC/Nga mice with atopic dermatitis. The expression of IL-31 mRNA in the skin of NC/Nga mice with scratching behavior was significantly higher than that in NC/Nga mice without scratching behavior.

Further animal models for atopic dermatitis were described, such as the NOA (Naruto Research Institute Otsuka Atrichia) mouse (Natori et al. 1999; Watanabe et al. 1999). The mice develop ulcerative skin lesions with accumulation of mast cells and increased serum IgE.

A spontaneous mutation characterized by chronic proliferative dermatitis in C57BL mice was described by HogenEsch et al. (1993).

Barton et al. (2000) reported that mice lacking the transcription factor ReIB develop T cell-dependent skin lesions similar to human atopic dermatitis.

Herz et al. (1998) developed a human-SCID mouse model to analyze the possible role of bacterial superantigens in human allergic immune responses under in vivo conditions.

Hossen et al. (2005) described the effect of loratadine on atopic-dermatitis-associated pruritus in ICR and hairless mice.

Sonkoly et al. (2006) described IL-31 as a new link between T cells and pruritus in atopic skin inflammation.

Chan et al. (2001) reported that the expression of IL-4 in the epidermis of **transgenic mice** results in a pruritic inflammatory skin disease. The mice spontaneously develop a skin disease reproducing all key features of human atopic dermatitis, including xerosis, conjunctivitis,

inflammatory skin lesions, *Staphylococcus aureus* infection, histopathology of chronic dermatitis with T cell, mast cell, macrophage-like mononuclear cell, and eosinophil infiltration, and elevation of total serum IgE and IgG1.

Konishi et al. (2002) demonstrated in IL-18-transgenic mice that IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions.

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Motheaten Mice

Mice homozygous for the autosomal recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Kovarik et al. 1994; Su et al. 1998). These mice show arthritis, patchy dermatitis, and hemorrhagic pneumonitis; the latter is considered to be the cause of the early death of me and me^v mice at the age of 3 and 9 weeks, respectively.

Homozygous me^v mice are identified first at the age of 3–4 days by focal depigmentation of the skin, followed by patchy absence of hair and by necrotic lesions on paws, tail, and ears.

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Spontaneous Erythema in Hairless Rats

The hairless rat (WBN/Kob-*Ht*) is a dominant mutant derived from the Wistar strain. With an incidence rate of about 4 % in both male and female animals, at an age of 20 weeks an erythema appears spontaneously on the dorsal skin, gradually becoming widespread and progressive in nature (Iwamoto et al. 1997; Tani et al. 1998). Histopathologically, erythema is characterized by dermatitis induced by an immunological reaction. Areas of erythema in the skin were decreased by treatment with dexamethasone (1 mg/kg) or cyclosporine (25 or 50 mg/kg) injected subcutaneously every other day for 2 weeks. The results suggested that erythema on the hairless rat could be used as an animal model of spontaneous dermatitis.

Modifications of the Method

Atopic dermatitis-like symptoms were reported in hypomagnesemic hairless rats by Chavaz et al. (1984) and Neckermann et al. (2000).

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Spontaneous Atopic Dermatitis in Dogs

Purpose and Rationale

Atopic dermatitis is a known chronic inflammatory skin disease in dogs (Scott et al. 2001).

Olivry et al. (2001) characterized the inflammatory infiltrate during IgE-mediated late-phase reactions in the skin of normal and atopic dogs with *Dermatophagoides farinae*-induced allergy.

Nuttall et al. (2002) studied the expression of Th1, Th2, and immunosuppressive gene transcripts in canine atopic dermatitis and found overproduction of IL-4. Canine atopic dermatitis was proposed as a possible animal model of human atopic dermatitis.

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Contact Dermatitis

Contact Hypersensitivity in Animals

Purpose and Rationale

The phenomenon of contact hypersensitivity in animals is thought to mirror atopic dermatitis or eczema in patients (Corsini et al. 1979; Cooper 1994; Leung 1997).

Contact Hypersensitivity in Mice

Procedure

CD1 mice are sensitized on the shaved abdomen with 20 µl of 0.5 % 2,4-dinitrofluorobenzene

(DNFB) in a vehicle consisting of 95 % acetone/5 % olive oil on days 0 and 1. On day 5, groups of 10 mice are challenged with 0.2 % DNFB with or without codissolved drug, 10 µl on both the internal and external surface of both ears. Mice are sacrificed 24 h post challenge, and a 7 mm diameter circle punched from each ear is weighed immediately on a microbalance.

Evaluation

The mean earplug weights from naïve mice challenged with 0.2 % DNFB are used as background control.

Percent inhibition is calculated using the following formula:

$$100 - \frac{\text{plug}_{\text{exp.}} - \text{plug}_{\text{mean naïve}}}{\text{plug}_{\text{mean sens.}} - \text{plug}_{\text{mean naïve}}} \times 100$$

plug_{exp} = experimental earplug weight
$\text{plug}_{\text{mean naïve}}$ = mean naïve earplug weight
$\text{plug}_{\text{mean sens}}$ = mean sensitized vehicle control plug weight

ED_{50} values are derived by simultaneous least squares regression analysis.

Modifications of the Method

Lowe et al. (1977) used oxazolone-sensitized Swiss Webster **mice** to evaluate anti-inflammatory properties of a prostaglandin antagonist, a corticosteroid, and indomethacin in experimental contact dermatitis.

Studies in mice on allergic contact dermatitis were performed by Ek and Theodorsson (1990), Fraginals et al. (1990), Katayama et al. (1990), Lavaud et al. (1991), Trenam et al. (1991), Stanley et al. (1991), Maguire (1996), and Corsini and Galli (1998).

Grabbe et al. (1995) showed that removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of contact hypersensitivity in BALB/c mice.

Koyama et al. (1998) used tenascin-C knockout mice and studied the effect on dinitrofluorobenzene-induced dermatitis.

Meingassner et al. (1997) sensitized female NMRI mice on the shaved abdomen with 50 µl of oxazolone (2 % in acetone). After 7 days, they were challenged with 10 µl of 2 % (for topical testing) or 0.5 % (for systemic testing) oxazolone on the inner surface of the right ear. Compounds were tested topically by a single application of 10 µl ethanolic solution to the challenge site 30 min after challenge. Compounds were tested for systemic activity by administration of a single subcutaneous injection immediately after challenge or by two oral doses (2 h before and immediately after challenge). The unchallenged left ears served as normal controls. Dermatitis was evaluated from the difference in pinnal weight, which was taken as a measure of inflammatory edema 24 h after challenge.

Traidl et al. (1999) reported inhibition of allergic contact dermatitis to dinitrochlorobenzene (DNCB) but not to oxazolone in interleukin-4-deficient mice.

Morita et al. (1999) reported that fur mites induce dermatitis associated with IgE hyperproduction in NC/Kuj mice. Four-week-old NC/Kuj mice were kept together with NC mice infected with fur mite (*Myocoptes musculinus*) for 2 weeks in isolated clean rooms and thereafter separated. Serum IgE levels were determined and the skin examined histologically.

Matsuoka et al. (2001) described a mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. The mites were cultured in a mixture of mouse diet and dried yeast in culture flasks. A crude extract in PBS was applied to the shaved back of NC/Nga mice or BALB/c mice three times a week for 8 weeks. In the NC/Nga group, dryness and scaling appeared on the skin, and scratching behavior increased at the 2nd week in the treated group. Skin erosion and hemorrhage occurred in the 4th week. The epidermis thickened and deepened into the upper dermis, in which mast cells were highly accumulated, corresponding with the skin lesions of atopic eczema/dermatitis syndrome in patients.

Matsui and Nishikawa (2002) used female specific-pathogen-free BALB/c mice at an age of 6–8 weeks. The animals were barrier disrupted by repeated applications of adhesive cellophane tape prior to percutaneous sensitization by topical administration of 100 µl of a solution of house-dust mite antigen. Seven days after the sensitization, the mice were challenged on the shaved dorsal skin near the ear by subcutaneous injection of 10 µl of mite antigen or lipoteichoic acid from *Staphylococcus aureus* for elicitation of localized skin inflammation. The cytokine response in the dorsal skin was investigated by reverse transcription polymerase chain reaction and immunohistological analysis.

Spergel et al. (1998) reported that epicutaneous sensitization with ovalbumin induced localized allergic dermatitis and hyper-responsiveness to methacholine after single exposure to aerosolized antigen in mice.

Spergel et al. (1999) used mice with targeted deletions of IL-4, IL-5, and interferon-γ cytokine genes to assess the role of these cytokines in a murine model of allergic dermatitis elicited by epicutaneous sensitization with ovalbumin.

Laouini et al. (2003) used IL-10$^{-/-}$ mice to examine the role of IL-10 in a mouse model of allergic dermatitis induced by epicutaneous sensitization with ovalbumin on tape-stripped skin.

Takeshita et al. (2004) underlined the essential role of MHC II-independent CD4^{+} T cells, IL-4, and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse and recommended fluorescein-isothiocyanate-induced contact hypersensitivity as a suitable animal model for atopic dermatitis.

Sasakawa et al. (2001) induced atopic dermatitis-like lesions by topical application of mite antigens in NC/Nga mice. Extracts of mite antigen were injected repeatedly at the ventral side of the ear. Clinical symptoms and thickness of the ear were measured. On day 18, blood and submandibular lymph nodes were collected to measure plasma immunoglobulins and to perform histochemical analysis.

Heishi et al. (2003) performed gene expression analysis of atopic dermatitis-like skin lesions induced in NC/Nga mice by mite antigen stimulation under specific-pathogen-free conditions. Mite Extract-Dp (LSL Japan) was injected intradermally into the right and left pinnae and into the skin of NC/Nga of BALB/c mice in two places once per 3 days, and the clinical symptoms and the ear thickness were measured. On day 14 or 28, plasma IgE was determined. Furthermore, mRNA transcripts in pinnae were analyzed using real-time quantitative PCR.

Food hypersensitivity plays a pathogenic role in patients with atopic dermatitis (Sicherer and Sampson 1999).

Li et al. (2001) reported a murine model of atopic dermatitis associated with food hypersensitivity. Female C3H/HeJ mice were sensitized orally to cow's milk or peanut with cholera toxin adjuvant and then subjected to low-grade allergen exposure. Histological examination of skin lesions, allergen-specific Ig levels, and allergen-induced T-cell proliferation were studied. Treatment of the eruption with topical corticosteroids led to decreased pruritus and resolution of the cutaneous eruption.

Contact Hypersensitivity in Rats

Meingassner et al. (1997) sensitized female Sprague Dawley rats by application of 80 µl of 2 % 2,4-dinitrofluorobenzene (DNFB, dissolved in acetone, DMSO, and olive oil 50:10:38 v/v/v), applied in 20 µl volumes to the inner surface of both ear lobes and to both shaved inguinal regions on day 1. Allergic contact dermatitis was elicited with 30 µl of 0.5 % DNFB applied to the test sites of ≈15 mm in diameter on both shaved flanks on day 12. Animals were treated twice by gavage 2 h before and immediately after challenge. Dermatitis was evaluated by measuring the thickness of the lifted skinfold at the test sites with a spring-loaded micrometer (Schnelltaster, Kröplin, Germany) before challenge and 24 h after challenge.

Contact Hypersensitivity in Guinea Pigs

Guinea pigs, six per group, are sensitized on the dorsal surface of one ear pinna with 50 µl of 10 % DBFB in 50 % acetone: 50 % olive oil on day 0 and then on the opposing ear on day 1. On day

5, animals are shaved and challenged with 0.5 % 1-chloro-2,4-dinitrochlorobenzene (DNCB) with or without codissolved drug, 15 µl per site, 4.8 µl per cm^2, on the dorsolateral surface (Hsieh et al. 1996). Naïve animals are challenged with DNCB and serve as nonspecific controls. The response is scored visually in a blinded fashion 24 h after challenge (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: mild, confluent erythema; 3: moderate erythema without edema or induration; 4: strong erythema with uniform induration or edema; 5: severe erythema with induration or edema, plus ulceration). Data are calculated as for mouse contact hypersensitivity.

Rosenqvist et al. (1991) studied the effects of cilazaprilat and enalaprilat on experimental dermatitis in guinea pigs.

Boyera et al. (1992) tested repeated application of dinitrochlorobenzene to the ears of sensitized guinea pigs as an animal model for contact eczema in humans.

The effect of a topical preparation of mycophenolic acid on experimental allergic contact dermatitis of guinea pigs induced by dinitrofluorobenzene was described by Shoji et al. (1994).

Contact Hypersensitivity in Pigs

Groups of 6–12 pigs are sensitized with 10 % DNFB in acetone/DMSO/olive oil (45:5:50 by volume) to the shaved outer aspect of both ears and bilateral sites of the lower abdomen, 100 µl per site, on day 0, with a second application of 5 % DNFB to the internal pinna and the lower thorax on day 3. On day 9, pigs are restrained on a webbed canvas cart and the test area carefully shaved with an electric clipper. A pilot challenge with 0.1, 0.15, and 0.2 % DNCB in acetone/olive oil (95:5 by volume), 3.8 µl per cm^2, is used to determine conditions for obtaining submaximal average response in each animal cohort. Pigs are scored by a blinded observer, 24 h post challenge on a scale from 0 to 4 (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: moderate erythema without induration or edema; 3: strong erythema with focal areas of edema or induration: 4: extreme erythema with uniform induration or edema). Pigs having a mean DNCB control score <1.5 are excluded (Hsieh et al. 1997). Based on their pilot response on day 10, animals are stratified into two groups having comparable mean scores, challenged on duplicate sites with DNCB in 95:5, with or without codissolved drug, and scored on day 11. Scores for each challenge site are compared with the average of the control spots treated with DNCB alone on the same pig, and expressed as percentage inhibition.

Bilski and Thomson (1984) recommended allergic contact dermatitis in the domestic pig as a model for evaluating the topical anti-inflammatory activity of drugs and their formulations.

Meingassner and Stütz (1992) sensitized female domestic pigs with a 10 % solution of DNFB dissolved in 50 % acetone, 10 % dimethylsulfoxide (DMSO), and 30 % olive oil applied in volumes of 100 µl onto both auricles (medial aspects) and groins on day 1. The animals were exposed again on the lateral aspects of both auricles to 100 µl of 2 % DNFB on day 4. Challenge was performed with a 1 % DNFB solution (without DMSO) on day 12 by applying 20 µl epicutaneously to each of 24 test sites (2 cm diameter) arranged in four craniocaudal lines on the dorsolateral back of each animal. The test sites were treated twice (0.5 and 6 h after challenge) with 20 µl of solutions of active compound or drug vehicle. Evaluation of the treatment-related effects was performed at the peak inflammatory response which was 24 h after challenge. Each test site was evaluated visually for (1) intensity, (2) extent of erythema, and (3) consistency using arbitrary scores from 0 to 4. In addition, skin changes were biophysically characterized by measuring microvascular perfusion (PeriFlux PF3 Laser Doppler Perfusion Monitor) and reflective color measurement (Minolta Chroma Meter CR 200).

Vana and Meingassner (2000) described morphological and immunohistochemical features of experimentally induced contact dermatitis in **Göttingen minipigs**.

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Nonimmunologic Contact Urticaria

Purpose and Rationale

Contact urticaria is defined as a wheal-and-flare reaction, appearing shortly after certain substances contact intact skin and disappearing within some

hours, leaving normal-appearing skin. Contact urticaria is divided into two main types, immunologic and nonimmunologic (Maibach and Johnson 1975). Immunologic contact urticaria is mediated at least partially by specific IgE antibodies attached to mast cell membranes. Vasoactive substances, released from mast cells, elicit erythema and edema of the skin. Nonimmunologic contact urticaria appears only on the contact area without previous sensitization. Specific antibodies against the causative substance are not found in serum. Lathi and Maibach (1984) investigated the suitability of the guinea pig for studies on nonimmunologic contact urticaria.

Procedure

Female Hartley strain guinea pigs weighing 350–500 g are used. Fifty microliters of test substances is applied with a micropipette to both sides of the earlobe. One ear of the animal is challenged with the contact urticant, while the other ear serves as control with ethyl alcohol. Earlobe thickness is measured three times on four different sites using a string micrometer with round touching plates, 6 mm in diameter. The string of the instrument is adjusted so that the moving plate does not squeeze the tissue but stops at once when it reaches the surface of the ear. The mean of 12 measurements is recorded as the preapplication thickness. All measurements after application of the test substance are performed once on the same four sites, and the mean is recorded as the postapplication thickness.

The thickness of the ear is measured 5 min after application and then every 10 min during the first h, every 15 min during the second, and every 30 min during the third h.

Evaluation

The differences between postapplication and preapplication values are recorded and plotted as time–response and dose–response curves.

Modifications of the Method

Lauerma et al. (1997) used the trimellitic anhydride-sensitive mouse as an animal model for contact urticaria. BALB/c mice were sensitized with trimellitic anhydride by topical applications and treated with glucocorticosteroids, antihistaminics, or nonsteroidal anti-inflammatory drugs. Ears were challenged with trimellitic anhydride, and ear thickness was measured at baseline and 1, 2, 4, 8, and 24 h after challenge. Trimellitic anhydride caused a biphasic ear swelling response. However, there was also an early swelling by trimellitic anhydride in nonsensitized mice, suggesting that nonimmunologic as well as immunologic mechanisms contribute to early swelling by trimellitic anhydride.

Irritant contact dermatitis was induced in mice by the application of 20 µl 0.01 % A23187 (calcium ionophore) or 0.005 % PMA (phenyl mercuric acetate) to both aspects of the right pinnae which were treated simultaneously (A23187) or after 30 min (PMA) with the test compound or vehicle (Meingassner and Stütz 1992). Topical efficacy was assessed by determination of the differences in weight of both auricles of treated and untreated mice 7.5 h (A23187) or 6 h (PMA) after application of the irritants.

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Immunological Models of Atopic Dermatitis

Mononuclear Cells from Atopic Dermatitis Donors

Purpose and Rationale

Rühl et al. (2003) studied the effects of PPAR-α and PPAR-β ligands on immunoglobulin synthesis and cytokine production by peripheral blood mononuclear cells from normal donors and patients with atopic dermatitis.

Procedure

Peripheral blood mononuclear cells were isolated from buffy coats of nonallergic healthy donors and atopic dermatitis patients by Ficoll Hypaque (D = 1.077) separation (350 *g*, 30 min, room temperature). B cells were purified by magnetic cell sorting using anti-CD19-coupled magnetic beads. Then 4×10^8 peripheral blood mononuclear cells in 800 µl PBS + 20 nM ethylenediaminetetraacetic acid + 0.2 % bovine serum albumin + 15 µl Beriglobin (Chinon Bering, Marburg, Germany) were incubated for 10 min in ice, followed by addition of 200 µl conjugated beads (1.5 ratio) and 10 min of incubation at 4 °C. After several washings, CD19-positive B cells were selected by magnetic positive selection with LS^+ column, and the cells were then resuspended in medium and counted. The same procedure was done for the purification of T cells and monocytes, respectively, using anti-CD3 and anti-CD14-coupled magnetic beads.

Peripheral blood mononuclear cells and purified B cells (10^6 cells per ml) were cultured in RPMI 1640 culture medium supplemented with l-glutamine (2 mm), penicillin (100 U per ml), streptomycin (100 µg/ml), and 10 % heat-inactivated fetal calf serum. All cell cultures were carried out at 37 °C in humidified air and 5 % CO_2 atmosphere.

For the immunoglobulin assays, immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with the isotope-specific anti-human Ig-Fc antibodies diluted in 0.1 m bicarbonate buffer. Blocking was performed by adding 2 % bovine serum albumin/Tris-buffered saline for 1 h, followed by several washings. Supernatants and internal standard were then added to the wells and incubated in duplicate for 2 h. After several washes, the alkaline phosphatase-conjugated antibodies (IgA, IgG, and IgM) or biotinylated anti-IgE MoAb was added. For IgE, plates were incubated for another hour with alkaline-phosphatase-conjugated streptavidin. Following the final reaction with phosphatase substrate (Sigma, Dreieich, Germany), plates were read in a microplate ELISA reader at 405 nm, and the amount of immunoglobulin was calculated according to the standard curve. Cytokines were determined by ELISA assays.

Evaluation

Wilcoxon's signed rank test for matched pairs was performed to compare the results within the donor groups.

Modifications of the Method

Furthermore, Rühl et al. (2003) performed in vivo experiments in ovalbumin-sensitized female NMRI mice confirming the in vitro findings and showing that the IL-4-mediated immune response was inhibited in PPAR-activator-treated mice.

Sperhake et al. (1998) studied the effects of recombinant human soluble interleukin-4 receptor on interleukin-4/staphylococcal enterotoxin B-stimulated peripheral mononuclear cells from patients with atopic eczema.

Hong et al. (2003) demonstrated the PPARγ-dependent anti-inflammatory action of rosiglitazone in human monocytes. Furthermore, it was shown that the suppression of tumor necrosis factor α (TNFα) secretion is not mediated by phosphatase and tensin homolog (PTEN) regulation.

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The SCID-hu Skin Mouse as Model for Atopic Dermatitis

Purpose and Rationale

Severe combined immunodeficiency (SCID) mice have a defect in the antigen recombinant system that prevents the development of mature B and T lymphocytes (Bosma et al. 1983) and therefore renders these mice tolerant to xenografts of human cells and tissues.

Carballido et al. (2000, 2003), Biedermann et al. (2002, 2004), Igney et al. (2004), and Lametschwandtner et al. (2004) reported the establishment of an in vivo mouse model that allows monitoring of human T cell migration into human skin. This model is based on the use of SCID mice transplanted with human skin (SCID-hu Skin mice). Adoptively transferred human T helper (Th)2 cells obtained from atopic dermatitis skin lesions or peripheral blood T cells selectively migrate to the human skin grafts of these SCID mice in response to defined chemokines locally injected in the human skin grafts. Homing of human T cells into the human skin on SCID-hu Skin mice is a specific process since it only occurs in response to chemokine ligands that are specific for the chemokine receptors expressed on the migrating T cells.

Procedure

Generation of SCID-hu Skin Mice

Human adult skin is obtained from breast or abdominal reduction surgery. The skin is perfused with cell culture media containing penicillin and streptomycin and fat tissues are removed. Subsequently, full-thickness skin is trimmed at the dermal side to obtain skin biopsy samples containing the full epidermis, the papillary dermis, and part of the reticular dermis. This skin tissue is cut into pieces of approximately 10 × 10 mm and kept at 4 °C in cell culture media until use. C.B-17/GbmsTac-*Prkdc*^{scid} *Lyst*^{bg} mice (M&B, Ry, Denmark, herein referred to as SCID mice) are anesthetized by i.p. injection of a solution containing ketamine and xylazine and their backs shaved. Subsequently, two pieces of skin of the same size as the human grafts are removed from the back of the mice and replaced by human skin pieces, which are fixed at their edges using surgical glue. At the end of the procedure, the human grafts are covered with a polyvinyl bandage to prevent scratching and to facilitate tissue engraftment. After transplantation, the human grafts undergo a period of human keratinocyte hyperproliferation resulting in the formation of hyperkeratotic crusts. During this process, human skin grafts fuse with the adjacent mouse skin. About 5 weeks after transplantation, the crusts fall off and reveal a skin tissue containing all the characteristic structures of normal human skin including a network of newly grown vessels connecting the graft with the underlying mouse tissues.

Generation and Characterization of Human T Cells for Skin-Homing Studies in Vivo

Th lymphocytes selected for in vivo migration studies in SCID-hu Skin mice are isolated from human atopic skin lesions in order to obtain Th cells with optimal skin-homing potential. Human Th cell lines are grown from biopsies of **atopic dermatitis** patients challenged with house-dust mite (HDM) antigens (Carballido et al. 1997). Th cell clones are isolated from the HDM-specific lines by limiting dilution and maintained by repeated stimulation with irradiated allogenic human peripheral blood mononuclear cells (PBMC) and phytohaem agglutinin (PHA), followed by expansion with human IL-2.

Atopic dermatitis is an inflammatory skin disease in which skin-infiltrating allergen-specific

Th2 cells play a crucial role (Robert and Kupper 1999). Accordingly, the majority of the human Th cell clones isolated from atopic skin biopsy samples are of the polarized Th2 phenotype. These cells produce large amounts of IL-4, IL-5, and IL-13 but low or undetectable levels of IL-2 and IFN-γ. All Th cells express CLA LFA-1 and CCR4. Based on these results and the observation that CCL22 (macrophage-derived chemokine, MDC) is abundantly produced in atopic dermatitis skin lesions, CCL22 is selected as a relevant ligand for the evaluation of skin-specific migration of human Th cells in the SCID-hu Skin mouse model. In order to demonstrate that CCL22 induces Th2 cell activation, Th2 clones are loaded with Fluo-4acetoxymethyl ester (Fluo-4/AM, Molecular Probes, Eugene, Ore., USA). Following the addition of CCL22 (300 ng/ml), changes in fluorescence intensity are analyzed every 5 s using a FACScan FL-1 channel (Becton Dickinson, San Jose, Calif., USA) (Biedermann et al. 2002). Comparable results were obtained with CCL17 (thymus-activation-regulated chemokine, TARC), which is the other ligand for CCR4 and is also abundantly expressed in atopic dermatitis lesions. These results demonstrate the relevance of CCL17 and CCL22 interactions with CCR4 for activation of Th2 cells derived from atopic dermatitis skin lesions. In addition, human peripheral T cells are analyzed for their skin-homing capacity in SCID-hu Skin mice. PBMC are isolated from healthy volunteers.

Recruitment of Human Th Cells to Human Skin Grafts in SCID-hu Skin Mice

To evaluate the potential of selected chemokines to recruit atopic-dermatitis-derived human Th2 cells and peripheral blood T cells to human skin in vivo, 1.5×10^8 Th2 cells or PBMC are adoptively transferred (i.p.) into SCID mice which have been previously transplanted (6–8 weeks) with two pieces of human skin (SCID-hu Skin mice). Following Th cell transfer, 300 ng of CCL22, CXCL10 (IFN-γ-inducible protein of 10 kDa, IP-10), or vehicle control is injected intradermally (i.d.) in the human skin grafts of the SCID-hu Skin mice. On day 8, mice are sacrificed, and human skin grafts are harvested and processed into single-cell suspensions using a mechanical tissue disaggregator. These cell suspensions are stained with human anti-CD3, anti-CD4, or anti-CD45 mAb conjugated to FITC and PE (Becton Dickinson) and analyzed in the FACScan. CCL22 effectively recruits human Th2 cells into human skin grafts of SCID-hu Skin mice, whereas CXCL10 is ineffective. These data are consistent with the expression of CCR4 on atopic-dermatitis-derived Th2 cells, whereas CXCR3, which is the receptor for CXCL10, is not expressed on these cells. Furthermore, treatment of Th2 cells prior to transfer into SCID mice with *Bordetella pertussis* toxin, an inhibitor of G-protein-coupled receptors, completely abolishes the migratory capacity of these Th2 cells indicating the requirement of an intact chemokine receptor signaling pathway (Biedermann et al. 2002). It has to be noted that CCR4-mediated human skin homing of Th2 cells is not restricted to CCL22 since the alternative CCR4-ligand, CCL17, also induces Th2 migration to human skin grafts, although to a lesser degree than CCL22.

Similarly to Th2 cells, T cells present in human PBMC can also be recruited into human skin grafts of SCID-hu Skin mice. These T cells migrate in response to both CCL22 and CXCL10 chemokines (300 ng injected i.d. into human skin), which is consistent with CCR4 and CXCR3 expression on these cells. Peripheral blood T cells also express functional CCR2, since the T cells migrate into human skin in response to the CCR2 ligand CCL2. From these data it can be concluded that both skin-derived Th2 cells and peripheral blood T cells can be recruited into human skin grafts provided that these T cells express the chemokine receptors specific for the recruiting chemokine.

Inhibition of Human T Cell Migration into Human Skin of SCID-hu Skin Mice

The present SCID-hu Skin mouse model has been validated for measuring various inhibitors of human T cell rolling, extravasation, and migration in vivo. It was shown that inhibition of CLA/E-Selectin interactions using an E-Selectin-specific mAb or a low molecular weight (LMW)

E-Selectin-specific antagonist strongly inhibited CCL22-mediated Th2 cell migration into the skin (Biedermann et al. 2002). Similarly, anti-LFA-1 mAbs administered at 5 mg/kg at day 1 prior to Th2 cell transfer and on day 4 strongly inhibits Th2 cell migration into the human skin. Th2 cell migration in response to CCL22 is also inhibited in a dose-dependent fashion by LMW LFA-1 inhibitors, given daily p.o. starting on the day of T cell transfer.

Critical Assessment of the Method

This mechanistic model allows analysis of the relevant steps involved in human T-lymphocyte migration into inflamed skin. In addition, it can be used for preclinical testing of drug candidates that are highly selective for human target molecules associated with the different steps of T cell migration in an environment that resembles the physiological or pathological conditions occurring in humans. The model allows the study of chemotactic responses of human cells toward specific, intracutaneously administered chemokines and testing of specific antagonists of human Th cell migration in vivo.

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Pruritus Models

Howard Maibach

Contents

Pruritus and Scratching Behavior in Mice

Purpose and Rationale

Pruritus is a common feature of many skin disorders. Since its pathogenesis in largely unknown, proper treatment is not available. The development of new treatments is hampered by the lack of animal models for studying pruritus (Woodward et al. 1985). Kuraishi et al. (1995) have proposed that pruritogenic but not algesiogenic agents stimulate the scratching activity in mice. Scratching is usually registered by counting the number of scratches from direct visual observation or from a video recording (Gmerek and Cowan 1983; Larsen et al. 1994; Thomas et al. 1994). Elliott et al. (2000) developed a method, which allows the automated registration of the scratching activity of the hind legs of mice for periods longer than 24 h.

Procedure

Mice with chronic proliferative dermatitis (cpdm/cpdm mice), a spontaneous mutation of C57BL/Ka mice showing a skin disorder accompanied by severe scratching (Gijbels et al. 1996, 2000), at an age of 6–12 weeks or normal C57BL/Ka mice treated subcutaneously with 100 μg/mouse compound 48/80 or 3 mg/mouse histamine hydrochloride, are used.

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Metal rings made of soft 1-mm-diameter aluminum wire are placed around both hind legs of the animal just above the ankle. The ring has to be of sufficient diameter that it is just free enough to rotate around the limb. Mice are housed individually in cages (12 × 18 × 13 cm), which are placed on a scratch detection unit consisting of a plastic outer casing containing the circuit board, and, inset into the top, four ferrite rods with copper coils.

As the mouse scratches, the movement of the metal rings (fitted around its hind legs) in the field generated by the coils elicits a signal that can be transformed into peaks of different frequencies using fast Fourier transformation. Data are downloaded to memory every 3 s, filtered and a power spectrum constructed. The power spectrum is analyzed for a scratch pattern every 1.5 s. Scratching is routinely classified as a signal that gives rise to peaks with maximal amplitudes of around 200 mV and frequencies greater than 15 Hz. The amplitude of general motor activity peaks (movement from bedding using hind limbs, drinking from water bottle) is usually greater than 500 mV and at frequencies of less than 10 Hz. In order to obtain the best discrimination between scratching and general motor activity, any contribution to the scratch frequencies of high energy/low frequency peaks is filtered out by setting the upper detection limit at 0.5 mV for scratching.

Evaluation

Data are expressed as mean ± SEM Statistical analysis is performed by one-way analysis of variance followed by Student's *t*-test.

Modifications of the Method

Inagaki et al. (2002, 2003) used new apparatus, MicroAct, to study mechanisms in the induction of scratching behavior in BALB/c mice by compound 48/80. A small magnet (1 mm in diameter, 3 mm long) was inserted subcutaneously in both hind paws under ether anesthesia. The mouse with magnets was placed in an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the hind paws was amplified and recorded. Characteristic waves reflecting scratching behavior were detected by a computer.

Brash et al. (2005) designed a repetitive movement detector used for automatic monitoring and quantification of scratching in mice. The system is based on a sensitive force transducer positioned below a recording platform holding a lightweight recording box in which the animal is placed. A programmed microcontroller is used to discriminate between nonspecific movement, grooming behavior, and scratching movements made by the animal's hind limb. Following subdermal injections of histamine receptor agonists into the neck of a mouse, dose-related scratching occurred which was detected and quantified.

Larsen et al. (1994) studied the influence of ultraviolet irradiation on scratching behavior in hairless mice. Especially the wavelengths 315–330 nm were more itch provoking than erythemogenic.

Several authors used the model of compound 48/80-induced scratching behavior in mice. Sugimoto et al. (1998) studied the effects of histamine H_1 receptor antagonists on compound 48/80-induced scratching behavior in mice. Rojavin et al. (1998) investigated the antipruritic effect of millimeter waves in mice treated with compound 48/80. Inagaki et al. (2002) studied the mechanisms of induction of scratching behavior in BALB/c mice by compound 48/80. Shinmei et al. (2004) studied the effect of Brazilian propolis on scratching behavior induced by compound 48/80 and histamine in mice. Oliveira et al. (2004) reported suppression of the scratching behavior induced by dextran 40 and compound 48/80 by treatment with pentacyclic triterpenoids. Inagaki et al. (1999, 2000) studied the participation of histamine H_1 and H_2 receptors in passive cutaneous anaphylaxis-induced scratching behavior in ICR mice and evaluated antiscratch properties of

drugs in BBLB/c, ICR, and ddY mice treated with dinitrofluorobenzene painting.

Hossen et al. (2003) reported involvement of histamine H_3 receptors in scratching behavior in mast-cell-deficient mice.

The involvement of the μ- and κ-opioid system in scratching behavior was investigated by Tohda et al. (1997), Kamei and Nagase (2001), and Umeuchi et al. (2003, 2005).

Tan-No et al. (2000) reported that intrathecally administered spermine produces scratching, biting, and licking behavior in mice.

Hansson et al. (2002) described a model of chronic itchy dermatitis in transgenic mice with epidermal overexpression of stratum corneum chymotryptic enzyme.

Andoh and Kuraishi (2002) described inhibitory effects of azelastine on the substance P-induced itch-associated response in mice.

Umeuchi et al. (2005) tested spontaneous scratching behavior in MRL/*lpr* mice, a possible model for pruritus in autoimmune diseases, and antipruritic activity of a κ-opioid receptor agonist and recommended MRL/*lpr* mice scratching behavior as a suitable model of pruritus that occurs in autoimmune diseases.

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Pruritic Dermatitis in Other Species

Gmerek and Cowan (1983) described an animal model for preclinical screening of systemic antipruritic agents in **rats**. Bombesin induces dose-related excessive scratching when administered intracerebroventricularly (i.c.v.) to rats. Scratching elicited by 0.10 μg bombesin i.c.v. was monitored with the help of a microcomputer.

Thomas and Hammond (1995) found that microinjections of morphine into the rat medullary dorsal horn produces a dose-dependent increase in facial scratching in Sprague Dawley rats.

Thomsen et al. (2001) proposed scratch induction in the rat by intradermal serotonin as a model of pruritus. Several compounds (histamine, compound 48/80, kallikrein, trypsin, papain, substance P and PAF) were injected into the rostral back of rats. Only serotonin induced excessive scratching at the site of injection. The model was recommended for research and development of antipruritics of the nonhistaminic type.

De Castro Costa (1987) tested scratching behavior in arthritic rats; Thomsen et al. (2002), in hairless rats; Ozaki et al. (2005), in beige rats with IgE hyperproduction.

Hayashi et al. (2001) studied the effects of a histamine H_1 antagonist on cutaneous hyperpermeability and scratching behavior induced by poly-L-arginine in rats.

Noshima and Carstens (2003) described quantitative assessment of directed hind limb scratching behavior in Sprague Dawley rats after

intradermal injection of serotonin into the nape of the neck.

Gingold and Bergasa (2003) found that a cannabinoid agonist increases nociception threshold in rats with cholestasis secondary to bile duct resection. The model was recommended when testing compounds for treatment of pruritus due to cholestasis.

Minami and Kamei (2004) published a chronic model for evaluating the itching associated with allergic conjunctivitis in rats. After subcutaneous and local sensitization with egg albumin, scratching behavior was observed after instillation of egg albumin into the eyes.

Butler et al. (1983) described pruritic dermatitis in asthmatic basenji-greyhound dogs, which was manifested as lichenified plaques and as inflammatory nodules and papules. The authors recommended this disease as a model for human atopic dermatitis.

Osifo (1991) used a dog model to study structure–activity relationships in the pruritogenicity of chloroquine and amodiaquine metabolites.

Patterson and Harris (1981) reported chronic pruritic dermatitis in asthmatic rhesus monkeys.

Ko and Naughton (2000) described an experimental itch model in rhesus monkeys.

Holden et al. (2003) found in this model that activation of κ-opioid receptors inhibits pruritus evoked by subcutaneous or intrathecal administration of morphine.

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Psoriasis Models

Howard Maibach

Contents

Psoriasis Models

General Considerations

Several authors reviewed the experimental models for psoriasis (Krueger and Jorgensen 1990; Boehncke 1997; Nickoloff 1999; Rosenberg et al. 1999; Schön 1999; Mizutani et al. 2003). In addition to studies in animals, various in vitro investigations were carried out with autopsy material from human psoriatic lesions. Several studies were devoted to the T cell hypothesis of psoriasis (Bos and de Rie 1999). Lymphocytes may change epidermal growth homeostasis, leading to increased keratinocyte proliferation and abnormal differentiation of these apoptotic cells that end their life cycle as corneocytes.

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In Vitro Studies with Isolated Cells

Cultured Keratinocytes

Purpose and Rationale

Cultured keratinocytes have been used to study the pathogenesis of psoriasis (Mils et al. 1994; Bata-Csörgö et al. 1993, 1995a, b; Ockenfels et al. 1996; Nylander-Lundqvist and Egelrud 1997a, b; Nylander-Lundqvist et al. 1998; Konger et al. 1998; Szabo et al. 1998; Dimon-Gadal et al. 2000; Karvonen et al. 2000; Segaert et al. 2000; Ting et al. 2000) and to evaluate antipsoriatic drugs (Chapman et al. 1990; Ockenfels et al. 1995; Medalie et al. 1996; Lin et al. 1999; Diaz et al. 2000; Farkas et al. 2001). Sampson et al. (2001) tested the in vitro keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins.

Procedure

The medium (FDMEM) consists of Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin, and 2 % streptomycin.

SVK-14 keratinocytes (Taylor-Papadimitriou et al. 1982) are cultured in FDMEM medium in flasks at 10 % CO_2 and 37 °C. When confluent, cells are washed with magnesium- and calcium-free phosphate buffered saline (PBS). PBS is decanted and cells detached by adding 5 ml of trypsin (0.05 %) in EDTA (0.02 %). PBS is added to a volume of 50 ml. The cell pellet obtained by centrifugation (1,000 *g*, 5 min) is resuspended in 10 ml of FDMEM. Cell counts in the final suspension are determined using a hemacytometer, and the cell density is adjusted to 25,000/ml with FDMEM.

For microtiter assays, cells (5,000 in 200 µl medium) are inoculated into the inner wells of 96-well plates. The outer edge wells of the plate contain 200 µl of 10 % fetal bovine serum in PBS. After 24 h at 37 °C, 10 % CO_2, plating medium is replaced with FDMEM containing test material or standard in 200 µl. Standard substances are madecassoside (50 nM–5 mM), asiaticoside (100 nM–10 mM) dissolved in methanol, and dithranol (0.33–170 µM) dissolved in DMSO. Each sample concentration is tested with six replicates on each of the three separate plates. Cells exposed to FDMEM alone provide 100 % growth control. Cells are incubated for 7 days at 37 °C, 10 % CO_2, prior to carrying out the sulforhodamine B assay.

For the sulforhodamine B (SRB) assay (Skehan et al. 1990), cells are fixed by layering 100 µl of ice-cold 50 % trichloroacetic acid on top of the growth medium. Cells are incubated at 4 °C for 1 h, after which plates are washed five times with cold water, excess water drained off, and the plates left to dry in air. SRB stain (50 µl; 0.4 % in 1 % acetic acid) (Sigma) is added to each well and left in contact with the cells for 10–30 min after which they are washed with 1 % acetic acid, rinsing four times until only the dye adhering to the cells is left. The plates are dried and 100 µl of 10 mM Tris buffer added to each well to solubilize the dye. The plates are shaken gently for 5 min on a plate reader and the absorbance is read at 550 nm using a Titertek Multiscan MCC/340 II plate reader.

Evaluation

Mean optical density (OD ± SD) is calculated for each concentration from the six replicate wells in a single plate. The data is used to plot a dose response curve from which IC_{50} values ± SD are obtained.

Modifications of the Method

Fogh et al. (1993) described an ex vivo skin model. Keratomized psoriatic skin samples were incubated in the presence of the calcium ionophore a23187 and arachidonic acid for 45 min at 37 °C. After extraction of lipids, eicosanoids were determined by reversed-phase high-performance

liquid chromatography in combination with specific radioimmunoassays.

Hager et al. (1999) described long-term culture of murine epidermal keratinocytes.

Hanley et al. (1998) found that the differentiation of keratinocytes in cultures from human foreskin was stimulated by activators of the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα).

Rivier et al. (1998) reported differential expression of PPAR subtypes during the differentiation of human keratinocytes. When normal human keratinocytes were induced to differentiate by shifting the culture medium to a high Ca^{2+} concentration, the expression of PPARα and PPARγ mRNA was increased, whereas that of PPARδ remained unchanged. In lesional compared with unlesional psoriatic epidermis, the expression of PPARα and PPARγ was reduced, indicating that these two subtypes are tightly linked in the epidermal differentiation process.

Naik et al. (1999) found that human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with proinflammatory mediators and dinitrochlorobenzene.

Muga et al. (2000) reported that 8S-lipoxygenase products activate PPARα and induce differentiation in murine keratinocytes. Transgenic mice with 8S-lipoxygenase targeted to keratinocytes were generated through the use of a loricrin promoter. In cultured keratinocytes, PPARα was identified as a crucial component of keratin-1 induction through transient transfection with expression vectors for PPARα, PPARγ, and a dominant-negative PPAR as well as through the use of known PPAR agonists.

Ellis et al. (2000) found that ligands for PPARγ inhibit keratinocyte proliferation. Troglitazone improved psoriasis and showed normalization in these models of proliferative skin disease.

Kömüves et al. (2000) studied keratinocyte differentiation in hyperproliferative epidermis and found that topical application of PPARα activators restores tissue homeostasis.

Rosenfield et al. (2000) reviewed PPARs and skin development.

Sakamoto et al. (2000) described activation of human PPAR subtypes by pioglitazone.

Tan et al. (2001) studied the roles of PPARβ/δ in the keratinocyte's response to inflammation. The authors showed by the use of cultures of primary keratinocytes from wild-type and PPAR$\beta/\delta^{-/-}$ mice that inflammatory signals, such as tumor necrosis factor α (TNFα) and interferon β (INFβ), induce keratinocyte differentiation. The proinflammatory cytokines initiate the production of endogenous PPARβ/δ ligands, which are essential for PPARβ/δ activation and action.

Thuillier et al. (2002) found inhibition of PPAR-mediated murine keratinocyte differentiation by lipoxygenase inhibitors. The effect was mediated primarily through PPARα and PPARγ.

Zhou et al. (2002) performed genetic analysis of four novel PPARγ splice variants in monkey macrophages.

Hong et al. (2003) described PPARγ-dependent anti-inflammatory action of rosiglitazone in human monocytes, whereby the suppression of TNFα secretion is not mediated by lipid phosphatase PTEN regulation.

Kojo et al. (2003) evaluated human PPAR subtype selectivity of a variety of anti-inflammatory drugs based on a novel assay for PPARδ (β).

Kuenzli and Saurat (2003) reviewed the role of PPARs in cutaneous biology.

Westergaard et al. (2003) studied expression and localization of PPARs and nuclear factor κB in normal and lesional psoriatic skin.

Gosh et al. (2004) found disruption of transformation growth factor signaling and profibrotic responses in normal skin fibroblasts by PPARγ.

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Effect on T Lymphocytes

Purpose and Rationale

Psoriasis vulgaris is considered as an inflammatory cell-mediated autoimmune disorder (Uyemura et al. 1993; Gottlieb et al. 1994; Bata-Csörgö et al. 1995; Austin et al. 1999). Lesional skin T cells contribute proinflammatory/type 1 (T1) cytokines to initiate and maintain the cell-mediated keratinocyte hyperplasia in inflammatory lesions. Atopic dermatitis is characterized by the presence of Th2 cell and their respective cytokines (Cooper 1994; Leung 1997). A therapeutic agent downregulating both Th1 and Th2 cytokine production would be desirable (Mollison et al. 1999; Santamaria et al. 1999).

Procedure

Human Mixed Leukocyte Reaction (MLR) Assay

Fifty milliliters of sodium-heparinized blood, obtained from normal, unrelated donors, is mixed with an equal volume of Dulbecco's phosphate buffered saline (DPBS). Peripheral blood mononuclear cells (PBMC) are isolated by density centrifugation at 400 *g* over Histopaque-1077. A sufficient number of PBMC are washed three times in RPMI-1640 medium and used as responder cells. The remaining PBMC (stimulator cells) are washed as above and treated with 25 μg/ml mitomycin C for 30 min at 37 °C in an atmosphere of 5 % CO_2 and 100 % humidity and then washed three times with RPMI-1640 medium. The stimulator cells are pooled at 0.5–1.0 × 10^6 cells per ml per donor in RPMI-1640 medium. Cells are cultured in medium consisting of RPMI-1640 supplemented with 4 mM L-glutamine, 500 μM 2-mercaptoethanol, 10 % fetal bovine serum, 25 units/ml penicillin, and 25 μg/ml streptomycin. Mixed leukocyte reactions are performed in 96-well flat-bottom plates (Corning, Acton MA) in a final volume of 220 μl, containing 20 μl of culture medium with or without test compound or standard (ascomycin, purified from a fermentation of *Streptomyces hygroscopicus* ssp. *ascomyceticus* ATCC 14891 and characterized according to Or et al. 1993), 100 μl of responder cells (1 × 10^6 cells per ml), and 100 μl of stimulator cells (2–4 × 10^6 cells per ml). Cultures are incubated at 37 °C in an atmosphere of 5 % CO_2 and 100 % humidity for 4 days. On day 4, 0.5 μCi of [^{3}H]thymidine is added to each well during the last 6 h of culture. Cultures are harvested onto glass-fiber mats using a 96-well harvester. [^{3}H]Thymidine uptake is measured by direct β-counting using a Matrix 9600 β-counter (Packard, Meriden, CT).

Inhibition of IL-2 Production in Human Whole Blood and PBMC

Fifty milliliters of venous blood from normal donors is drawn into tubes containing sodium heparin. Twenty-five milliliters of blood is used directly. PBMC are isolated from the remaining sample as described above and cultured at 1 × 10^6

cells per ml with supplemented RPMI-1640. Whole blood or PBMC are induced to secrete IL-2 with 50 ng PMA per ml plus 1 μg ionomycin per ml. Immunosuppressive potency of test compound versus standard is determined by measuring the inhibition of IL-2 secretion. Assays are performed in 96-well flat-bottom plates in a volume of 210 μl, which includes 190 μl of whole blood or PBMC (1×10^6 cells per ml), 10 μl of PMA (1 μg/ml) and ionomycin (20 μg/ml) mixture, and 10 μl of serially diluted test compound. Plasma or tissue culture supernatants are collected 24 h later and IL-2 concentrations determined by ELISA.

Porcine MLR Assay

Fifty milliliters of sodium-heparinized venous blood is drawn from pigs and diluted with an equal volume of 0.9 % saline. PBMC are isolated by density centrifugation for preparation of responder and stimulator cells as described above. MLR are performed as described for human MLR using 100 μl of responder cells (2×10^6 cells per ml) and 100 μl of stimulator cells ($0.5–4 \times 10^6$ cells per ml).

Rat MLR Assay

Popliteal, inguinal, and mesenteric lymph nodes from newly sacrificed Lewis rats and spleens from brown Norway rats are aseptically removed. Single-cell suspensions of splenocytes and lymphocytes are prepared using forceps and a hemostat to macerate the tissues. Red blood cells in the splenocyte suspension are lysed by 2 min incubation at ambient temperature in red blood cell lysis buffer containing 0.14 M NH_4Cl in 0.0167 M Tris–HCl, pH 7.2. Responder cells from Lewis rat lymph nodes and stimulator cells from brown Norway rat spleens are washed three times in RPMI-1640 medium and then sedimented at 400 *g* for 10 min. Stimulator cells are prepared as for human, and the assay is conducted in serum-free AIM-V medium supplemented with 1 % antibiotic–antimycotic solution and 50 μM 2-mercaptoethanol, using an optimized cell ratio of 100 μl of responder cells (2×10^6 cells per ml) and 100 μl of stimulator cells ($1–2 \times 10^6$ cells per ml) and an incubation period of 4 days.

Mouse MLR Assay

Spleens are aseptically removed from newly sacrificed $C_3H(C_3H/HeNCrIBR)$ and BALB/c mice. Splenocytes are prepared and the assay conducted as for rat, using an optimized cell ratio of 100 μl of responder cells (4×10^6 cells per ml) and 100 μl of stimulator cells (1×10^6 cells per ml).

Concanavalin A-Induced Guinea Pig Lymphocyte Proliferation Assay

Spleens are aseptically removed from newly sacrificed guinea pigs. Single-cell suspensions of splenocytes are prepared as for rat using 4 min incubation in lysis buffer to remove red blood cells. Splenocytes are washed three times in RPMI-1640 medium and adjusted to 6.25×10^5 cells per ml in culture medium consisting of RPMI-1640 supplemented as described for human MLR. Concanavalin A-induced proliferation reactions are performed in a volume of 200 μl, containing 20 μl of culture medium with or without test compound or standard, 160 μl of guinea pig splenocytes, and 20 μl concanavalin A (20 μg/ml). Cultures are incubated for 3 days, pulse labeled on the last day with [^{3}H]thymidine, and harvested and counted as described.

Inhibition of Cytokine Secretion

Serially diluted test compound or standard is added to 96-well plates with PBMC in RPMI-1640 medium at 1×10^6 cells per ml. PMA and ionomycin are added to effect 10 ng per ml and 500 ng per ml concentrations, respectively. Supernatants from these cultures are collected 24 h later after centrifugation and stored at –80 °C until use. Concentrations of IL-2 and IFN-γ in the supernatants are determined by ELISA. For assessing inhibition of IL-4 and IL-5 secretion in T cells by the test compound, $CD4^+$ cells are isolated from PBMC using a T cell subset enrichment column (R&D Systems, Minneapolis, MN). Purified $CD4^+$ cells are cultured with serially diluted test compound and PMA at 10 ng per ml in plates previously coated with 100 ng per ml solution of anti-CD3 antibody (Immunotech, Westbrook, ME). Supernatants are collected after

centrifugation 40 h later. IL-4 and IL-5 concentrations in the supernatants are determined by ELISA.

Evaluation

Dose–response curves for test compound and standard are established and IC_{50} values for inhibition calculated.

Modifications of the Method

Gillitzer et al. (1996) studied neutrophil migration in psoriatic lesions as a model for neutrophil chemotaxis.

Kunstfeld et al. (1997) investigated the migration of inflammatory T cells from psoriasis through superficial vascular plexus and through deep vascular plexus endothelium. Superficial and deep plexus human skin was placed separately into SCID mice.

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Psoriasis Models in Normal Animals

Mouse Tail Model for Psoriasis

Purpose and Rationale

The mouse-tail test was introduced by Jarrett and Spearman (1964). The model is based on the induction of orthokeratosis in those parts of the adult mouse tail, which have normally a parakeratotic differentiation. Quantification of orthokeratosis was achieved by morphometric evaluation of the scales with the aid of a

semiautomatic evaluation unit (Bosman et al. 1992; Bosman 1994; Sebök et al. 1996).

Procedure

Male albino NMRI mice weighing 25–27 g are used.

The tails are treated locally with 0.1 ml ointment applied to the proximal part of the tail. For the contact time of 2 h, a plastic cylinder is slipped over the tail and fixed with adhesive tape. At the end of contact time, the cylinders are removed and the tails washed. Animals are treated once daily, five times a week, for 2 weeks. Five to eight animals are used per dosage group. Two hours after the last treatment, the animals are sacrificed and the tails prepared histologically (fixation in 4 % formalin, paraplastic embedding). Longitudinal sections of about 5 μm thickness are prepared and stained with hematoxylin–eosin.

The epidermal thickness is measured as the distance between the dermoepidermal borderline and the beginning of the horny layer. Five measurements per animal are made in each of 10 scales. Out of these 50 measurements, the mean for the individual animals is calculated.

The sections are examined for the presence of a granular layer or isolated granular cells induced in the previously parakeratotic skin areas (10 sequential scales per animal). The measurements are carried out at the border of the scale with a semiautomatic image evaluation unit (VIDS III, AI TEKTRON). The distances are obtained in pixels (1 pixel = 1.2120 μm).

Quantitative values of orthokeratosis are obtained by measuring the length of the granular layer per scale (A) and the whole scale length (B). The whole scale length is defined as the length of the scale lying between two adjacent hair follicles, beginning and ending at the turning point between hair follicle and scale. Percent keratosis is calculated by the formula:

$$(A/B) \times 100.$$

Evaluation

Ten sequential scales per animal are measured and the results given in % orthokeratosis per scale. Five to eight animals are taken for one drug concentration or control group. Thus, 50–80 individual orthokeratosis values are obtained per test group. Mean and standard error of the mean are calculated per animal and per group. From the individual orthokeratosis values per dosage group (50–80 scales), a frequency distribution is constructed. Therefore, the values (ranging from 0 to 100 % orthokeratosis) are grouped into classes with a constant class interval of 10 % (class 1, 0–10 %; class 2, 10.1–20 %; class 10, 90.1–100 % orthokeratosis). The frequency per class is calculated in %:

$$\text{Class frequency} = \frac{\text{No.of scales in the class}}{\text{Total no. of scales}} \times 100.$$

For every class the cumulative frequency is constructed by adding the frequencies of all foregoing classes.

Due to a non-Gaussian distribution of the orthokeratotic values (100 % is the maximal effect), the Mann–Whitney U-test is used.

The efficacy of test compounds on epidermal differentiation is calculated from the mean length of orthokeratosis after treatment with the substrate (Ok_s) and with salicylic acid as control (Ok_c) using the formula:

$$(Ok_s - Ok_c)/(100 - Ok_c) \times 100.$$

Modifications of the Method

Nagano et al. (1990) studied the effect of tumor necrosis factor in the mouse-tail model of psoriasis.

Beyaert et al. (1992) induced a psoriasiform inflammatory reaction in mice by subcutaneous injection of a combination of tumor necrosis factor and lithium chloride.

Sebök et al. (2000) used the mouse-tail test to compare tazarotene, a receptor-specific retinoid, with the classical topical antipsoriatic compound dithranol.

Worm et al. (2001) studied the effects of retinoids on in vitro and in vivo IgE production in ovalbumin-sensitized BALB/c mice.

Several studies were performed in **guinea pigs** (Miller and Ziboh 1990; Kumar et al. 1992; Mani et al. 1999). Tuzun et al. (1993) reported psoriasis lesions in guinea pigs receiving propranolol; however, their results could not be confirmed by Wolf et al. (1994).

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Rat Ultraviolet Ray B Photodermatitis Model for Psoriasis

Purpose and Rationale

Rat ultraviolet ray B photodermatitis has been proposed as an experimental model of psoriasis vulgaris by Nagakuma et al. (1995).

Procedure

Male Wistar rats weighing around 300 g are used. Hair on the dorsal skin is clipped and carefully shaved. An area (1.5 × 2.5 cm) on one side of the flank is irradiated for 15 min (1.5 J/cm^2) at a vertical distance of 20 cm with UVB lamps. A biphasic erythema is observed. Immediately after irradiation, initial faint erythema appears, disappearing within 30 min. The second phase of erythema starts 6 h after the irradiation and gradually increases, peaking between 24 and 48 h. The color is brownish-red, and the reaction is confined to the exposed area with a sharp boundary. By 48–72 h after irradiation, dark-brown scale is formed on the erythematous lesion. Pieces of the scale are relatively thick. The scale separates and the erythema decreases daily. The skin sites return to normal about 10 days after irradiation.

The irradiated rats are sacrificed after various time intervals by decapitation under ether anesthesia. Skin biopsies are taken immediately, fixed in 10 % formalin, and embedded in paraffin. Tissue sections (4 μm thick) are stained with hematoxylin and eosin. The numbers of the keratinocyte layers, including the basal layer, are counted by direct microscopy.

For DNA labeling (Morimoto et al. 1991), 20 mg/kg 5-bromo-2-deoxyuridine (BrdU) is administered i.p. 8 h before decapitation. Frozen tissue sections (8 μm) are prepared with a cryostat. The sections are then fixed in acetone for 10 min, 4 % paraformaldehyde containing 1 % $CaCl_2$ (pH 7.0) for 10 min, and 1 % glutaraldehyde for 5 min. After fixation, sections are digested with 0.006 % pepsin in 10 mM HCl for 10 min at 37 °C and incubated in 4 M HCl for 30 min at ambient temperature to denature DNA. They are then incubated with anti-BrdU mouse monoclonal antibody and then with alkaline phosphatase-conjugated anti-mouse IgG sheep IgG antibody. Antibody-binding sites are visualized with naphthol and fast red at pH 9 in the dark. The numbers of labeled as well as unlabeled cells at the epidermal basal layer or outer root sheath cell layers of the hair follicles are counted in five high-power fields to a total of at least 100 cells. The intensity of BrdU incorporation is demonstrated as a labeling index (%) with the following formula:

$$\text{Labeling index} = \frac{\text{No. of labelled cells}}{\text{Total cell no. counted}} \times 100$$

Evaluation

Chronological changes of thickness of keratinocytes at the epidermis and the hair follicles as well as of DNA labeling indices are plotted as time-response curves.

Modifications of the Method

Kippenberger et al. (2001) found that activators of peroxisome proliferator-activated receptors (PPARs) protect human skin from ultraviolet-B-light-induced inflammation. In an in vitro model with human keratinocytes, inflammation was mimicked by irradiation with ultraviolet light. Activators of PPARα were shown to reverse ultraviolet-B-light-mediated expression of inflammatory cytokines.

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Hyperproliferative Epidermis in Hairless Mice

Purpose and Rationale

Epidermal differentiation and barrier development in vivo are influenced by nuclear hormone receptors (Hanley et al. 1997, 1998, 1999; Kömüves et al. 1998; Rosenfield et al. 2000; Smith et al. 2001a, b; Kuenzli and Saurat 2003a, b). Kömüves et al. (2000) studied the effect of PPARα activators on keratinocyte differentiation in hyperproliferative epidermis in hairless mice.

Procedure

Subacute epidermal hyperproliferation is induced by repeated barrier abrogation with acetone treatment (Denda et al. 1996). Animals are treated twice a day with acetone until the transepidermal water loss reaches 8–10 mg per cm^2 per h (measured on four to five different spots) as determined by an electrolyte water analyzer.

Chronic epidermal hyperproliferation due to essential fatty acids deficiency is achieved by feeding an isocaloric diet free of essential fatty acids after weaning for 2–3 months (Man et al. 1993).

To test the effect of PPARα activators, mice are treated on one flank side with solutions of the drug dissolved in propylene glycol/ethanol (3/7) twice a day for 3 days. After sacrifice, tissues are collected for histology, in situ hybridization, or immunohistochemistry.

Evaluation

Data are presented as mean ± SEM. Statistical differences are determined using Student's *t*-test.

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Psoriasiform Skin Diseases in Spontaneous Mice Mutations

Purpose and Rationale

Nearly 100 mouse mutations have been described as causing some type of abnormality of the skin or hair. These include "asebia," a mildly hyperkeratotic disorder with sebaceous gland hyperplasia; "ichthyosis," an example of abnormal hair growth associated with hyperkeratosis; "rhino" and "hairless," two related examples of congenital follicular malformations; and "flaky skin," a potential animal model of eruptive psoriasis.

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Flaky Skin (*fsn*) Mouse

Purpose and Rationale

Flaky skin (*fsn*) is an autosomal recessive mouse mutation that causes pathologic changes in the skin, yielding a papulosquamous disease

resembling human psoriasis (Sundberg et al. 1993). In addition, this mutation, assigned to distal chromosome 17, causes anemia and gastric forestomach hyperplasia (Beamer et al. 1995).

The *fsn* mutation arose spontaneously in the A/J inbred strain at The Jackson Laboratory (Beamer et al. 1986). Psoriasiform skin lesions are first evident as focal epidermal hyperplasia and inflammation at 2 weeks of age. These lesions become confluent and diffuse by 3–4 weeks of age and are associated with marked dermal infiltration of lymphocytes and small numbers of neutrophils and macrophages. Mast cell numbers increase significantly in the dermis from 2 weeks of age onward. Diffuse dermal neovascularization accompanies these cutaneous changes (Sundberg et al. 1997). Systemic lesions include progressive and massive papillomatosis of the stratified squamous epithelium of the forestomach, hyperplasia and dysplasia of the glandular stomach, increased apoptosis of cecal enterocytes, renal glomerulopathy associated with inflammatory cell infiltrates and fibrosis around the portal triads in the liver, splenomegaly due to massive erythropoiesis, and granulomatous lymphadenitis.

Scanning microscope examination (Morita et al. 1995) reveals a greatly thickened epidermis and sparsity of hairs and scale accumulations in the epidermal surface. Hair shafts have conspicuous pits, striations, and exophytic protrusions. Nails are bent at a 90° angle with surface irregularities and accumulation of scale at the nail base. Transmission electron microscopic examination shows increased epidermal thickness, mitochondrial aberrations, and intraepidermal invasion by neutrophils. Keratin abnormalities are detected using immunocytochemical staining for profilaggrin. At the dermal–epidermal junction, numerous macrophages and mast cells are seen in close proximity to focal dissolutions of the basement membrane. A high density of collagen fibers and cellular infiltrates is evident in the papillary dermis.

Besides lymphadenopathy and mast cell accumulation, elevated serum IgE levels (>7,000-fold increase compared with normal littermates) and autoimmunity (evidenced by glomerulonephritis with immune complex deposition in the kidneys) are observed in flaky skin mutant mice (Pelsue et al. 1998).

Peripheral lymph nodes of adult mutant (*fsn*/*fsn*) mice were found to contain almost tenfold more leukocytes than peripheral lymph nodes from phenotypically normal littermates. Analysis of peripheral lymph node cells using mAbs and flow cytometry revealed that this predominantly lymphoid hyperplasia is characterized by approximately equivalent increases of $CD3^+$ T cells and $C19^+$ B cells (Abernethy et al. 2000a). A dysregulated expression of CD69 and IL-2 receptor alpha and beta chains was found on $CD8^+$ T lymphocytes (Abernethy et al. 2000b). Expression and function of IL-1 beta is increased in psoriasiform skin lesions of flaky skin (*fsn*/*fsn*) mice (Schön et al. 2001).

However, neutrophils also played a critical role for the generation of psoriasiform skin lesions in flaky skin mice (Schön et al. 2000). Intraperitoneal injection of the neutrophil-depleting RB6–85C monoclonal antibody resulted in a dramatic reduction of the epidermal thickness.

Similar to active human psoriatic lesions, an increase of epidermal growth factor receptors was observed in *fsn*/*fsn* mice (Nanney et al. 1996).

Sundberg et al. (1994a) transplanted full-thickness skin grafts from flaky skin mice on the dorsal skin of genetically athymic nude (*nu*/*nu*) mice. The grafts maintained the psoriasiform phenotype of the donors.

Backcrosses to different mouse strains suggested several modifier genes affecting the *fsn* phenotype (Sundberg et al. 1994b). As cyclosporine A, in contrast to glucocorticoids, was not effective when used for topical or systemic treatment of *fsn* lesions, it seems that there is no immunologic basis for these lesions. Therefore, it remains uncertain whether the flaky skin mouse can be used to test potential therapeutic compounds.

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Asebia (*ab*/*ab*) Mouse

Purpose and Rationale

The asebia mouse was first described by Gates and Kasarek (1965) as hereditary absence of sebaceous glands. The asebia mouse represents a spontaneous mutation in BALB/c mice leading to hyperplasia of the epidermis and chronic inflammatory dermal changes, including enhanced cellularity, edema, and elevated mast cell numbers (Josefowicz and Hardy 1978; Brown and Hardy 1988). The circadian rhythms in cell proliferation are suppressed in the chronically hyperproliferative epidermis of the asebia mouse (Brown et al. 1988a). UVB radiation and anthralin and tar with UVB further stimulate proliferation in the already hyperproliferative epidermis of the asebia mouse (Brown et al. 1988b, 1989). The gene for the enzyme stearoyl-CoA desaturase 1, which is expressed in sebaceous glands, is disrupted in the asebia mouse (Parimoo et al. 1999; Zheng et al. 1999; Miyazaki et al. 2000). Besides Sdc1 and Sdc2, Zheng et al. (2001) identified Scd3 – a novel gene of the stearoyl-CoA desaturase family with restricted expression in the skin. Since T cell and neutrophil infiltrates are not observed in asebia mice, a pathogenesis distinct from psoriasis has been suggested (Schön 1999).

Asebia-2J (Scd1(ab2J)), a new allele and a model for scarring alopecia, was described by Sundberg et al. (2000).

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Psoriasiform Skin Diseases in Genetically Modified Animals

Purpose and Rationale

Based on expression in inflammatory skin disorders, several factors, such as cytokines, are considered to play a crucial role in the pathogenesis of psoriasis and other skin diseases. Transgenic animals are tools to analyze these factors (Meyer 1990; Rothnagel et al. 1990; Sellheyer 1996; Rosenberg et al. 1999).

Bullard et al. (1996) described a polygenic mouse model of psoriasiform skin disease in **CD18-deficient mice**. CD18 deficiency in patients results in recurrent microbial infections, leukocytosis, impaired wound healing, failure of granulocyte emigration, and lack of pus formation (Anderson et al. 1995). A hypomorphic mutation for CD18 was introduced by Wilson et al. (1993) into mice with homozygotes displaying mild leukocytosis, an impaired response to chemically induced peritonitis, and delays in transplantation rejection. When this CD18 mutation was crossed back onto PL/J strain of mice, the development of an inflammatory skin disorder was observed (Bullard et al. 1996). The disease is characterized by erythema, hair loss, and the development of crusts. The histopathology reveals hyperplasia of the epidermis, subcorneal microabscesses, orthohyperkeratosis, parakeratosis, and lymphocyte exocytosis similar to human psoriasis and other hyperproliferative skin disorders. The dermatitis rapidly resolved after subcutaneous administration of dexamethasone.

Dermal infiltrates of macrophages/monocytes within the dermis of clinically uninvolved skin were seen in transgenic mice with epidermal overexpression of K14/IL-1α, suggesting a role of IL-1α for macrophage attraction (Groves

et al. 1995). In severely affected animals, inflammation reactions occur that are characterized by a mixed inflammatory infiltrate and by acanthosis and parakeratosis. **IL-1α transgenic mice** support a primary role of IL-1α as an inducer of cutaneous inflammation, which may be helpful for clarifying pathogenesis of psoriasis.

Wilson et al. (1990) reported that expression of the **BNLF-1 oncogene** of Epstein–Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6.

Cook et al. (1997, 1999) found that overexpression of the heparin-binding EGF-related ligand amphiregulin in the epidermis of transgenic mice induces a psoriasis-like cutaneous phenotype. **Transgenic mice with a K14 enhancer/promoter-driven amphiregulin gene** targeted to the epidermis displayed a macroscopic phenotype that included extensive areas of scaling and erythematous skin with marked alopecia. Histological examination revealed hyperkeratosis, focal parakeratosis, acanthosis, mixed leukocytic infiltration that included both CD3-positive T cells in the dermis and epidermis, and a tortuous vasculature.

Two completely opposite phenotypes were observed in **mice expressing K10/BMP-6** (bone morphogenetic protein-6, a member of the TGF-β superfamily) within the epidermis (Blessing et al. 1996). Whereas keratinocyte proliferation was severely reduced in animals with strong and homogenous expression of the transgene, weaker and patchy expression led to marked hyperproliferation.

Rodriguez-Villanueva et al. (1998) reported that **human keratin-1.bcl-2 mice** aberrantly express keratin 6, exhibit reduced sensitivity to keratinocyte cell death induction, and are sensible to skin tumor formation.

The concept of vascular endothelial growth factor (VEGF) being an important angiogenic factor in psoriatic skin (Detmar et al. 1994) was supported by **transgenic mice with constitutive epidermal K14/VEGF expression**. These animals exhibited dilated and contorted dermal microvessels (Detmar et al. 1998). There was also an increased number of dermal mast cells, and leukocyte adhesion and extravasation was enhanced in VEGF transgenic mice. The transgenic delivery of VEGF to mouse skin results in a profound inflammatory condition with many of the cellular and molecular features of psoriasis, including the characteristic vascular changes, epidermal alterations, and inflammatory infiltrates. These symptoms can be effectively reversed by the addition of VEGF Trap, a potent VEGF antagonist (Xia et al. 2003). VEGF-A also promoted lymphatic vessel proliferation and enlargement. Combined systemic treatment with blocking antibodies against VEGF receptor-1 (VEGFR-1) and VEGFR-2 potently inhibited inflammation and also decreased lymphatic vessel size (Kunstfeld et al. 2004).

Klement et al. (1996) found that **IκBα deficiency** results in a sustained NF-κB response and widespread dermatitis in mice.

Seitz et al. (1998) reported that alterations on NF-κB function in transgenic epithelial tissue demonstrate a growth inhibitory role for **NF-κB**.

To determine the role of κB-α-deficient immunocytes in the pathogenesis of skin disease in adult mice, Chen et al. (2000) utilized the RAG2-deficient blastocyst complementation system to generate **RAG2–/–, IκB-α–/– chimeras**. These animals display a psoriasiform dermatitis characterized by hyperplastic epidermal keratinocytes and dermal infiltration of immunocytes, including lymphocytes. Skin grafts transferred from diseased chimeras to recipient nude mice produce hyperproliferative epidermal keratinocytes in response to stimulation.

Transgenic mice overexpressing suprabasal integrins α_2, α_5, and β_1 demonstrate a phenotype similar to psoriasis with cycles of flaking and inflamed skin, suggesting that disrupted keratinocyte integrin-ligand interactions may play a role in hyperproliferative states (Carroll et al. 1995).

Robles et al. (1996) found that **expression of cyclin D1 in epithelial tissue** of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia.

Carroll et al. (1997) used the **involucrin promoter to overexpress** IFN_γ in the suprabasal layers of transgenic mouse epidermis. The mice exhibited striking hypopigmentation of the hair

due to reduction of DOPA-positive melanocytes. Severely affected mice had reddened skin, growth retardation, hair loss, and flaky skin lesions. The skin was characterized by a dermal infiltrate of T lymphocytes and macrophages/monocytes.

When **scid/scid mice were reconstituted with MHC-matched, but minor histocompatibility mismatched $CD4^+/CD45RB^{hi}$T lymphocytes**, almost all of the animals developed skin lesions similar to human psoriasis within 4–8 weeks after transfer (Schön et al. 1997). The psoriasiform skin lesions did not develop in recipients of unfractionated splenocytes or $CD4^+/CD45RB^{lo}$ T cells, indicating that T cell dysregulation is the primary pathogenic factor in this model. When recipients of $CD4^+/CD45RB^{hi}$ T cells were treated with either cyclosporine A or UVB irradiation, the psoriasiform lesions were dramatically improved demonstrating that immunosuppressive therapies are efficacious. The model was proposed as a T cell-initiated murine model of inflammatory skin lesions (Schön and Parker 1997).

Mann et al. (1993) found that mice with a **null mutation of the TGF-a gene** have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation.

Turksen et al. (1992) described psoriasiform changes in the skin of transgenic mice with **overexpression of interleukin 6**.

Guo et al. (1993) reported that targeting expression of **keratinocyte growth factor** to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice.

Hong et al. (1999) showed that IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like disorder. Only a few *scid/scid* mice develop skin lesions when $CD4^+/CD45RB^{hi}$ are transferred alone. Coadministration of LPS plus IL-12 or staphylococcal enterotoxin B in *scid/scid* mice one day after **$CD4^+/CD45RB^{hi}$cell transfer** greatly enhances disease penetrance and severity.

Schön et al. (2000) reported a cutaneous inflammatory disorder in **integrin alpha E (CD103)-deficient mice**. Skin inflammation correlated with alpha E deficiency in mice with a mixed 129/Sv × BALB/c background.

Transgenic rats expressing human HLA-B27 and β_2-microglobulin develop psoriasiform skin changes as part of a multiorgan inflammatory disease (Hammer et al. 1990). In the most severely affected lines, psoriasiform lesions first occurred at about 20 weeks of life and were observed in 10–80 % of animals. Male appeared to be more prone to skin changes than females (Taurog et al. 1993, 1994, 1999). Similar to human pathogenesis, T cells seem to be the most important factor in HLA-B27 transgenic rats (Breban et al. 1996).

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Xenotransplantation of Human Psoriatic Skin

Purpose and Rationale

Xenotransplantation models for psoriasis involve the transfer of human psoriatic skin to animals. First studies were performed by transplantation of involved psoriatic and non-psoriatic human skin onto congenitally athymic (nude) mice (Krueger et al. 1975, 1985; Baker et al. 1992). More recently, human psoriatic skin grafts were transplanted onto SCID mice (Nickoloff et al. 1995; Boehncke et al. 1994, 1997, 1999; Gilhar et al. 1997; Sugai et al. 1998; Dam et al. 1999; Raychaudhuri et al. 2001) allowing screening of antipsoriatic drugs.

Procedure

Keratome biopsies ($7 \times 2 \times 0.05$ cm containing both dermis and epidermis) are obtained from clinically symptomless skin of patients with psoriasis or from psoriatic plaques. Prior to the procedure, the skin is defined and infiltrated with 1 % lidocaine and epinephrine 1:200. The full-thickness skin biopsy is dissected into 12 grafts. CB-17 SCID mice at an age of 6–8 weeks are anesthetized with an i.p. injection of 1.56 mg phenobarbital before transplantation of the human xenografts on the flank area. The grafts are sutured with absorbable 6–0 Vicryl Rapide suture and covered with Xeroform dressings for 1 week. Animals transplanted with the psoriatic plaque are then randomized into groups receiving PBS as negative controls or 0.15 mg cyclosporine A or the test compound intradermally into the xenografts. Within 4 weeks, the animals are sacrificed by CO_2 asphyxiation and 4 mm punch biopsies are obtained from each xenograft. Biopsies are fixed in 10 % neutral-buffered formalin for paraffin embedding.

Evaluation

A semiquantitative scale is used to indicate the extent of new vessel formation (angiogenesis) that can be seen between the xenograft and the underlying fascia muscularis. A calibrated eyepiece microscope is used for estimating the epidermal thickness in the vertical sections. Statistical

differences are calculated using Student's *t*-test for multiple comparisons.

Modifications of the Method

Using this model, Raychaudhuri et al. (2004) found that a high-affinity nerve growth factor receptor blocker improves psoriasis.

Nickoloff et al. (1995) studied severe combined immunodeficiency mouse and human psoriatic skin chimeras. Autologous blood-derived $CD4^+$ cells injected into symptomless transplanted psoriatic skin engrafted onto SCID mice produced full-fledged psoriatic lesions (Wrone-Smith and Nickoloff 1996; Sugai et al. 1998; Nickoloff and Wrone-Smith 1999; Nickoloff et al. 1999; Nickoloff 2000).

Yamamoto et al. (1998) studied the effects of superantigen-driven peripheral blood mononuclear cells on the persistence of psoriasiform epidermis and on the cytokine gene expression of grafted psoriatic skin. Staphylococcal enterotoxin B-stimulated peripheral blood mononuclear cells from psoriatic patients were repeatedly injected under the grafted full-thickness involved psoriatic skin onto severe combined immunodeficient mice. A persistence of the psoriasiform epidermis was found after 5 weeks. E-selectin expression was observed on endothelial cells in the upper epidermis of the mice.

Tissue specificity of E- and P-selectin ligands in Th1-mediated chronic inflammation was studied by Chu et al. (1999).

Zeigler et al. (2001) used the human psoriatic skin-SCID mouse transplant model to compare the effect of antibody to CD11a with ciclosporin and clobetasol propionate.

Zollner et al. (2002) used a SCID-hu model and found that proteasome inhibition reduces superantigen-mediated T cell activation and the severity of psoriasis.

Boyman et al. (2004) presented an animal model in which skin lesions developed spontaneously when symptomless prepsoriatic human skin was engrafted onto AGR129 mice, deficient in type I and type II interferon receptors, and for the recombination activating gene 2. Upon engraftment, resident human T cells in prepsoriatic skin underwent local proliferation.

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Scleroderma Models

Howard Maibach

Contents

Scleroderma Models

Scleroderma Models in Chicken

Scleroderma is an autoimmune disorder which occurs in the severe systemic form, a localized scleroderma (morphea) and as lichen sclerosus et atrophicus. Chickens of the University of California line 200 (**UCD-200 chickens**) developed an inherited inflammatory fibrotic disease, closely resembling human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Boyd et al. 1991; Gruschwitz et al. 1991, 1993; Herold et al. 1992; Needleman 1992; Brezinschek et al. 1993; Ausserlechner et al. 1997; Nguyen et al. 2000). An acute inflammatory stage started at an age of about 60 days after hatching, leading to fibrosis with fast progression with severe lymphocytic infiltration and excessive accumulation of collagen in skin and internal organs. A sequential increase of type VI, type I, and type II procollagen transcripts and a progressive increase of autoantibodies to histone, to ssDNA, and to dsDNA were found.

A further strain of chickens with progressive systemic sclerosis (UCD 206) has been identified (Duncan et al. 1995; Sgonc et al. 1995).

Worda et al. (2003) analyzed the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay.

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Scleroderma Models in Mice

Jimenez and Christner (2002) gave a survey on murine animal models of systemic sclerosis.

The **tight-skin mouse** is regarded as an experimental model for scleroderma (Walker et al. 1990; Muryoi et al. 1992; Delany and Brinkerhoff 1993; Kasturi et al. 1993, 1994; Wallace et al. 1994; Pablos et al. 1995; Frondoza et al. 1996). This mutant mouse develops autoantigens specific for scleroderma target tissues. The tight-skin disease was first discovered in 1967 at the Jackson Laboratory in the B10.D2 (58 N)/Sn mouse strain. The disease occurred spontaneously. Green et al. (1976) have identified the tsk locus and shown it to be linked to pallid locus. The tight-skin character is transmitted as an autosomal dominant trait and homozygous tsk/tsk mice die in utero. The striking feature of the disease is the presence of thickened skin, firmly bound to subcutaneous and deep muscular tissue, with excessive accumulation of collagen in the skin and internal organs (Menton et al. 1978;

Osborn et al. 1983). Synthesis and accumulation of type I collagen is markedly increased (Jimenez et al. 1986; Ong et al. 1998). Biochemical studies have shown that the prolyl hydroxylase and glycosaminoglycan content of the affected tissue is increased (Ross et al. 1987).

Dermal fibrosis in the tight-skin mouse was reduced after local application of halofuginone, an inhibitor of collagen type I synthesis (Pines et al. 2001).

Additionally, the **tight-skin 2 mouse** has been described as an animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration (Christner et al. 1995, 1996; Wooley et al. 1998; Sgonc et al. 1999).

Bleomycin-induced scleroderma in genetically mast cell-deficient **WBB6F1-W/WV mice** was described as an animal model of sclerotic skin (Yamamoto et al. 1999a, b, c, d, 2000; Yamamoto and Nishioka 2001, 2002; Yamamoto 2002).

Murine sclerodermatous graft-versus-host disease is considered as a model for human scleroderma (Claman 1990; Schiltz et al. 1994; McCormick et al. 1999; Zhang et al. 2002, 2003; Atamas and White 2003). Fibrosis in sclerodermatous graft-versus-host disease is driven by infiltrating TGF-β_1-producing mononuclear cells. Zhang et al. (2002) characterized the origin and types of cutaneous effector cells, the cytokine and chemokine environments, and the effects of anti-TGF-β antibodies on skin fibrosis, immune cell activation markers, and collagen and cytokine synthesis.

Dong et al. (2002) transplanted the skin from scleroderma patients to SCID mice and investigated the skin xenografts for factors downstream of TGF-β. Deficient expression of Smad7 was found as a putative molecular defect in scleroderma.

A syndrome resembling human systemic sclerosis (scleroderma) in **MRL/lpr mice lacking interferon receptor** was described by Le Hir et al. (1999).

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Pemphigus Models

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Contents

Pemphigus Models

Experimentally Induced Pemphigus in Mice

Purpose and Rationale

Pemphigus is defined as a group of spontaneously occurring disorders of skin and mucosae in which blisters and erosions form within the epidermis due to loss of intercellular contact (Marks 1987). Pemphigus occurs also spontaneously in domestic animals, such as dogs, cats, and horses (Hurvitz 1980; Scott et al. 1983; Sueki et al. 1997). Various attempts were made to study experimentally induced pemphigus in mice (Buschard et al. 1981; Takahashi et al. 1985; Anhalt et al. 1986a, b; Rock et al. 1990; Juhasz et al. 1993; Allbritton et al. 1997; Koch et al. 1997; Fan et al. 1999; Mahoney et al. 1999; Amagai et al. 2000; Zillikens et al. 2001, Rädisch et al. 2002; Hashimoto 2003).

Fan et al. (1999) screened four strains of female mice (BALB/c, DBA/1, SJL/J, and HRS/J) for their ability to produce pathogenic anti-desmoglein 3 antibodies. Only BLB/c mice immunized with full-length desmoglein 3 can produce pathogenic antibodies capable of causing acantholysis in human foreskin in culture and blistering in neonatal mice.

Procedure

Six- to 8-week-old female BALB/c, DBA/1, SJL/J, and HRS/J are immunized four times with

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20 μg/mouse of purified desmoglein 3 protein in CFA (on days 1, 10, 20, and 30), four times with 20 μg/mouse of extracellular domain of desmoglein 3 (on days 40, 50, 60, and 70), and twice with 20 μg/mouse of refolded desmoglein 3 (on days 80 and 90), and then they are boosted twice more with 20 μg/mouse of extracellular domain of desmoglein 3 in IFA by i.p. inoculation (on days 97 and 104). Control groups of mice are similarly immunized with BSA.

IGs from 10 ml of pooled serum from mice immunized with desmoglein 3 are precipitated with 40 % ammonium sulfate, dialyzed against PBS twice, lyophilized, and reconstituted in water to 1 ml. One hundred μl of the reconstituted antibodies/mouse is injected into neonatal BALB/c mice.

Evaluation

Mice are examined 18–24 h postinjection for blister formation. Cross sections containing the blister and comparable areas in control animals are biopsied and frozen sections are prepared for routine histological examination.

Modifications of the Method

Amagai et al. (2000) and Ohyama et al. (2002) developed an active autoimmune disease model for pemphigus using autoantigen-knockout mice.

Xu et al. (2001) characterized BALB/c mice B lymphocyte autoimmune responses to skin basement membrane component type XVII collagen, the target antigen of the autoimmune skin disease bullous pemphigoid. The mice were immunized with peptides of the human and/or the murine-equivalent BPAg2 pathogenic NC16A domain.

Using keratinocytes from plakoglobin-knockout mice, Caldelari et al. (2001) studied the role of the Armadillo protein plakoglobin in pemphigus vulgaris.

Nguyen et al. (2004) reported that pemphigus vulgaris IgG and methylprednisolone exhibit reciprocal effects on keratinocytes. The therapeutic effects of methylprednisolone may be due to both the upregulated synthesis and the posttranslational modifications of keratinocyte adhesion molecules.

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Ichthyosis Models

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Contents

Ichthyosis Vulgaris Models

Experimentally Induced Ichthyosis in Mice

Purpose and Rationale

Ichthyosis vulgaris is a heterogeneous autosomal skin disease characterized by dry, scaly skin, mild hyperkeratosis, and a decreased or absent granular layer that either lacks or contains morphologically abnormal, keratohyalin granula (Anton-Lamprecht and Hofbauer 1972). Both the skin of ichthyosis vulgaris patients and keratinocytes cultured from affected individuals exhibit reduced or absent profilaggrin mRNA and protein levels (Sybert et al. 1985). The symptoms and the genetics of the ichthyotic (*ic*/*ic*) mouse were described by Spearman (1960), Green et al. (1974), Jensen and Esterly (1977), and Holbrook (1989). Presland et al. (2000) demonstrated loss of normal profilaggrin and filaggrin in flaky tail (*ft*/*ft*) mice and proposed this as an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris.

Elias et al. (1983) and Chung et al. (1984) induced ichthyosis in the hairless mouse by treatment with diazacholesterol and used this as an assay for comparative potency of topical retinoids.

Procedure

Male hairless mice, 2–3 months old, are fed either a normal laboratory diet supplemented with

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60 mg/kg/day of 20,25-diazacholesterol or normal laboratory diet. Diazacholesterol blocks the conversion of Δ^{24}-reduction of desmosterol to cholesterol (Anderson and Martt 1965), and as a result, desmosterol accumulates in the stratum corneum as lipids rather than as cholesterol. Ichthyotic changes generally become apparent after 8–12 weeks and are most pronounced over the back and tail. With the exception of some reduction in body weight in comparison with controls, the animals appear healthy. Since the tail manifests the most exaggerated scaling, this site is used for topical drug applications. As the animals become ichthyotic, the daily dose of diazacholesterol can be lowered to 30 mg/kg and maintained at that level.

The test substances (retinoids) are first solubilized in a small volume of dimethyl sulfoxide and then dissolved in Cremophor EL. A volume of ~100 μl of each test substance is applied once daily to the circumscribed areas of the tail. Treatment groups consist of three animals each, and each animal serves as its own control. The drop is first placed on an investigator's gloved index finger and is then spread evenly around a designated band of the tail. Each animal is treated with two concentrations of the test drug. The most proximal and most distal portions of the tail are left untreated as control regions. Applications are continued for 2 weeks. At three- or four-day intervals, and at the termination of the experiment, the clinical response is graded from 0 to 4+, with 0 indicating no response and 4+ indicating removal of all visible scale, leaving a glistening surface.

Prior to biopsy, the skin surface is coated with a thin film of flexible collodion to prevent fragmentation during frozen sectioning. Perpendicular sections of biopsy samples are stained with aqueous 8-anilino-2-naphthalene sulfonic acid, which on fluorescence microscopy depict selectively stratum corneum hydrophobic membrane domains.

Evaluation

Both the control and drug-treated sections are measured in a double-blind manner. The mean and SE from a minimum of five separate regions are tabulated. Significant differences are determined by Student's *t*-test.

Modifications of the Method

Harlequin Ichthyosis (ichq): a juvenile lethal mouse mutation with ichthyosiform dermatitis was described by Sundberg et al. (1997).

Shultz et al. (2003) described mutations at the mouse ichthyosis locus within the lamin B-receptor gene as a single gene model for human Pelger-Huet anomaly.

Knox and Lister-Rosenoer (1998) described an infantile ichthyosis in **rats** and proposed this as a new model of hyperkeratotic skin disease.

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Xeroderma Models

Howard Maibach

Contents

Xeroderma Models

Experimentally Induced Xeroderma in Mice

Purpose and Rationale

Xeroderma pigmentosum is an autosomal recessive disorder characterized by hyperphotosensitivity and multiple cancers in association with abnormal DNA repair (Robbins et al. 1974; Satokata et al. 1992). Xeroderma pigmentosum group A (XPA) gene-deficient mice cannot repair UV-induced DNA damage and easily develop skin cancers by UV irradiation (Nakane et al. 1995; Miyauchi-Hashimoto et al. 1996, 1999). Kuwamoto et al. (2000) tested the involvement of enhanced prostaglandin E_2 production in the photosensitivity in xeroderma pigmentosum group A model mice and the influence of a prostaglandin synthesis inhibitor.

Procedure

XPA gene-deficient homogeneous mice, age 8–12 weeks, are used (Nakane et al. 1995). The UVB source consists of a bank of fluorescent sunlamps with an emission spectrum from 270 to 375 nm, peaking at 305 nm. The mice are anesthetized by i.p. injection of phenobarbital to keep them immobile during exposure. The ears are irradiated with 250 mJ per cm^2 of UVB. Immediately after irradiation, the test drug (20 µl of 1 % solution of the prostaglandin synthesis inhibitor indomethacin) is applied to the ears. Ear thickness is measured with

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a dial thickness gauge immediately before irradiation and 1–4 days after irradiation. The amounts of PGD_2, PGE_2, and PGF_{2a} in mouse ears at 0, 24, 48, and 72 hr after UVB irradiation are determined by enzyme immunoassay.

Evaluation

Student's *t*-test is employed to determine the statistical difference between means.

Modifications of the Method

De Boer et al. (1999) described a mouse model for the DNA repair/basal transcription disorder trichothiodystrophy, revealing cancer predisposition.

Sun et al. (2003) described a genetic mouse model carrying the nonfunctional xeroderma pigmentosum group G gene. A disrupted XPG allele was generated by insertion of neo cassette sequences into exon 3 of the XPG gene by using embryonic stem cell techniques.

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Vitiligo Models

Howard Maibach

Contents

Vitiligo Models

Vitiligo in Mice

Purpose and Rationale

A **mouse model for vitiligo** was described by Lerner et al. (1986), designated C57BL/6J-vit/vit. The vitiligo mouse has congenital dorsal and ventral white spots as well as progressive replacement of pigmented hairs with each spontaneous molt or after plucking.

Sidman et al. (1996) described pigment epithelial and retinal phenotypes in the vitiligo mivit, mutant mouse.

Tang et al. (1997) studied abnormalities of the electroretinogram in relation to histopathological findings in vitiligo mutant mice.

Iwamoto et al. (1991) established a mouse model for melanoma in which metallothionein/ret transgenic mice express the ret oncogene fused to the metallothionein promoter. Lengagne et al. (2004) reported the occurrence of spontaneous vitiligo in this animal model for human melanoma.

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Smyth Line Chickens

Purpose and Rationale

Smyth et al. (1981) developed a mutant line of chickens (DAM line) that is characterized by a high incidence of spontaneous, postnatal, cutaneous amelanosis as a result of an autoimmune phenomenon (Smyth 1989). An intrinsic melanocyte defect, characterized by melanosomes with abnormal irregular surfaces, predisposes these chickens to the pigment disorder (Boissy et al. 1986). Both B-cell and T-cell compartments of the immune system are involved in the pathology of the disease (Lamont et al. 1982; Erf et al. 1995). Melanocyte-specific antibodies have been detected in the serum of Smyth line chickens (Austin et al. 1992). These autoantibodies cross-react with mouse and human melanocytes and are able to bind pigment within tissues (Searle et al. 1993). Mammalian tyrosinase-related protein-1 is recognized by autoantibodies from vitiliginous Smyth chickens (Austin and Boissy 1995). The Smyth chicken model for spontaneous autoimmune vitiligo shows various incidences and degrees of alopecia ranging from alopecia areata-like to universalis-like integumental changes (Smyth and McNeil 1999). Sreekumar et al. (2000) analyzed the effect of endogenous viral genes in the Smyth line chicken model for autoimmune vitiligo. Erf et al. (2001) reported a strong connection between herpesvirus infection and the expression of autoimmune vitiligo in Smyth line chickens. Sreekumar et al. (2001) performed molecular characterization of the Smyth chicken sublines and their parental controls by restriction fragment length analysis and DNA fingerprint analysis. Wang and Erf (2003, 2004) investigated the melanocyte-specific cell-mediated immune response in vitiliginous Smyth line chickens and studied apoptosis in feathers of Smyth line chickens with autoimmune vitiligo.

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Erythropoitic Protoporphyria

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Contents

Erythropoietic Protoporphyria

General Considerations

Purpose and Rationale

Erythropoietic protoporphyria (EPP) is an inherited disease that is associated with deficiency of the last enzyme of heme biosynthesis, ferrochelatase, which catalyzes the insertion of ferrous iron into protoporphyrin. Ferrochelatase deficiency is accompanied by photosensitivity syndrome (Bottomley et al. 1975; Cox 1997; Lim and Cohen 1999). It is characteristically manifested by a severe burning pain associated with edema and erythema within a few minutes of exposure to visible light (Cox 1997). Some of these symptoms are related to the extravasation of intravascular contents, such as blood plasma, caused by pronounced damage to endothelial cells (Brun and Sandberg 1991). The defect in ferrochelatase results in the accumulation of protoporphyrin (Bottomley et al. 1975; Cox 1997; Lim and Cohen 1999), which may be produced in immature red blood cells, released into the plasma, and may accumulate in the cell membranes of endothelial cells due to its hydrophobicity (Brun and Sandberg 1991).

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Animal Models for Erythropoietic Protoporphyria

Purpose and Rationale

Many animal models on erythropoietic protoporphyria are published. Berenson et al. (1992) described a new model of protoporphyric hepatopathy using protoporphyrin overload in unrestrained rats.

Most studies that have been performed using mice (Gschnait et al. 1975; Konrad et al. 1975; Cantoni et al. 1983; Tanaka et al. 1993; Libbrecht et al. 2003; Bellingham et al. 1995) described experimental murine protoporphyria induced by griseofulvin. Smith et al. (1997) induced protoporphyria by the orally active iron chelator 1,2-diethyl-3-hydroxypyridin-4-one in C57BL/10ScSn mice.

Many authors (Boulechfar et al. 1993; De Verneul et al. 1995; Meerman et al. 1999; Fontanellas et al. 2001; Abitbol et al. 2005; Han et al. 2005; Navarro et al. 2005) have used genetically altered mice with a recessive inherited ferrochelatase deficiency, originally described by Tutois et al. (1991). Magness et al. (1998) analyzed the human ferrochelatase promoter in transgenic mice.

Pawliuk et al. (2005) reported prevention of murine erythropoietic protoporphyria-associated skin photosensitivity and liver disease by dermal and hepatic ferrochelatase.

Takeshita et al. (2004) performed an L-band electron spin resonance (ESR) study to investigate in vivo oxygen radical generation in the skin of the protoporphyria mouse model with visible light exposure.

Procedure

Chemiluminescence techniques have been used to detect $^1O_2$ and O_2^{*-} using the emission of $^1O_2$ itself or a chemiluminescent probe (Khan 1981; Nakano et al. 1986). As the luminescence arising from reactive oxygen species is usually very weak compared with the irradiating light, it is technically hard to evaluate reactive oxygen species generation during photodynamic reactions using this method. In vivo generation of reactive oxygen species was detected in the skin in erythropoietic protoporphyria disease during irradiation with light, using a technique other than chemiluminescence.

In vivo electron spin resonance (ESR) spectroscopy was used operating at low microwave frequencies as an alternative to chemiluminescence techniques. This technique has enabled the noninvasive measurement of durable free radicals, including a nitroxyl radical, in living experimental animals (Subczynski et al. 1986; Bacic et al, 1989; Ishida et al. 1989; Ferrari et al, 1990; Utsumi et al. 1993). The nitroxyl radical loses its ESR signal on reaction with transient free radicals, such as $^*OH$ and O_2^{*-} (Takeshita et al. 2004). Free radical reactions were evaluated in various animal models, including hyperoxia (Miura et al. 1992), ischemia–reperfusion (Utsumi et al. 1993), X-ray irradiation (Miura et al. 1997), and streptozotocin-induced diabetes (Sano et al. 1998) using L-band (1–1.2 GHz) and 300 MHz ESR spectrometers with a nitroxyl radical probe (a spin probe). In these experiments, the disease model animal was set in the loop-gap resonator of the ESR spectrometer. Loop-gap type resonators are unsuitable for measuring radicals in the skin of experimental animals, because they detect radicals distributed throughout the body. By contrast, a surface-coil-type resonator (surface resonator) has been used to measure radicals in limited positions, such as the skin (Bacic et al. 1989; Swartz et al. 1994; Kuppusamy et al. 1998; He et al. 2001; Fuchs et al. 1997). Furthermore, ESR measurement with a surface resonator does not interfere with light irradiation, since only the single-turn coil portion of the resonator is attached to the skin.

This study used ESR with a surface resonator to detect in vivo ROS generation with light exposure in the skin of griseofulvin-induced

protoporphyria model mice, a standard model for EPP (Gschnait et al. 1975; Konrad et al. 1975; Wolff et al. 1975; Plosch et al. 2002).

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Acne Models

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Activity on Sebaceous Glands of Rats

Purpose and Rationale

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965) and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. Early transformations, which take place in the morphology of the organelles in sebaceous cells, can be observed by electron microscopy. In the cytoplasm of intermediate cells, a large number of vesicular elements derived from the smooth endoplasmic reticulum are formed, participating in the synthesis of lipids which appear as droplets of varying size (Karasek 1968; Morohashi 1968). Following an increase of lipid droplets, the cells increase in size, become totally differentiated, and are pushed toward the apex of the gland where they break up and release their content (sebum) into the infundibulum. This effect is used for morphometric evaluation of topical antiandrogens.

Procedure

Groups of five adult male Sprague-Dawley rats weighing 180–220 g are shaved in the interscapular area. Twenty-four hours later, the test preparation or the standard (cyproterone acetate) is applied locally to the shaved area at increasing doses (0.05, 0.5, and 5 mg/cm^2) in 20-µl ethanol. The treatment is continued for 3 weeks. Controls receive ethanol only. The animals are sacrificed 24 h after the last administration. Pieces of skin from the interscapular region are excised and processed for evaluation by electron microscopy. The volume density of the smooth endoplasmic reticulum vesicles is measured.

Evaluation

Dose-response curves are established for volume density of vesicles of the smooth endoplasmic reticulum after various doses of antiandrogen and testosterone standard in order to calculate activity ratios. Statistical comparison with untreated controls allows calculation of threshold doses.

Modifications of the Method

Ebling and Petrow (1993) tested 19-aldehydo-4-androstene-3,17-dione, an estrogen precursor that inhibits sebaceous secretion in ovariectomized testosterone-treated rats.

De Young et al. (1984, 1985) described intradermal injection of *Propionibacterium acnes* as a model of inflammation relevant to acne. Intradermal injection of killed *P. acnes* into the rat ear induces a chronic acne-like inflammation characterized by edema and cell infiltration of several months' duration, formation of comedones, hypersensitization, and transepithelial elimination.

Shamoto et al. (1999) investigated the dermal histology and the regional draining superficial lymph node of a new mutant strain of hairless rats (ISh). The homocygote ISh rat was characterized as having naked and wrinkled skin. The comedo-like casts in the skin resembled human acne.

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Activity on Sebaceous Glands of the Fuzzy Rat

Purpose and Rationale

Fuzzy rats, a genetic mutant between hairless and haired albino rats, exhibit sexual dimorphism in the skin (Ferguson et al. 1979). A brown-colored, thick seborrheic coating on the entire back is a characteristic of male rats while the female rats show clear skin with white fuzzy hairs. In male rats, the hair follicles resemble human sebaceous follicles associated with hyperplastic glandular lobules, dilatation, and microcomedo (Plewig and Kligman 1975). Castration caused a reduction in size of the sebaceous glands and ducts, and a testosterone implant in castrated rats resurrected glandular hyperplasia and ductal dilatation (Uno et al. 1990).

This rodent model for androgen-dependent hyperplasia of the sebaceous glands is useful for the study of many pharmacological aspects, comprising the rate of percutaneous absorption, stability, and affinity to target organs of the testing compounds and selection of adequate vehicles for topical application (Ye et al. 1997).

Procedure

Peripubertal male fuzzy rats at an age of 25 days weighing about 40 g are kept in single cages at a temperature of 24 °C and a 12-h light and dark cycle. They are divided into several groups for local treatment with 5α-reductase inhibitors or androgen receptor antagonists. One group serving as control is castrated at an age of 25 days in anesthesia. Approximately, 0.5 ml of a 1 % solution of test compounds dissolved in 30 % propylene glycol, 50 % alcohol, and 20 % water are applied to the other groups in a 4 × 4 cm area on the lower back once per day 5 days per week. Vehicle solution alone is applied to the control and castrate groups. After 8 weeks of treatment, the animals are euthanized by anesthesia with ketamine HCl, 100 g/ml; xylazine HCl, 120 g/ml; or acepromazine, 5 g/ml (1 ml/kg) intramuscularly.

Photographs of the backs are taken pretreatment and every 2 weeks after the start of treatment.

Eight weeks after starting treatment, the animals in all groups are given an intraperitoneal injection of 200-mg/kg bromodeoxyuridine 2 h before euthanasia. A blood sample is then collected from the right ventricle of the heart under anesthesia with the ketamine-xylazine-acepromazine mixture. After euthanasia with an overdose of the same anesthesia solution, fresh skin tissues are taken by a punch (4-mm diameter) from the marked area of the back and immediately incubated with EDTA solution for the split-skin preparation. The rest of the skin in the marked area is cut and fixed with 10 % neutral buffered formalin solution for morphometric analysis of the sebaceous glandular lobules. The ventral lobes of the prostate are dissected and the weight is measured.

For morphometric analysis of the sebaceous glands, the size of sebaceous glandular lobules is first determined. Using formalin-fixed skin, small skin samples are taken by punch (4-mm diameter), and serial frozen sections (40-μm thick), horizontal to the epidermal surface, are cut and collected in water-filled wells. Four to five free-floating sections containing sebaceous glandular lobules are selected and stained with 1 % osmium and 2.5 % potassium dichromate solution for 2–3 min. The sections are washed with distilled water and mounted on a glass slide with aqua mount. Darkly stained globular lobules are distinctively seen under the microscope or on microvideo images. The largest lobular area in each glandular image is measured by a computer-assisted microimage apparatus, using a program of image analysis.

Split preparations of the pilosebaceous organ are used to measure the size of the sebaceous lobule and duct and the number of DNA synthesis sebocytes. Fresh skin samples, 4-mm punched, are incubated with 17-mM EDTA in phosphate buffer (0.1 M, pH 7.4) for 2 h at 27 °C. The pilosebaceous organs attached to the epidermis are manually split from the dermis. Following fixation with 10 % buffered formalin, free-floating split tissues are stained by the immunocytochemical method, using a monoclonal antibody against bromodeoxyuridine with the avidin-biotin complex method.

On viewing the split tissue under a stereomicroscope, the sebaceous glandular lobules with a duct attached to the follicular shaft are clearly visible. After mounting on a slide glass, these in situ images of the sebaceous glands are observed on a computer monitor with a microvideo apparatus, and the area of the lobes, the diameter of the duct, and a number of bromodeoxyuridine-stained nuclei in the sebocytes are measured.

Evaluation

Group data are expressed as means ± standard error. The results are analyzed using the *t*-test.

Modifications of the Method

Marit et al. (1995) presented anatomical and physiological parameters of the fuzzy rat, collected for each sex at five ages, including histological and clinical biochemical profiles, organ and body weights, and a characterization of gross and histopathological findings.

Yourick and Bronaugh (2000) studied percutaneous penetration and metabolism of 2-nitro-*p*-phenylenediamine in human and fuzzy rat skin.

Salcido et al. (1995) described an animal model and computer-controlled surface pressure delivery system for the production of pressure ulcers. A method for inducing dermal pressure lesions on the fuzzy rat was developed using a computer-controlled displacement column, which produced a constant tissue interface pressure.

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Activity on Ear Sebaceous Glands of Syrian Hamsters

Purpose and Rationale

The ear sebaceous glands of the Syrian hamster have been proposed as a model system for human sebaceous glands because of the similarities in morphology and in turnover time (Hamilton and Montagna 1950; Plewig and Luderschmidt 1977). As an androgen-sensitive structure, the ear sebaceous glands have been used to determine the effect of antiandrogenic compounds by the use of histoplanimetry on projections of sagittal sections of the ear (Luderschmidt and Plewig 1977). Matias and Orentreich (1983) developed a stripped skin planimetric method to measure the ear sebaceous gland areas in Syrian golden hamsters.

Seki et al. (1995) determined the effects of topically applied spironolactone on androgen-stimulated sebaceous glands in the hamster pinna.

Procedure

Adult female Syrian golden hamsters, 9–10 weeks of age and 110–120 g in weight, are kept at constant temperature and humidity and fed commercially prepared hamster food and water. The hamsters are divided at random into three groups. Testosterone propionate (80 μg dissolved in 1-ml sesame oil) is administered to two groups every other day over a 2-week period to stimulate androgen responses. Hamsters in the third group are injected with 1-ml sesame oil only as controls. The hamsters receive once daily applications of 0.1 g of a colorless clear hydrophilic gel preparation of 5 % spironolactone, containing ethanol and isopropyl alcohol on the ventral side of the right pinna for 2 weeks. The left side is left untreated as control. On day 15, 4-mm punch biopsy specimens from each hamster are obtained from the central region of the bilateral pinnas where sebaceous glands are most developed.

The sebaceous gland size is measured by the whole mount technique (Motoyoshi 1988). After the biopsy specimens are immersed in physiological saline at 4 °C for 18 h, cartilages are removed. Then, each specimen is immersed in 2-N NaBr at 37 °C for 1 h, and the epidermis is peeled off with fine forceps. The obtained dermis sheet containing sebaceous glands is stained with Susan III and mounted on a glass slide.

Evaluation

An area of five or more sebaceous glands, including all the sebaceous acini attached to one pilosebaceous unit in each biopsy specimen, is measured with a computerized image analyzer (Olympus CIA-102) and shown as a mean value ± standard deviation ($mm^2/100$). The values are compared using Student's paired *t*-test.

Modifications of the Method

Gollnick (1990, 1992) evaluated azelaic acid proposed for acne treatment, using comedo formation

in the hamster ear model and recommended it as a new substance in the spectrum of antiacne agents.

Matias and Gaillard (1995) studied the local inhibition of sebaceous gland growth in the ventral ear pinna of sexually mature male Syrian hamsters by topically applied androgen receptor inhibitors.

Seki and Morohashi (1993) investigated the effect of some alkaloids, flavonoids, and triterpenoids on the lipogenesis of sebaceous glands of the hamster ear. Lipogenesis was assayed by determining ^{14}C incorporation into sebaceous lipids extracted from the sebaceous glands, which were incubated with ^{14}C-acetate.

Morgan et al. (1993) studied the occurrence of zinc-induced synthesis of metallothionein in skin after topical application of the antiacne drug Zineryt lotion in hamster ears. The dinitrophenyl hapten-sandwich immunohistochemical method involving a monoclonal anti-metallothionein antibody was used to detect and localize zinc-binding metallothionein in the treated and untreated hamster skin.

Burkhart et al. (2000) studied the effects of benzoyl peroxide on lipogenesis in sebaceous glands using the flank organs of female golden Syrian hamsters.

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Activity on Ear Sebaceous Glands of Rabbits

Purpose and Rationale

Several authors used the rabbit ear model to study comedo formation (Weirich and Longauer 1974; Mills and Kligman 1975; Motoyoshi 1983a; Kligman 1989; Ito et al. 1985, 1991; Maeda 1991) in order to assess the comedogenicity of cosmetics, toiletries, and drugs and to evaluate potential antiacne drugs. Sebaceous follicles in the inner surface of rabbit ears are sensitive to many substances called comedogenes, which, when applied topically, induce comedo formation.

This comedo induction takes place after about 2 weeks of repeated topical application of a chemical comedogen such as 1 % coal tar, 50 % oleic acid, or 50 % tetradecane.

Procedure

Male rabbits weighing 2.5–3.5 kg are used. Tetradecane, testosterone, and dimethyl sulfoxide are separately inuncted on rabbit pinnas once a day for 4 weeks. The pinnas are biopsied on days 1, 3, 7, and 28. Untreated pinnas and squalene-treated pinnas serve as controls. Three-dimensional images of sebaceous glands are reconstructed from their serial histological sections using a computer-image analysis system.

Evaluation

The volumes of the sebaceous glands, the number of acini, and the volume of individual acini are compared between groups.

Modifications of the Method

Motoyoshi (1983b) studied the correlation between surface microscopy and dermal histology in tetradecane-induced comedones in rabbit ear skin.

Kligman and Kligman (1994) used the rabbit ear to assay the comedogenic potentialities of an array of known tumorigens. Complete carcinogens and some tumor promoters were invariably strongly comedogenic at concentrations of 1.0 % and below. The rabbit ear model was recommended to be an easy and reliable way to screen for carcinogenicity.

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Activity on the Hamster Flank Organ

Purpose and Rationale

The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemical or topical antiandrogens.

Procedure

Female Syrian golden hamsters (*Mesocricetus auratus*), weighing 80–110 g, are kept at constant temperature on a commercial diet and water ad libitum. They are castrated 24 h prior to the experiment. The costovertebral region is shaved; the horny layer is stripped the day prior to the test and then every 3 days. For stimulation, animals receive a subcutaneous dose of 250-µg testosterone propionate in 25-µl peanut oil in the dorsal neck fold, for 3 weeks (weekdays only or continually). On the same days, the antiandrogen dissolved in water/ethanol (1:4, v/v) is applied locally to the left flank organ at increasing doses, using a micropipette under a continuous airstream to enhance the evaporation of the solvent. At least five animals are used per group. Controls receive vehicle only, and another group, testosterone propionate only. After treatment, the animals are sacrificed under ether anesthesia. The two major perpendicular axes of the pigmented spot overlying each flank organ are measured and multiplied to obtain the surface area index. The flank organs are excised and divided into two halves along the major axis, immediately fixed in 10 % formalin and embedded in paraffin. The 5-µm-thick sections are stained with hematoxylin-eosin. Sebaceous glands and hairs are measured in the first two sections of each half of the specimen, using a semiautomatic computerized image analyzer. The sebaceous gland area in each field is quantified in square millimeters. The diameter of all hairs under each flank organ is measured in micrometers.

Evaluation

The local (topical) antiandrogenic activity of the test compound is estimated by the ability to inhibit the effects in the ipsilateral treated flank organ whereas the systemic activity is evaluated by the inhibition on the untreated contralateral flank organ. The values for surface area index, sebaceous gland area, and average of diameter of the hairs on the flank organ of the treated left and the untreated right side are compared for antiandrogen-treated animals with controls using two-way analysis of variance and *t*-test.

Modifications of the Method

Wuest and Lucky (1989) studied the differential effect of testosterone on pigmented spot, sebaceous glands, and hair follicles in the Syrian hamster flank organ.

Noto et al. (1991) quantified the antiandrogenic activity of topically applied canrenoic acid in the hamster flank organ. The flank organs of female Syrian hamsters were stimulated by subcutaneous administration of testosterone propionate. Sebaceous glands and hair follicles were measured by a computerized image analyzer. Using this method, the same authors (Noto et al. 1992) compared the activity of some topical antiandrogens.

Aricò et al. (1993) found no antiandrogenic effects of topical bifonazole on sebaceous glands and hair in the hamster flank organ.

Lucky et al. (1995) studied the autoradiographic localization of tritiated dihydrotestosterone in the flank organ of the albino hamster.

Foreman et al. (1984) found that in the hairless hamster, progesterone can antagonize dihydrotestosterone-mediated hypertrophy of the sebaceous gland.

Critical Assessment of the Method

The relevance of the hamster flank organ model to man has been challenged by Franz et al. (1989).

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Activity on the Skin of the Rhino Mouse

Purpose and Rationale

The rhino mouse has been widely used as an experimental acne model to evaluate topically active comedolytic and anti-keratinizing agents (Kligman and Kligman 1979; Ashton et al. 1984; Mezick et al. 1984; Chatelus et al. 1989; Bernerd et al. 1991; Bouclier et al. 1991; Tramposch et al. 1992; Zheng et al. 1993; Sundberg 1994; Fort-Lacoste et al. 1999).

Rhino mice are hairless mutants with a rhinoceros-like appearance, which carry the rhino gene, a recessive allele of the hairless gene (*hrrhhrrh*) (Howard 1941). This recessive mutation on chromosome 14 results in a mouse with wrinkled skin devoid of body hair by age of 25 days. At that time, the end of the first hair cycle, the follicular papillae fail to follow the regressing hair follicles and become isolated in the dermis. The papillae do not reassociate with the follicular epithelium to initiate a new hair follicle cycle. The upper remnants of the hair follicles are filled with sloughed, cornified cells and form utriculi with a small sebaceous gland at their base, resembling an open comedone. The rhino skin becomes progressively loose, forming folds and ridges, due to the expansion of the surface, secondary to abortive hair follicles filling with cornified debris. The utriculi progressively enlarge, forming pillory cysts

(pseudocomedones), which are dilated follicular infundibula filled with cornified debris (Mann 1971). The dermal cysts of the rhino mouse develop into unopened sebaceous glands (Bernerd et al. 1996).

Seiberg et al. (1997) studied the effects of trypsin on apoptosis, utriculi size, and skin elasticity in the rhino mouse. González et al. (2000) measured the effect of graduated local doses of all-*trans* retinoic acid applied to the skin of rhino mice with fluorescence excitation spectroscopy and compared the data with histological findings.

Procedure

Seven-week-old female rhino mice (*hrrhhrrh*) obtained from Charles River Laboratories are divided into groups receiving graduated doses of test compound (all-*trans* retinoic acid) dissolved in an ethanol/propylene glycol mixture (70:30) or the solvent as controls. The solution is applied once per day in doses of 100 µl on the entire dorsal skin, for five consecutive days each week, for a total period of 2 weeks. Animals in all groups are sacrificed 24 h after the last treatment, and biopsies are obtained immediately following sacrifice. Fluorescence excitation spectra are collected from dorsal skin on a daily basis during treatment. During the measurements, the animals are sedated with inhalation of methoxyflurane.

Fluorescence excitation spectra are obtained in vivo with a fluorescence spectrophotometer (SkinSkan, SPEX, Edison NJ) equipped with a 450-W xenon lamp, double monochromators on the excitation and emission, a photomultiplier detector (R928P, Hamamatsu, Japan) connected to a single photon counting system, and a bifurcated quartz fiber bundle (Model 1950; SPEX Ind., Edison, NJ) for light delivery and collection. The individual fibers are 100 µm in diameter and are randomly arranged to form a bundle 6 mm in diameter. The resolution is 4 nm, the intensity of the excitation radiation is in the range of 1–20 mW/cm^2, and the total delivered radiation dose is below the erythema threshold. Each fluorescence measurement consists of a set of eight serial excitation spectra collected by positioning the emission monochromator from 340 to 480 nm in increments of 20 nm and scanning the excitation monochromator from 260 to within 20 nm of the emission monochromator setting. Light from the excitation monochromator is focused into one leg of the bifurcated fiber bundle. The other leg of the fiber bundle is focused into the input of the emission monochromator. The joined end of the fiber bundle is brought into direct contact with the skin site measured. Care is taken to clean properly the fiber bundle end with an alcohol swab between measurements on different animals, and that gentle pressure is applied to the animals during measurement.

Significant changes are observed in the fluorescence spectra as a result of the application of comedolytic agents. The first peak, located approximately at 295 nm, which is related to tryptophan, increases significantly, whereas the second peak at 340–370 nm, which is attributed to collagen links, exhibits a dramatic decrease.

For histology, skin samples approximately 5 mm in diameter are obtained from the skin of each animal following sacrifice, from the midline of the anterior portion of the skin. All specimens are fixed in 10 % buffered formaldehyde for 24 h and later embedded in paraffin. Tissue sections 5-µm thick are cut perpendicularly to the epidermal surface and stained with hematoxylin and eosin for light microscopic examination. For each animal, the epidermal and dermal thickness, utriculi diameter, and number of capillaries containing more than five erythrocytes are assessed with a micrometer eyepiece adapted to a microscope (Leitz SM-LUX, Ernst Leitz, Wetzlar, Germany) using a 40× objective for the measurement of epidermal and dermal thickness, and utriculi diameter, and a 20× objective for the count of dilated capillaries.

Evaluation

Time-response curves and dose-response curves of the changes of fluorescence spectra are established compared with the changes of histological data.

Modifications of the Method

In addition to image analysis, Seiberg et al. (1997) tested the effects of trypsin on skin elasticity and elastin expression in the rhino mouse.

Beehler et al. (1995) studied gene expression of retinoic acid receptors and cellular retinoic acid-binding proteins in rhino and hairless mouse skin.

Imakado et al. (1995) found that targeting expression of a dominant-negative retinoic acid receptor (RAR) mutant in the epidermis of transgenic mice results in loss of barrier function.

Feng et al. (1997) reported that suprabasal expression of a dominant-negative RXR alpha mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids.

González et al. (1997) investigated DNA ploidy changes in rhino mouse skin after treatment with all-*trans* retinoic acid and retinol.

Petersen et al. (1984) developed an animal model using human face skin onto the nude mouse to study human sebaceous glands. The effects of androgens were evaluated.

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Activity on the Skin of the Mexican Hairless Dog

Purpose and Rationale

The Mexican hairless dog has been recommended as a model for the comedolytic and morphogenic activity of retinoids and other antiacne agents (Loux et al. 1974; Schwartzman et al. 1996; Kimura and Doi 1996). A semilethal mutation of an autosomal dominant "L" gene produces an animal with multiple developmental defects, including poor dentition, early degeneration of the thymus, hairlessness, and numerous comedones (Yankell et al. 1970a; González-Diddi et al. 1971; Goto et al. 1987; Fukuda et al. 1991). The Mexican hairless dog is nearly bald, with sparse, flimsy hair most abundant on the limbs and head. In most hairy species, the primary hair is surrounded by seven to ten accessory hairs. In this species, the follicles are mainly single and not in clusters. The skin is tan colored. A brownish, waxy material unevenly coats the surface; this can be removed easily by soap and lipid solvents. A great number of comedones cover the surface, and these are nearly all of the open variety. The majority is rather small (a few millimeters in diameter) and may be viewed as horn-filled shallow invaginations. Scattered among these, especially on the face, neck, and thighs, are larger, black-tipped open comedones. These are hard, deep, horny impactions, which are expelled with difficulty. A few small, closed comedones occur on the neck and lateral aspect of the chest.

Papulopustules are rare and are found mainly on the metacarpus and metatarsus. They apparently do not develop from the rupture of comedones as in humans. Thus, this animal mimics human acne only with regard to comedones. These, unlike the human variety, do not originate from preexisting sebaceous follicles. They arise de novo and completely lack sebaceous glands.

Procedure

About 1-year-old female animals are used. Areas of 4–5 cm^2 on the dorsolateral aspects of the trunk are treated once daily (5 working days) for 14 weeks with test formulations or tretinoin formulations (0.025–0.1 %) as standard.

Biopsies are taken from each site under local anesthesia at 5 weeks and again at 14 weeks.

Evaluation

The clinical picture and the histology of biopsies are compared before, during, and after treatment.

Modifications of the Method

Yankell et al. (1970b) used the Mexican hairless dog for sunscreen recovery studies.

Hunziker et al. (1978) compared percutaneous penetration of benzoic acid, progesterone, and testosterone between Mexican hairless dogs and man.

Matsumura et al. (1992) described a burn wound-healing model in the hairless descendant of the Mexican hairless dog.

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In Vitro Sebocyte Model

Purpose and Rationale

Cultured human sebocytes have been used by several authors to study sebum formation and to evaluate potential antiacne drugs (Zouboulis et al. 1991a, b, 1993, 1994, 1998; Rosenfield 1989; Doran and Shapiro 1990; Doran et al. 1991; Akamatsu et al. 1993; Guy et al. 1996a, b; Rosenfield et al. 1998; Wauben-Penris et al. 1998; Tsukada et al. 2000; Fritsch et al. 2001).

Procedure

Human sebaceous glands are isolated from facial skin and seeded on monolayer 3T3 cells (Xia et al. 1989). Primary sebocyte cultures are derived from the periphery of the gland lobules and are maintained to confluence before subcultivation. All experiments are performed using secondary sebocyte cultures, which consist of cells undergoing sebocytic differentiation.

Human sebocytes are seeded in 96-well culture plates at a concentration of 10^4 cells/well and are left to attach for 2 days at 37 °C with 5 % CO_2 in culture medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) supplemented with 8 % fetal calf serum, 2 % human serum, 10 ng/ml epidermal growth factor, 10^{-9}-M cholera toxin, 3.4-mM L-glutamine, 100-IU/ml penicillin, and 100-µg/ml streptomycin. The medium is then aspirated, and serum-free keratinocyte basal media (KBM) (Clonetics, San Diego, CA) without additives, supplemented with

steroids, e.g., testosterone (10^{-8}–10^{-5} M), 5a-dihydrotestosterone (10^{-8}–10^{-5} M), or spironolactone (10^{-12}–10^{-7} M) or their combinations, are added to six wells at each concentration. KBM is concomitantly added to another six wells serving as controls. The plates are incubated at 37 °C for 10 days before evaluation. KBM with and without compounds is changed every 2 days.

Cell numbers of treated human sebocytes in 96-well culture plates are assessed over 9 days by counting single-cell suspensions in Neubauer chambers and compared with the absolute fluorescence units (AUF) of parallel wells obtained using the 4-methylumbelliferyl heptanoate (MUH)-fluorescence assay (Stadler et al. 1989). This assay is based on the hydrolysis of the fluorogenic substrate MUH by esterases of proliferating cells. A stock solution of 10-mg/ml MUH is prepared in DMSO and kept frozen at –20 °C until use. On the day of assessment, KBM is removed, and the cells are washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (pH 7.2). The MUH stock solution is diluted in phosphate-buffered saline up to 100 µg/ml, and 100 µl of the final solution is added to each well. The plates are then incubated for 30 min at 37 °C and read automatically on a Titertek Fluoroscan II (Flow, Meckenheim, Germany).

Evaluation

The results are given as absolute fluorescence units using 355-nm excitation and 460-nm emission filters. Statistical significance of the differences between the means is assessed by Student's *t*-test.

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Skin Mycosis

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Contents

Skin Mycosis

General Considerations

Purpose and Rationale

Fungal infections of the skin account for a large number of consultations to general practitioners and dermatologists. They are caused by dermatophytes, such as *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Epidermophyton floccosum*, *Microsporum gypseum*, and *Trichophyton verrucosum* (Sinski and Kelley 1991).

Most fungal skin infections, such as tinea pedis and tinea cruris, respond to topical therapy, although widespread or chronic infections that do not respond to local measures may require systemic treatment, for example, with griseofulvin, ketoconazole, or terbinafine. Traditional topical products such as compound benzoic acid ointment (Whitfield's ointment) and tolnaftate preparations have largely been superseded by other topical-active antifungal agents, such as the imidazoles (e.g., clotrimazole, miconazole, or econazole) or hydroxypyridones (e.g., ciclopirox), which are well tolerated and rapidly effective (Gupta et al. 1994). Fungal infections also affect the appendices, such as hair and nails. To evaluate antifungal agents not only the antimicrobial spectrum but also the penetration plays a decisive role.

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In Vitro Inhibitory Activity

Purpose and Rationale

In vitro tests are performed to investigate whether the test compound in comparison to standards covers the most relevant pathogens of dermal mycoses. The test conditions are rendered more difficult by the addition of protein since the main infection site for fungi is the horny layer of the epidermis, which has a high protein content.

Procedure

The studies are performed by means of conventional serial dilution procedures in test medium without and with addition of 4 % bovine albumin. The test medium is Sabouraud dextrose broth containing 1 % Neopeptone Difco (Difco Laboratories, Detroit, Mich., USA) and 2 % glucose. The basic medium is sterilized in an autoclave at 121 °C for 15 min. The pH is adjusted to 6.5 with 1 N NaOH. The medium containing albumin is sterilized by filtration through a membrane filter. For the preparation of the test series, the inhibiting substances are dissolved in methanol and then rapidly diluted with slightly warmed test medium, so that series with a continuous dilution factor of 2 are obtained: 125–0.03 µg/ml in medium without protein, 500–1 µg/ml in medium with protein. Each test tube contains 3 ml. The test organisms are various strains of dermatophytes (*Trichophyton mentagrophytes*, *T. rubrum*, *T. verrucosum*, *T. equinum*, *T. gallinum*, *Microsporum canis*, *Microsporum gypseum*) and yeasts (*Candida albicans*, *Ca. tropicalis*, *Ca. pseudotropicalis*, *Ca. krusei*, *Ca. parapsilosis*, *Ca. lipolytica*, *Ca. brumpti*, *Ca. utilis*, *Torulopsis glabrata*). The organisms are pre-cultured on a modified Grütz agar at 28 °C for periods of 1–4 weeks. The suspensions are adjusted by photometry that about 10^5 microconidia of dermatophytes and 10^4 yeast cells per ml are obtained in each inoculated test tube. The minimal inhibitory concentrations are measured after 14 days incubation at 28 °C.

Evaluation

The percentage of strains of *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Candida albicans* is plotted against dilution steps for each test compound with and without albumin and IC_{50} values are calculated.

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In Vivo Activity in the Guinea Pig Trichophytosis Model

Purpose and Rationale

The guinea pig trichophytosis model has been used by several authors to evaluate antimycotic compounds (Millberger and Gillert 1973; Dittmar et al. 1981; Plempel et al. 1983; Petranyi et al. 1987; Schaude et al. 1990; Garcia Rafanell et al. 1992; Arika et al. 1993).

Procedure

Male albino guinea pigs (Pirbright White), bred mycosis-free, weighing 450–550 g, are fed Altromin pellets and tap water ad libitum. On both sides of the back, areas of 5 × 12 cm are shorn to a fur length of 1 mm. Three areas with a diameter of 3 mm are inoculated with a pipette on

either side. Per injection site, 10^4 spores of *Trichophyton mentagrophytes* 2114 in 0.05 ml suspension in physiological saline solution are inoculated. Three days after inoculation, infections with redding and scale formations are observed. From days 3–7 after the infection, 1 ml of the test preparation or standard is applied onto the right animal sides and rubbed in once daily. The diameters (mm) of all alopecias are measured with a ruler 3.5 weeks after the infection.

Evaluation

The values of alopecias, separated according to the treated group and animal side, are determined and statistically evaluated using Duncan's new multiple range test.

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Skin Penetration

Purpose and Rationale

Cutaneous pathogenic fungi like dermatophytes or Candida ssp. present a particular affinity for the stratum corneum. Therefore, the bioavailability of antimycotic drugs in the thick horny layer of the skin is an important element of treatment success. These studies can be performed in human cadaver skin (Dittmar 1981; Kligman et al. 1987; Hänel et al. 1988).

Procedure

Skin pieces, measuring 10 × 12 cm, are removed from the back of corpses not later than 24 h after death. The subcutis is removed and the skin piece divided three to five parts. The edges (4 mm) of each individual piece are then taped with tesafilm (adhesive tape) and the remaining surface is treated with the antimycotic compound. 0.05 ml of compound preparation or solution is rubbed into the skin for 10 s. For a period of one h, the pieces are placed on water agar, allowing for free admission of air. After this time, the compound is carefully wiped off with filter paper and water.

The skin area treated with the antimycotic is then stripped with tesafilm tape as follows: (A) one third of the area remains unstripped; (B) one third of the area is stripped three times; (C) one third of the area is stripped six times. Then each piece of skin is placed into a Petri dish, which is heated in a water bath to 52 °C for 15 min. The epidermis is removed from all the skin pieces, which are then placed on slides (with the base turned upwards) and put into the upper parts of plastic Petri dishes. Each of the epidermis pieces is inoculated in 20 sites with a microconidia

suspension of the dermatophyte *Trichophyton mentagrophytes* 109-FHM 1a. The spores had been rinsed off from 3-week-old slant cultures, using distilled water. Approximately 50–100 viable spores are deposited at each inoculation site.

The upper parts of the Petri dishes (containing the slides with the epidermis pieces) are then covered with the bottom parts of Petri dishes, which contain 2 % aqueous agar for producing a wet chamber. Holes in the covers serve to avoid excess humidity in the chamber. The chambers are incubated at 28 °C for 5 days. The fungal growth is recorded daily according to a three-point score system.

Evaluation

Growth is evaluated by visual scoring from days 2–5 after inoculation and calculation of AUCs from growth curves.

Inhibition values (%) are calculated according to the formula:

$$\text{inhibition} = \frac{\text{growth on controls} - \text{growth after treatment}}{\text{growth on contols} \times 100}$$

Modifications of the Method

Ceschin-Roques et al. (1991) performed penetration studies on excised skin from the back of slaughtered pigs.

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Biomechanics of Skin

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Contents

General Considerations

Several attempts were made to describe the mechanical properties of skin by mathematical models (Ridge and Wright 1965; Harkness 1971; Hirsch and Sonnerup 1968; Jamison et al. 1968; Viidik 1968, 1969, 1973, 1979; Frisén et al. 1969a, b; Veronda and Westman 1970; Danielson 1973; Soong and Huang 1973; Wilkes et al. 1973; Jenkins and Little 1974; Lanir and Fung 1974; Vogel 1976, 1986; Barbanel and Evans 1981; Barbanel et al. 1978; Lanir 1979; Barbanel and Payne 1980; Burlin 1980, 1981; Fung 1981; Sanjeevi 1982; Potts and Breuer 1983).

Most of these authors used models derived from studies in polymers (Ferry 1970). The simplest mechanical model analogous to a viscoelastic system is a spring combined with a dashpot, either in series (**Maxwell element**) or in parallel (**Voigt or Kelvin element**). Combinations of these elements were used to explain the mechanical phenomena in connective tissue, such as stress–strain behavior, relaxation and mechanical recovery, hysteresis and creep phenomena (Jamison et al. 1968; Frisén et al. 1969a, b; Hirsch and Sonnerup 1968; Vogel 1976, 1993; Riedl and Nemetscheck 1977; Vogel and Hilgner 1979; Viidik 1968, 1969, 1973, 1977, 1979). Larrabee (1986), Larrabee and Sutton (1986).

Larrabee and Galt (1986) reviewed the theoretical and experimental mechanics of skin and soft tissue and proposed a mathematical model of skin

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deformation based on the finite element method. A finite element based method to determine the properties of planar soft tissue was also described by Flynn et al. (1998).

Unfortunately, none of these models has been found to be sufficient to describe all properties of human and animal skin, including the mechanical history before measurement and the time-dependence during measurement. There is no comprehensive and unequivocally accepted model to describe completely the biorheology of skin. Therefore, several methods are used in order to get insight into the physical properties of skin.

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In Vitro (ex Vivo) Experiments

Stress–strain Behavior

Measurement of Skin Thickness, Ultimate Load, Tensile Strength, Ultimate Strain, and Modulus of Elasticity

Purpose and Rationale

Animal experiments are preferable when studying the biomechanical properties of the dermis, since only in animals can the values at higher extension degrees be studied, ex vivo or in vivo under anesthesia, whereas studies in humans are limited by pain threshold or to tests in cadaver skin. Skin thickness, ultimate load, tensile strength, ultimate strain, and ultimate modulus of elasticity are the most informative parameters, which describe the mechanical properties of the dermis.

Procedure

Groups of at least 10 male Sprague–Dawley rats with an initial weight of 120 ± 5 g are treated subcutaneously or orally with test drugs or saline. The duration of treatment is usually 5 days; however, for special studies treatment can be prolonged up to 3 months. The animals are sacrificed under anesthesia. The back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness. In this way, **skin thickness** can be measured reliably by calipers with an accuracy of 0.1 mm. Perpendicular to the body axis, two dumbbell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1989, 1993b). The samples are kept in Petri dishes until testing at room temperature on filter paper soaked with saline solution. The specimens are fixed between the clamps of an INSTRON instrument at gauge length of 30 mm. All measurements are carried out within at least 1 h. For long lasting test procedures, such as relaxation or cyclic loading, the samples are wrapped with saline soaked filter paper (Vogel 1976a, b, 1989, 1993a, b).

Stress–strain curves are registered at an extension rate of 5 cm/min, showing a characteristic shape. During low strain values, there is a gradual increase of load; the curve has a concave part. The stress–strain curve ascends according to an exponential function (Vogel and Hilgner 1977). Afterwards an almost straight part is reached indicating the dependence on Hook's law. At this part, the **ultimate modulus of elasticity** (Young's modulus) can be calculated (increase of load divided by the cross sectional area). Then some yielding of the curve occurs which ends in a sudden break of the specimen. This point indicates **ultimate strain** and **ultimate load**.

Evaluation

From ultimate load divided by the cross sectional area (specimen width times original skin thickness measured at the beginning of the experiment), **tensile strength** can be calculated. The mean values of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity of treated animals are compared with controls using ANOVA and Student's *t*-test.

Critical Assessment of the Method

The parameters **skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity** are influenced by many factors, such as hormones and desmotropic drugs. The most pronounced changes are seen after glucocorticosteroids, both after systemic and local application (Vogel 1969, 1970, 1974a). Several studies were performed on age dependence of mechanical parameters in rat skin. Ultimate load, tensile strength, and modulus of elasticity show a very sharp increase during puberty, a maximum at 12 months and a slight decrease thereafter (Vogel et al. 1970, 1976b, 1978, 1983, 1988, 1989, 1993a). Studies on age dependence show a similar pattern for skin thickness, ultimate load, tensile strength, ultimate extension, modulus of elasticity, hysteresis, relaxation, creep behavior, and biochemical data both in animals and men (Holzmann et al. 1971; Vogel 1987a, b).

Therefore, extrapolations from animal studies to behavior of human skin are justified.

However, due to the anatomical conditions (haired skin in animals), the biomechanics of the epidermis can be studied better in human experiments than in animals.

Modifications of the Method

Measurement of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity can be used in the evaluation of topical corticosteroids (Schröder et al. 1974; Alpermann et al. 1982; Vogel and Petri 1985).

Similarly, Töpert et al. (1990) measured skin atrophy and tensile strength of skin after 30 days local administration of corticosteroids in rats.

Oxlund and Manthorpe (1982) found an increase of strength and a decrease of extensibility of skin strips after long-term glucocorticoid treatment of rats. In agreement with studies by Vogel (1974b, 1978), these results were explained by a change in collagen cross-linking pattern.

Jørgensen et al. (1989) found a dose-dependent increase of mechanical strength in intact rat skin after treatment with biosynthetic human growth hormone.

Andreassen et al. (1981) studied the biomechanical properties of skin in rats with streptozotocin-induced diabetes. An increased stiffness and strength was found: maximal stiffness was increased by 20 % and the strain rate at maximum stress was decreased by 10 %.

Oxlund et al. (1980) found that the stiffness of rat skin was increased in the early postpartum period. This increase was also found in adrenalectomized animals.

Foutz et al. (1992) studied the effects of freezing on mechanical properties of rat skin.

Among desmotropic drugs, *lathyrogenic* compounds, such as amino-acetonitrile and *D-penicillamine*, decrease ultimate load and tensile strength without major influence on skin thickness (Vogel 1971a, b, 1974a). A decrease of the strength of skin strips in rats after treatment with D-penicillamine was also found by Fiedrich et al. (1975) and by Oxlund et al. (1984).

A dose-dependent increase of tensile strength in skin after treatment was found with **nonsteroidal anti-inflammatory drugs** (Vogel 1977).

Strain rate influences the values of ultimate load, tensile strength, and modulus of elasticity, but not the effect of age and corticosteroids (Vogel 1972b). Changes in tensile strength are correlated with the content of insoluble collagen (Vogel 1974b).

Fry et al. (1964) prepared skin rings from the lower part of the leg in rats and studied the age-dependence of the mechanical properties.

Nimni et al. (1966) measured tensile strength of excised skin samples in rabbits during aging.

Pan et al. (1998) studied ultrasound, viscoelastic, and mechanical properties in rabbit skin, including stress relaxation, creep, and Young's modulus as a function of strain.

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Measurement of Mechanical Properties at Low Extension Degrees

Purpose and Rationale

At low strain values, stress–strain curves of skin samples show a gradual increase of load, the curve has a concave part. The stress–strain curve ascends according to an exponential function (Vogel and Hilgner 1977).

Procedure

Groups of at least 10 male Sprague–Dawley rats with an initial weight of 120 ± 5 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia; the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis, two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1981, 1989, 1993). The specimens are fixed between the clamps of an INSTRON instrument at a gauge length of 30 mm. An extension rate of 5 cm/min is chosen. Tension is registered at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, and eventually 90 % elongation. The registration is started with high sensitivity and switched to one-tenth of the sensitivity in order to register the steep end of the curve.

Evaluation

During registration with high sensitivity, the curve reaches an almost straight part. At this part, a tangent is drawn which is used for calculation of E_2. The distance from the start to the cross-point of this tangent with the baseline is measured and denominated as extension until the first rise (D). The angle between the tangent and the baseline is halved. At the cross-point with the stress–strain curve, another tangent is drawn and used for calculation of E_1. At the second part of the curve registered with low sensitivity, a further tangent is drawn for calculation of E_3, which is identical to the ultimate modulus of elasticity.

Mean values for $\boldsymbol{E_1}$, $\boldsymbol{E_2}$, $\boldsymbol{E_3}$, and ultimate stress of skin strips from treated animals are compared with controls using Student's t-test.

Modifications of the Method

Belkoff and Haut (1991) developed a structural model to evaluate the changing microstructure of maturing rat skin.

Foutz et al. (1994) developed a phenomenological model to characterize the nonlinear portion of stress–strain curves of rat skin.

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Step Phenomenon

Purpose and Rationale

Analysis of the low parts of the stress–strain curve revealed a "step" phenomenon (Vogel and Hilgner 1977, 1979a, b; Vogel 1988). If samples obtained perpendicular to the body axis are extended, a gradual increase of load is observed at low degrees of extension, which is suddenly interrupted by a decrease of the registered curve. Then the curve increases again, being interrupted by a second or third step.

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out. The ends of the skin strips are fixed in the clamps of an INSTRON instrument resulting in a gauge length of 30 mm. The specimen is stretched with a strain rate of 50 mm/min. The first part of the stress–strain curve is registered with 10 times higher amplification than the second part. The steps are evaluated as follows.

Evaluation

Tangents to the curves are drawn before and after each step. Then a vertical line is drawn in the middle of the step. The distance between the cross-points of this line with the tangents is measured and calculated as stress loss (ΔS). Furthermore, in the middle of each vertical line a horizontal line is drawn. The distance between the cross-point with the curves is measured and calculated as elongation due to the step (ΔE). Total stress loss is calculated by adding all values of ΔS and dividing by the number of specimens.

If this parameter is considered not only by itself but also in connection with tensile strength it can be calculated as percentage of ultimate stress (= % of breaking strength). In addition, the stress value, which would have been achieved without the step (= value *S*) and the elongation at which the step occurred (= value *E*), is registered. If stress loss at one step is multiplied with the elongation at this point, an indication of work loss is achieved (value ΔS times *E*). Taking into account the corresponding parameters indicating total work input (ultimate stress times ultimate strain), the relative work loss can be calculated.

Furthermore, elongation gain due to the steps is calculated by adding all values of ΔE and by dividing by the number of specimens.

Mean values of these parameters from treated animals are compared with controls using Student's *t*-test.

Critical Assessment of the Method

The step phenomenon can be explained by the different orientation of collagen fibers in the dermis and by the presence of a muscular layer in rat skin. The muscular fibers are in a direction longitudinal to the body axis. If samples are obtained

perpendicular to the body axis, the muscle bundles are cut transversally. In further studies, the muscle layer was removed in one specimen and compared with a control still having the muscle layer. Investigation of the directional variation showed that the step phenomenon is mainly due to the muscular layer oriented longitudinal to the body axis and the connective tissue between the muscle bundles, whereas the anisotropic behavior of extensibility and ultimate strain is caused by the directional variation of the collagenous bundles in the dermis.

Modifications of the Method

The step phenomenon could also be found in creep experiments (Vogel and Hilgner 1979b).

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Anisotropy of Skin

Purpose and Rationale

As is the case with human skin indicated by Langer's lines (Langer 1861; Gibson et al. 1969; Wright 1971; Stark et al. 1977; Daly 1982), the skin of rats exhibits **directional differences** (Hussein 1972, 1973; Vogel and Hilgner 1979a, b; Vogel 1981, 1983a, 1985a, b, 1988; Belkoff and Haut 1991). Stress–strain curves of rat skin showed a different shape if excised perpendicularly or longitudinally to the body axis. Directional variations of mechanical parameters in rat skin were studied depending on maturation and age (Vogel 1981).

Procedure

Male Sprague–Dawley rats are used at different age groups from 1 week up to 24 months. Young animals (1, 2 and 3 weeks) are delivered with their mothers from the breeder. Each age group is randomly divided into two blocks which are assigned as "perpendicular to body axis" or "longitudinal to body axis." The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. From each rat, two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out either perpendicular or longitudinal to the body axis, allowing a gauge length between the clamps of an INSTRON instrument of 30 mm. Stress–strain curves are measured at a strain rate of 50 mm/min, whereby the first part of the curve is registered with 10-fold amplification. Besides ultimate values, the extension is measured at given load interval for each curve:

$$0.05,\ 0.1,\ 0.2,\ 0.5,\ 1,\ 2,\ 10,\ 20,\ \text{and } 50\ \text{N}$$

and at given stresses:

$$0.01, 0.02, 0.05, 0.1, 0.5, 1, 2, 5, \text{and } 10\ \text{N/cm}^2.$$

Evaluation

Each parameter is measured longitudinal and perpendicular to the body axis. The mean values are statistically compared within each age group using Student's *t*-test.

At low loads, extension is higher in the longitudinal than in the perpendicular direction. The situation is reversed at medium and high stress values. Ultimate extension shows remarkable differences between longitudinal and perpendicular samples. Specimens obtained perpendicular to the body axis showed an increase during maturation, a maximum at 4 months of age, and a decrease during further aging. The behavior of samples obtained longitudinal to the body axis was quite different. After an initial rise, a maximum was found at 3 weeks. Afterwards a slight decrease

was noted. Between 1 and 4 weeks, all values of ultimate extension were significantly higher in samples punched out longitudinally to the body axis; between 4 and 12, months they were considerably lower (Vogel 1981).

Critical Assessment of the Method

The data indicate the importance of directional variations in all studies of biomechanics of skin.

Modifications of the Method

Directional variations of rat skin were also found in hysteresis experiments (Vogel 1983a) and in relaxation experiments (Vogel 1985a).

Directional variations of the stress–strain curves were also described in the skin of tight-skin mutant mice (Menton et al. 1978).

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Relaxation Phenomenon

Purpose and Rationale

In the relaxation experiment, the viscous properties of rat skin are measured (Vogel 1973, 1976a, b, 1983, 1985, 1993a, b).

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 $\pm$ 10 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed in anesthesia, the back skin is shaved, and a flap of 5 $\times$ 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body

axis, two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin strips are fastened between the clamps of an INSTRON instrument and extended with the high strain rate of 1,000 mm/min up to 20 % extension. This extension is kept constant for 5 min. The chart speed is initially 1,000 mm/min, then 10 mm/min. In this way, the **initial tension** and the **stress values at 0.001, 0.01, 0.1, 1, and 5 min** can be measured. Due to relaxation, the stress values drop down roughly with the logarithm of time.

Furthermore, the **residual stress** after 5 min relaxation period is measured and calculated as percentage of the original stress. After 5 min, the sample is returned to 90 % of the original strain, for example, from 20 % to 18 %. The stress following such unloading is recorded and again calculated as percentage of the original stress. Immediately after unloading, the measured stress values rise again spontaneously, which is called **mechanical recovery**. Mechanical recovery is calculated as percentage of initial tension and as percentage of stress after unloading. The relaxation experiment is repeated with increasing degrees of extension of 40 %, 60 %, 80 %, and eventually 100 % until the specimen breaks.

Evaluation

For each sample, the relaxation is calculated according to the formula

$$\sigma(t) = A_1 + A_2 \times \log t$$

resulting in two constants (A_1 and A_2) for each sample whereby A_1 is the stress at $t = 0$, and A_2 the slope of the relaxation curve.

The **ratio between the constants A1 and A2** has to be considered as the most characteristic parameter of the relaxation experiment.

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

Modifications of the Method

In studies of age dependence in rats skin, a definitive decrease of the **ratio A2/A1** was found at 40 % and 60 % extension degrees indicating a decrease of plasticity with age. Mechanical recovery, as an indicator of secondary elasticity, was better in old animals at medium extension degrees than in young individuals. Stress relaxation was decreased after corticosteroids and increased after thyroid hormones and D-penicillamine (Vogel 1973, 1993a, b).

Purslow et al. (1998) suggested that relaxation processes within the collagen fibers or at the fiber-matrix interface might be responsible for the viscoelastic behavior of skin.

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Hysteresis Experiments

Purpose and Rationale

In the hysteresis experiment not only the elastic but also the viscous properties of skin are measured (Vogel 1978, 1983).

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved, and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. Perpendicular to the body axis, two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Using the INSTRON instrument, the samples are stretched up to a given extension degree (e.g., 20 %) with an extension rate of 20 mm/min. When the given extension is achieved, the crosshead is immediately moved back to the starting position with the same velocity. From the upward curve, the stress and the modulus of elasticity at the end of the loading phase at the given strain indicating the elastic properties can be measured. When the sample is unstretched, the unloading curve shows a different pattern reaching the baseline much earlier than the curve left it during the upward phase. From this point, the **residual extension** can be measured. Immediately after the first hysteresis cycle, the experiment is repeated up to an extension degree of 30 %, than to 40 % and 50 % and finally up to 60 %.

Evaluation

By planimetry of the area below the upward curve the **energy input** and of the area between the hysteresis loop the **energy dissipation** can be calculated as well as the **ratio between energy dissipation and energy input** at each hysteresis cycle indicating the viscous properties. Stress and modulus of elasticity at the end of the hysteresis loop, energy input, energy dissipation, and the ratio between energy dissipation and energy input at each hysteresis cycle are compared between treated animals and controls using Student's *t*-test.

Modifications of the Method

In rat skin, a maximum of the ratio between energy dissipation and energy input was found at 30–40 % extension. This ratio is influenced by age. At low extension degrees, there was an increase with age, whereas at high extension degrees an age-dependent decrease was noted. The ratio dissipation/input was slightly decreased by prednisolone, but definitively increased by D-penicillamine (Vogel 1993).

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Isorheological Point

Purpose and Rationale

Buss et al. (1976) demonstrated the determination of the isorheological point to be a valuable parameter for the mechanics of connective tissue. This method has been modified and elaborated for the skin strips of rats (Vogel 1984, 1985, 1987).

Procedure

Groups of 20 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated subcutaneously or orally with test drugs or saline. The groups are divided randomly into groups of 10 animals for the examination of skin samples either perpendicular or longitudinal to the body axis. The animals are sacrificed under anesthesia, the back skin is shaved, and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Being fastened between the clamps of an INSTRON instrument, the specimen is expanded rapidly up to 2 N and the corresponding strain is measured. Keeping this strain constant, the load decay (relaxation) is measured for 5 min. Then the sample is again loaded up to 2 N and a second relaxation period of 5 min is evaluated. In the third cycle, the sample is unloaded to 50 % of the load observed after the 5-min relaxation period in the second cycle. The phenomenon of **mechanical recovery** is observed. With the crosshead driven up and down, the point is sought where neither immediate relaxation nor mechanical recovery can be observed. The load and strain at this point define the **isorheological point**, which is characterized by the fact that under isometric conditions, the measured load is constant for several minutes. Increasing and decreasing the load by 10 % produces a saw-tooth-shaped curve from which the **modulus of elasticity at the isorheological point** can be calculated. The same procedure is performed at higher initial loads such as 10 N and 50 N. The product of percentage of strain multiplied by stress at the isorheological point indicates energy density.

Evaluation

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

The values showed a decrease during maturation, a minimum at 12 months, and an increase during senescence. In studies with desmotropic compounds, the decreased viscosity after treatment with prednisolone acetate was more evident at the isorheological points than at the ultimate values. Likewise, the higher extensibility after treatment with D-penicillamine was indicated more clearly by the isorheological points than by the ultimate strain.

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Creep Experiments

Purpose and Rationale

In creep experiments, viscous behavior of skin is studied. The strain under constant load is also denominated as retardation behavior (Vogel 1977, 1987).

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved, and a flap of 5×5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis, three dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

In a special apparatus, skin specimens are suddenly loaded with 100, 200, or 500 g and the extension degree measured. An immediate extension occurs which is followed by a slow and almost continuous creep being measured as **ultimate extension rate**. Furthermore, **extension achieved after 1 h** is registered.

Evaluation

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

An age-dependent decrease of these parameters was found in rat and human specimens indicating a decrease of viscosity or plasticity with maturation and age. Ultimate extension rate was decreased by prednisolone and increased by D-penicillamine.

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Repeated Strain

Purpose and Rationale

By the method of using repeated strain, mainly the viscous properties of skin are measured (Vogel and Hilgner 1978; Vogel 1987).

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved, and a flap of 5 × 5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis, two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin specimens are fastened between the clamps of an INSTRON instrument, extended with a strain rate of 100 mm/min up to 20 % extension and immediately unloaded followed by further cycles with the same strain rate and extension degree. The peak of the second cycle is considerably lower than the first, followed by a further decrease in the next cycles. The number of cycles is counted until the stress value is only one half of that of the first cycle. Immediately afterwards, the degree of extension is increased to 30 %. Again the number of cycles is counted until the stress value was only one half of that of the first cycle. In this way, the **number of cycles indicating the half-life of tension** due to relaxation is counted at each step of 20 %, 30 %, 40 %, 50 %, 50 %, 70 %, 80 %, 90 %, and eventually 100 % extension. The number of cycles decreased from the first step (20 %) to the third step (40 %) and increased continuously until the last step. This increase was almost an exponential function of the number of steps.

Evaluation

The means of the number of cycles indicating the half-life of tension due to relaxation at each step are compared between treated animals and controls using Student's *t*-test.

The parameters measured with this method are influenced by several factors: An increase was noted from an age of 1–24 months. The values were decreased by D-penicillamine and increased by prednisolone treatment. Again this method showed that plasticity of skin is decreased by age and by corticosteroids and increased by D-penicillamine.

Modifications of the Method

Lafrance et al. (1998) tested mechanical properties of human skin equivalents submitted to cyclic forces. The in vitro production of disk-shaped (25.4 mm diameter) skin equivalents was based on the culture, under submerged conditions, of keratinocytes seeded on anchorage-based dermal equivalents, a human type I + III collagen gel supplemented with elastin and glycosaminoglycans. The specimens were submitted to quasi-static ramp-deflection cycles induced by means of an actuated hemispherical head. The effects of repeated loading were studied by monitoring the indentation load versus deflection and the relaxation of load over 1,000 s.

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Correlation Between Biomechanical and Biochemical Parameters

Purpose and Rationale

Mechanical parameters of skin, such as skin thickness, ultimate load, tensile strength, ultimate strain, and ultimate load of elasticity as well as hysteresis, relaxation, isorheological point, creep behavior, and values after repeated strain are clearly dependent on maturation and age. They are also influenced by drugs, such as corticosteroids and desmotropic compounds. Therefore, it was of interest to elucidate the correlations of these parameters with biochemical values such as collagen content, collagen fraction, glycosaminoglycans, and elastin (Vogel 1973, 1976, 1980, 1987, 1988).

Procedure

Mechanical Parameters

Male Sprague–Dawley rats are sacrificed under anesthesia; the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis, two dumbbell-shaped specimens with a width of 4 mm in the middle of the sample are punched out. The specimens are fixed between the clamps of an INSTRON instrument at a gauge length of 30 mm. An extension rate of 5 cm/min is chosen. Tension is recorded at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, and eventually 90 % elongation. The recording starts with high sensitivity and is switched to one-tenth of the sensitivity in order to record the steep end of the curve. Stress–strain curves are determined at an extension rate of 5 cm/min, showing a characteristic shape. During low strain values, there is a gradual increase of load; the curve has a concave part. The stress–strain curve ascends according to an exponential function (Vogel and Hilgner 1977). Afterwards, an almost straight part is reached indicating the dependence on Hook's law. At this part, the ultimate modulus of elasticity (Young's modulus) can be calculated (increase of load divided by the cross-sectional area). Then some yielding of the curve occurs, which ends in a sudden break of the specimen. This point indicates ultimate strain and ultimate load.

Biochemical Parameters

Total collagen content was measured as mg hydroxyproline/g wet weight of skin according to the method of Stegemann (1958). Collagen was further analyzed according to solubility at 4 °C.

Fraction I	Soluble in 0.15 mol NaCl solution
Fraction II	Soluble in 0.5 mol NaCl solution
Fraction II	Soluble in citrate buffer solution
Fraction IV	Insoluble

Glycosaminoglycan content and the fractions hyaluronic acid, chondroitin sulfates, and heparan sulfate were determined by column chromatography. The results were expressed as glucuronic acid per g wet weight. Glucuronic acid was determined according to Bitter and Muir (1960).

Elastin was determined according to Naum and Morgan (1973). The specimen was homogenized in deep-frozen state. A sample of 50 mg was extracted with 0.15 M NaCl solution, later on twice by 5 M guanidine hydrochloride solution at 25 °C. After hydrolyzation in an autoclave at 1 atm excess pressure for 45 min, the residue was digested with 0.01 % elastase solution (Elastase, Merck, Darmstadt) at 37 °C for 2 h. Bovine ligamentum nuchae was used as standard. Elastin was determined in the supernatant as protein according to Lowry et al. (1951). Results were expressed as mg elastin per g wet weight.

Evaluation

A clear correlation between tensile strength and the content of insoluble collagen was found in all studies for age dependence and the effect of corticosteroids and desmotropic compounds such as D-penicillamine (correlation coefficient 0.9901; Vogel 1974). The values of glycosaminoglycans and elastin showed age-related dependence and changes after drug treatment, but no significant correlation with the mechanical parameters.

Modifications of the Method

Oxlund et al. (1988) studied the role of elastin in the mechanical properties of rat skin. They concluded that elastin plays a role in the mechanical behavior of rat skin at small stress values and small deformations.

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Thermocontraction

Purpose and Rationale

If collagenous material such as skin or tendon is heated in a water bath, thermocontraction occurs. This phenomenon was already described by Wöhlisch (Wöhlisch and du Mesnil de Rochemont 1927; Wöhlisch 1932). Verzár and other authors used the phenomenon of thermocontraction of tendons and skin strips extensively to study the aging process (Verzár 1955, 1957; Lerch 1951; Rasmussen et al. 1964; Boros-Farkas and Everitt 1967; Viidik 1969, 1977, 1979; Vogel 1969). With increasing temperature, a sudden increase of isometric force is found, which is followed by a decrease. To evaluate this phenomenon, either the **shrinkage temperature** or the **stress at and above the shrinkage temperature** can be measured. Furthermore, the decrease of sample strength can be measured.

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved, and a flap of 5 × 5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

The samples are attached to the clamps of a specifically modified INSTRON instrument which allows the submersion of the test specimen into a beaker filled with 0.9 % saline solution. This solution is kept at 58 °C or 60 °C by a thermostat. Immediately after immersion, the exerted tension is measured and the **maximum of tension** and **time until maximal tension** are recorded.

Evaluation

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

Modifications of the Method

Joseph and Bose (1962) tested the shrinkage temperature of skin in newborn rats, at 10 months and 2 years and found an increase from 51.2 °C to 61.1 °C.

Alain et al. (1977, 1980) tested pieces of dorsal skin of rats. Temperature of maximum tension was decreased from birth to 1 month, and then very slowly increased with age. A rapid relaxation was observed in young rats and in non-senescent adult rats.

Rundgren (1976) found changes of thermal contractility in skin of young and old female rats, and also changes due to repeated pregnancies.

Blackett and Hall (1980) found an increase of thermal shrinkage temperature in two strains of mice during the aging period.

Danielsen (1981) determined thermal stability measured as area shrinkage without tension during heating for membranes of collagen fibrils, reconstituted from solutions of highly purified rat skin collagen.

Allain et al. (1980) built a device, which measured not only hydrothermal shrinking but also swelling in rat skin.

Le Lous et al. (1982a, b, 1983), Flandin et al. (1984) applied the technique of differential scanning calorimetry to evaluate the denaturation process of collagen in rat skin.

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In Vivo Experiments

Stress–strain Curves in Vivo

Purpose and Rationale

In contrast to studies in human beings, animal experiments allow measurement of mechanical properties both in vivo under anesthesia and later on in vitro (ex vivo) at the same site. For this purpose, special methods had to be developed (Barbanel and Payne 1981; Vogel 1981a, b, 1982; Vogel and Denkel 1982, 1985; Denkel 1983).

Procedure

Groups of 10 male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline.

The rats are anesthetized with 60 mg/kg Nembutal i.p. The back skin is shaved mechanically. Skin thickness is measured by use of calipers on an elevated skin fold. Four small (14 × 14 mm) metal plates bearing a hook are used as tabs and are glued on the skin in both longitudinal and perpendicular direction at a distance of 25 mm with a cyanoacrylate preparation. An operation table is mounted on the crosshead of an INSTRON instrument. The table can be turned to allow stretching of the skin in both perpendicular and longitudinal directions relative to the body axis. The anesthetized animals are fastened by their legs to the operation table. A triangle is attached to the load cell. At the end of the triangle, threads are fastened which are conducted by reels and hooked to the tabs. The cross head is moved down manually until the threads are stretched, however, no tension is measured yet. Then the crosshead is driven downwards with a rate of 50 cm/min, what means that the actual extension rate is 100 cm/min. The load is measured only to limited values in order to prevent damage of the skin. In each rat, the stress–strain curve is recorded in both directions whereby the order (first longitudinal or first perpendicular) is changed from animal to animal. In this way, the influence of the first extension on the results of the second extension is eliminated. Stress–strain curves are recorded up to an elongation of 80 %. Modulus of elasticity is calculated from the almost straight part of the curve.

Evaluation

Average stress–strain curves with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

Critical Assessment of the Method

In spite of similar conditions, differences between the stress–strain curves from in vitro and in vivo experiments were found. These findings confirmed the statements of other authors (Barbanel and Payne 1981; Wijn 1980) of the importance of tab distance and tab geometry (Vogel 1981b). Analysis of the data showed that the higher the ratio of distance between the tabs to the area below the tabs, the higher were the stress values. These findings can be explained by

the fact that skin consists of several layers. Only the upper layer (epidermis) is fastened to the tabs in the in vivo experiment. The forces transmitted to the lower layers are transmitted by a larger area if the area under the tabs is larger. The lower layer can slide over a larger area and is therefore less extended resulting in lower stress values. No sliding is possible if the sample is fastened from both sides as it is performed in the in vitro experiments. Taking into account all experimental conditions including the strain rate, the in vivo results are comparable with those obtained in vitro. This holds true for the age-dependence as well as for the influence of desmotropic compounds (Vogel and Denkel 1985).

Modifications of the Method

Cook et al. (1977) compared tension/extension ratio curves in vivo and in vitro in **rats** using the suction-cup method. Baker et al. (1988) described an apparatus for testing mechanical properties of normal and irradiated **pig** skin in vivo. In this system, the pads were attached to the skin of the pig rump with double-sided tape. They were moved apart at a predetermined rate using a motorized unit. Force was assessed using a S-shaped, center point, double beam load cell mounted on a movable crosshead. Displacement of the pads was measured using a floating core linear variable displacement transducer. Using this system, Baker et al. (1989) studied the effect of single doses of X-rays on the mechanical properties of pig skin in vivo.

Zeng et al. (2001) studied the biorheological characteristics of skin after expansion in **dogs**.

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Repeated Strain in Vivo

Purpose and Rationale

A special method has been developed to study the mechanical properties of rat skin after repeated strain in vivo and the course of recovery during different time intervals (Denkel 1983; Vogel and Denkel 1985; Vogel 1988).

Procedure

Male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

Tabs are fastened on the shaved back skin of anesthetized rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 50 % strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, 10th, 20th, 25th, and 30th cycles are recorded with faster paper speed in order to facilitate the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress–strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

Evaluation

Average curves of stress versus number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

Critical Assessment of the Method

Stress values decreased after repeated loading approximately with the logarithm of number of cycles. Stress values and the area under the curve were higher perpendicular than longitudinal to the body axis, as found in other in vivo experiments (Vogel and Denkel 1985). During the experiment, modulus of elasticity increased in both directions from the first to the fifth cycle. This may be explained by the so-called "conditioning" of connective tissue (Nementscheck et al. 1980). From the 5th to the 30th cycle, a decay of modulus of elasticity approximately with the logarithm of number of cycles was noted. The area under the curve calculated from stress values resembled closely the pattern of initial stress, indicating that this value dominates for the area under the curve and that the decay is only of secondary importance.

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In Vivo Recovery After Repeated Strain

Purpose and Rationale

A method was developed to study the in vivo recovery of mechanical properties of rat skin after repeated strain (Denkel 1983; Vogel and Denkel 1985; Vogel 1988, 1990, 1993a, b). Full recovery, i.e., ***restitutio ad integrum***, can be observed only by doing in vivo experiments but not by in vitro experiments.

Procedure

Male Sprague–Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

As in the experiment of repeated strain, tabs are fastened on the shaved back skin of anesthetized

rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 40 % strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, and 30th cycles are recorded with faster paper speed in order to facilitate the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress–strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

After the first run, the animals are returned to their cages with the tabs still in position. A second run of repeated strain is applied after different time intervals at 1, 6, and 16 h. The stress values in the second run are definitively lower than in the first run.

When calculated as percentage of the first run, the differences diminish with extended time intervals. By this in vivo method not only the mechanical recovery, which can also be observed in vitro, but also the biological recovery, i.e., the restitutio ad integrum, can be measured. Almost full recovery is found after 16 h.

Evaluation

Average curves of stress versus number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test. Stress of the first and 30th cycle as well as the area under the curve at the second run is expressed as percentage of the first run after the 1, 6, or 16 h interval. Furthermore, the hours to reach certain percent levels up to 100 % are calculated for the stress values during the first cycle and the area under the curve both longitudinally and perpendicularly to the body axis.

Critical Assessment of the Method

In the late phase of recovery, an even better restitutio ad integrum was found in animals treated with 300 mg/kg p.o. D-Penicillamine or 10 mg/kg s.c. prednisolone acetate. In contrast to other biomechanical parameters, the restoration process was found to be barely influenced by treatment with desmotropic compounds.

Surprisingly in studies on age dependence, restitutio ad integrum was the fastened in old animals. The ability of the dermis to reconstitute the fibrous structure is apparently not influenced negatively by age.

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Healing of Skin Wounds

Purpose and Rationale

Healing of skin wounds is a multiphasic process. The effect of drugs on the healing process was studied by measuring the mechanical strength at various time intervals after incision of the skin (Vogel 1970).

Procedure

Groups of 10–20 male Sprague–Dawley rats weighing 120 ± 5 g are used for each dosage or control and for each test interval. Under anesthesia, the dorsal skin is shaved and an approximately 3 cm long incision is made down to the fascia in a cranio-caudal direction in the dorso-lumbar region. Immediately afterwards, the wound is closed with wound clips. The rats are treated subcutaneously with test drugs beginning on the day of surgery. The clips are removed the day before the tensile strength is tested or on the 10th post-operative day at the latest.

For the measurement of wound tensile strength, the rats are sacrificed under anesthesia on days 3, 6, 9, or 12 after surgery. Wound clips are fastened in situ on each side of the incision and connected by means of threads with the load cell and the crosshead of an INSTRON-Instrument. Stress–strain curves are recorded at an extension rate of 5 cm/min. **Dehiscence of the wound** results in a sudden drop of the registered load. For experiments up to 3 weeks, bell-shaped skin strips are punched and tensile strength is tested, as described for evaluation of tensile strength in normal skin.

Evaluation

Mean values of tensile strength of skin wounds in drug treated groups at each time interval are compared with controls using Student's *t*-test. To visualize the influence of drugs on the healing process, the changes following treatment with drugs are expressed as percentage of vehicle treated controls.

A dose-dependent decrease following treatment with corticosteroids is found after immediate post-operative treatment and a dose-dependent increase in prolonged experiments. This data serve as comparative parameters for new test compounds

Modifications of the Method

Many authors measured tensile strength of skin wound to follow the course of wound healing under various conditions. Most experiments were performed in **rats** (Struck et al. 1967; Corps 1969; Holm-Pedersen and Zederfeldt 1971; Holm-Pedersen and Viidik 1972a, b; Andreassen et al. 1977; Andreassen and Oxlund 1987; Greenwald et al. 1993; Seyer-Hansen et al. 1993; Jyung et al. 1994; Adamson et al. 1996; Brunius and Ahren 1996; Oxlund et al. 1996; Paul et al. 1997; Taylor et al. 1997; Quirinia and Viidik 1991, 1998; Canturk et al. 1999; Gupta et al. 1999), with some of them also using the INSTRON-Instrument (Phillips et al. 1993; Maxwell et al. 1998; Jiminez and Rampy 1999; Kim and Pomeranz 1999).

A biphasic effect of corticosteroids on wound healing in rats was also found by Oxlund et al. (1979).

Forslund et al. (2006) published a comparative dose–response study of cartilage-derived morphogenetic protein (CDMP)-1, -2, and -3 for tendon healing in rats.

Furthermore, **mice** (Butler et al. 1991; Celebi et al. 1994; Kashyap et al. 1995; Vegesna et al. 1995; Gonul et al. 1998; Matsuda et al. 1998), **guinea pigs** (Bernstein et al. 1991; Drucker et al. 1998; Silverstein and Landsman 1999), **rabbits** (Sandblom 1957; Wu and Mustoe 1995; Pandit et al. 1998, 1999; Knabl et al. 1999; Xia et al. 1999), **dogs** (Howes et al. 1929; Al Sadi and Gourley 1977; Scardino et al. 1999), **pigs** (Langrana et al. 1983; Higashiyama et al. 1992; Chang et al. 1998; Fung et al. 1999), or **Yukatan miniature pigs** (Van Dorp et al. 1998) were used.

Using PPARα, β, and γ mutant mice, Michalik et al. (2001) demonstrated that PPARα and β are important for the rapid epithelialization of a skin wound and that each of them plays a specific role in this process. PPARα is mainly involved in the early inflammatory phase of the healing, whereas PPARβ is implicated in the control of keratinocyte proliferation.

PPARβ is discussed as a target for wound healing drugs (Tan et al. 2003).

Smith et al. (2001a, b) discussed the role of peroxisomes in dermatology.

In some studies simultaneously polyvinyl alcohol sponges were implanted in which collagen

accumulation was determined (Albina et al. 1993; Schaffer et al. 1996; Koshizuka et al. 1997; Bitar 1998; DaCosta et al. 1998; Witte et al. 1998).

Ågren and Mertz (1994) found excessive granulation tissue formation and retarded wound contraction in wounds in tight-skin mice.

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Protection Against UV Light

Howard Maibach

Contents

Protection Against UV Light

Purpose and Rationale

The mechanical parameters' skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity were used to evaluate the effects of UV irradiation and the prevention of skin damage in hairless mice (Alpermann and Vogel 1978; Vogel et al. 1981).

Procedure

Female hairless mice (strain mutant h/h) with an initial weight of 20 ± 2 g are used. Groups of 20 animals are treated topically with 0.025 ml of the sunscreen product or the base. One other group is not treated topically, but irradiated. A further group is neither treated nor exposed to UVB.

A special light source (Osram Ultra-Vitalux No. 2) is used. This light source is almost free from UVC. For UV exposure groups of 5, mice are immobilized under a fine wire net of 14 × 14 cm at a distance from the lamp, resulting in an irradiation energy of 14 mW/cm^2 for UVB and 33 mW/cm^2 for UVA. Irradiation is performed once a day except Saturday and Sunday during 4 weeks. Exposure time is 45 s in the first week, 60 s in the second, 90 s in the third, and 120 s in the fourth week. The animals are sacrificed under anesthesia for 72 h after the last irradiation. A flap

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of back skin is removed; skin thickness measured by use of calipers and two dumbbell-shaped samples punched with a width of 4 mm in the middle of the specimen. Stress–strain curves are recorded with an Instron instrument at a strain rate of 5 cm/min. Ultimate load and ultimate strain are recorded, and tensile strength as well as ultimate modulus of elasticity is calculated.

Furthermore, samples of skin are deep frozen for chemical analysis of collagen and soluble collagen fractions as well as of elastin (Vogel 1978).

Evaluation

The mean data from animals treated with sunscreen products are compared with those of animals treated with ointment base, irradiated and nonirradiated controls using ANOVA and Student's *t*-test.

Modifications of the Method

The effect of UV irradiation on skin has been studied by several authors both in man and in animals.

Wolska (1974) recommended the hairless mouse as an experimental model for evaluating the effectiveness of sunscreen preparations.

Cook et al. (1979) investigated the changes in the mechanical properties of intact **guinea pig** skin resulting from ultraviolet irradiation.

Lowe and Breeding (1986) evaluated several sunscreen preparations by UVB irradiation of mice. Sunscreen solutions were applied to the back of 5–8-week-old female skh/HR-1 mice one h before irradiation with FS40 sunlamps. Epidermal DNA synthesis assay was used to measure sunscreen efficacy. The amount of UVB required to achieve a 50 % suppression of radiolabeled thymidine incorporation in treated and untreated mice was compared as a ratio to determine the protective factor. Furthermore, epidermal ornithine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine 24 h after a single UVB exposure. Edema was estimated by the increase in double skin-fold thickness measured by a caliper at 24-h postirradiation.

Benrath et al. (1995) reported that substance P and nitric oxide mediate wound healing of ultraviolet photodamaged skin in **rats**.

Muizzudim et al. (1998) studied the effect of topical application of antioxidants and free radical scavengers on protection of hairless mouse skin, exposed to suberythemal doses of ultraviolet B three times a week and measured epidermal thickness by microscopy.

Fullerton and Keiding (1997) quantified UVB-induced erythema in depilated Hartley-strain albino male guinea pigs and compared the results with a tristimulus colorimeter (Minolta ChromaMeter CR-200) and two spectrophotometers (Minolta Spectrophotometer CM-508i and CM-2002). With the tristimulus colorimeter, the color is expressed in a three-dimensional color space, which simulates the perception of color by the human eye.

Nishimori et al. (2001) described degenerative alterations of dermal collagen fiber bundles in photodamaged human skin and UV-irradiated hairless mouse skin and the effects on decreasing skin mechanical properties and appearance of wrinkles.

Oba and Edwards (2006) studied the relationships between changes in mechanical parameters of the skin, wrinkling, and destruction of dermal fiber bundles caused by photoaging on hairless mice irradiated with UVB light.

For further description of ultraviolet erythema in animals, see chapter "▶ Anti-Inflammatory Activity."

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Transepidermal Water Loss

Howard Maibach

Contents

Transepidermal Water Loss (TEWL)

Purpose and Rationale

The physical basis for the measurement of transepidermal water loss (TEWL) is the diffusion law discovered by A. Fick in 1855.

$$dm/dt = -D \times A \times dp/dx$$

where:

A = surface (m^2)
m = water transported (g)
t = time (h)
D = diffusion constant (=0.0877 g/m × h × mm Hg)
p = vapor pressure of the atmosphere (mm Hg)
x = distance from skin surface to point of measurement

Most of the recent studies are performed with commercially available instruments, such as Tewameter TM 210 (Courage and Khazaka, Cologne, Germany) and Evaporimeter EP1 (Servo Med AB, Vallingby, Sweden), primarily designed for use in human beings.

Several studies were performed in **rats** (e.g., in hairless rats (Doucet et al. 1991; Vanbever et al. 1998), neonatal rats (Wickett et al. 1995) and in rats with experimentally induced condition of essential fatty acid deficiency (Basnayake and Sinclair 1956; Prottey et al. 1976; Hartop and

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Prottey 1976; Hartop et al. 1978; Penneys 1992; Yamaguchi et al. 1998; Meguro et al. 2000)); **hairless mice** (Grubauer et al. 1989; Mortz et al. 1997; Sato et al. 1998), essential fatty acid-deficient hairless mice (Menton 1968; Lowe and Stoughton 1977), platelet-type 12-lipoxygenase-deficient mice (Johnson et al. 1999), and keratin 10-deficient mice (Jensen et al. 2000); **guinea pigs** (Frosch et al. 1993; Fuchs et al. 1998; Sagiv et al. 2000); **pigs** (Zhao and Singh 1999); and **Yucatan micro-swine** (Gendimenico et al. 1995).

Löffler et al. (2001) evaluated irritant skin reaction in mice by measurement of auricular transepithelial water loss.

Procedure

BALB/c mice aged 10–16 weeks are sensitized on day 0 by applying 50 µl 2,4-dinitroflurobenzene (DNFB) solution (0.5 % diluted in acetone/olive oil 4:1) to the shaved dorsal neck region. On day 5, the dorsal surface of one ear is challenged by applying 10 µl DNFB 0.3 %; the other side is treated by acetone/olive oil alone.

Transepithelial water loss is measured with an evaporimeter (Tewameter TM 210, Courage and Khazaka, Cologne, Germany) with a measuring cylinder into which the whole ear of the mouse can be placed. The measurements are performed under isoflurane inhalation anesthesia. Measurements are performed before and 24 h after challenge.

Evaluation

The comparison between the treated and untreated ears is calculated by the Wilcoxon test. The comparison between the tested groups is calculated by the Kruskal–Wallis *H* test, after the Mann–Whitney *U* test.

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Skin Hydration

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Contents

Skin Hydration

Purpose and Rationale

Techniques to characterize the barrier function of the skin include a number of noninvasive methods to measure moisture content and loss through the skin surface. One of these measurements is the determination of skin hydration using a method known as corneometry (Bilchmann and Serup 1988). This technique determines the capacitance of the skin due to its behavior as a dielectric medium and assesses a 10–20-μm thickness of the stratum corneum. The method has been compared with other techniques (Van Neste 1991; Fluhr et al. 2001) and applied to evaluate dermatological and cosmetic products in human volunteers (Zuang et al. 1997; Singh et al. 2001; Yilmaz and Borchert 2006).

Jensen et al. (2000) studied skin hydration in keratin-10-deficient mice. Kappes et al. (2004) used this method to investigate the quality of human xenografts on Severe Combined Immunodeficiency (SCID) mice. Hester et al. (2004) evaluated this method in dogs.

Procedure

Normal adult dogs (female Beagle and male hound-cross dogs) were used. Hair was clipped from the left inguinal region of each dog 1 week before measurements to minimize any effects of

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recent hair clipping. Because hair grows sparsely in the inguinal region, only a minimal amount of clipping was necessary. To measure skin hydration, a Corneometer 825 m (Courage + Khazaka Electronic) was used. Animals were gently restrained in a right-lateral recumbency on a padded floor and were given time to relax so that movements could be minimized during data collection. The probes were held in place manually, and readings were taken for 20 s each. The data were stored electronically using a laptop computer and appropriate software. Values representing an average of at least ten determinations were used to calculate average values.

Evaluation

Statistical analyses were conducted by repeated-measures ANOVA for main time-and-breed effects and time × breed interactions with Tukey's multiple comparisons performed at P values <0.05.

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Influence on Hair Growth

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Contents

Influence on Hair Growth

Purpose and Rationale

Several mutations affecting hair growth or quality have been reported in the laboratory mouse (Holland 1988), among them "alopecia" (Dicke 1955), "alopecia periodica" (Tutikawa 1952), "crinkled" (Falconer et al. 1951), "frizzy" (Falconer and Snell 1952), "fuzzy" (Mann 1964), "rhino" (Mann 1971), "naked" (Raphael et al. 1982), "nude" (Flenagen 1966; Buhl et al. 1990; Militzer 2001), "ragged" (Slee 1962), androchronogenetic alopecia (AGA) mouse (Matias et al. 1989), aging C3H/HeJ mice (Sundberg et al. 1994), SPF-ASH mice (Shimada et al. 1994), "nackt" (Benavides et al. 1998), and transgenic mice overexpressing homeobox gene MSX-2 (Jiang et al. 1999). Kligman (1988, 1998) used the Skh-hairless mouse as a model for evaluating promoters of hair growth.

Procedure

Six litters of Skh-hairless-1 albino mice with 5–8 animals are housed, with their mothers, in individual plastic cages. Food and water are supplied ad libitum. Beginning at 1 week of age, topical treatment is applied: two litters dorsal application of low dose (e.g., 0.2 % minoxidil), two litters dorsal application of high dose, and two litters abdominal application of high dose. Application

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is performed once daily five times a week at a rate of 20 µl/cm^2 of skin using a digital micropipette. Two control litters receive no treatment. All treatments continue for up to 17 weeks, after which mice are sacrificed and grossly examined for signs of toxicity.

Mice are observed daily for hair growth and weighed each week. Photographs are taken when hair growth appears to be maximum. At the time of maximal hair growth, two or three representative neonates from the treated and untreated groups are sacrificed for skin biopsies; all remaining mice continue on treatment to assess the extent of a third pelage. Biopsy specimens are fixed in formalin and stained with hematoxylin and eosin.

Evaluation

In transverse sections, all active hair matrices or viable follicles, which contain keratinized located in the subcutis, are counted. Fourteen to 16 contiguous fields are examined over a surface distance of 1 cm at 250× magnification. Mean values of the groups are compared using Student's *t*-test.

Modifications of the Method

De Brouwer et al. (1997) studied the effects of a nonsteroidal antiandrogen on human hair production by balding scalp grafts maintained in testosterone-conditioned **nude mice**. Samples of balding human scalp were grafted onto the left flank of nude mice (bal/c, nu/nu). All mice received a topical application of 300 µg/10 µl testosterone propionate on the non-grafted flank. Testosterone conditioning started 5–7 weeks after grafting and went on until the end of the experiment, once daily 5 days a week. Antiandrogen/placebo treatment was started before hair production was visible. Hair production potential was assessed according the number and diameter of the hairs.

A hair loss mutation on mouse chromosome 19, called **scraggly**, was described by Herron et al. (1999).

Several authors studied **chemotherapy-induced alopecia in mice, rats, and rabbits** (Powis and Kooistra 1987; Paus et al. 1994a, b; Cece et al. 1996; Sredni et al. 1996).

The **Dundee experimental bald rat (DEBR)** was used as model for alopecia areata (Oliver and Lowe 1995; McElwee et al. 1997).

Kimura (1996) underlined the usefulness of studies in **hairless descendants of Mexican hairless dogs** in dermatological science.

The **balding stump-tailed macaque** is recommended as a model for androgenetic alopecia (Brigham et al. 1988; Diani et al. 1995; Pan et al. 1998).

The stimulating effect of drugs on hair growth has been studied using in vitro *methods*.

Kurata et al. (1996) investigated the effect of hypertrichotic agents on follicular from macaque and human skin and nonfollicular cells (normal keratinocytes and dermal fibroblasts) in vitro. Minoxidil induced a significant increase in all follicular cells in a dose-specific manner, whereas nonfollicular cells showed no response.

Lachgar et al. (1996) found inhibitory effects of Basic fibroblast growth factor (bFGF), Vascular endothelial growth factor (VEGF), and minoxidil on collagen synthesis by cultured hair dermal papilla cells from rat vibrissa follicles.

Boyera et al. (1997) described biphasic effects of minoxidil on the proliferation and differentiation of normal human keratinocytes obtained from microdissected hairs or from plucked hairs. Minoxidil stimulated human keratinocyte proliferation at micromolar doses, while antiproliferative, pro-differentiative, and partially cytotoxic effects were observed with millimolar concentrations.

Sato et al. (1999) reported that minoxidil increases 17β-hydroxysteroid dehydrogenase and 5α-reductase of cultured human dermal papilla cells from balding scalp.

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Cutaneous Microcirculation

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Contents

General Considerations

Purpose and Rationale

Various techniques are used to determine cutaneous blood flow, such as radioactive microspheres, xenon clearances, plethysmography, laser Doppler velocimetry (Flagrell 1986; Guy et al. 1985), and reflectance spectrophotometry (Kimura et al. 1988; Kakizoe et al. 1992).

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Laser Doppler Velocimetry

Purpose and Rationale

Laser Doppler velocimetry and laser Doppler flowmetry are essentially identical procedures. With these methods, light is transmitted from a helium-neon laser source in the instrument to the skin via an optical fiber. The laser provides light of single frequency (wavelength 632.9 nm) and allows the Doppler effect to be exploited. The incident radiation enters the skin tissue and is multiply scattered and reflected by non-moving components and by the mobile red blood cells that are encountered as the radiation penetrates to a depth of 1–1.5 mm. A portion of scattered/reflected incident radiation exits the skin and is collected by second or third optical fibers that carry the light back to the instrument. The returning radiation falls on a photodetector and is converted to an electrical signal. Stationary skin tissue reflects and backscatters light at the same frequency as the incident source. Erythrocytes moving with certain velocity, however, reflect radiation that is slightly frequency shifted, the shift increasing with increasing velocity.

Hirkaler and Rosenberger (1989) described simultaneous two-probe laser Doppler velocimetric assessment of topically applied drugs as a simple, non-invasive method for the determination of cutaneous blood flow in anesthetized rats.

Procedure

Male Sprague–Dawley rats (400–500 g) are anesthetized with urethane (1.6 g/kg), the abdominal region carefully clipped and the remaining hair removed with a commercially available depilatory. Phthalate buffer (pH 4.0) is gently applied to neutralize the effects of the depilatory cream. The rat is placed on its back and allowed to stabilize for approximately 15 min. Cutaneous blood flow is measured using two standard Medpacific LD 5000 capillary perfusion monitors and probes (1.9 cm diameter). The probes are modified to allow application of the drug without removal of the probe. This is achieved by enlarging the center opening of the adhesive pad to 1 cm, which creates a drug well of approximately 5 µl vol. The drug is introduced through a short length of polyethylene tubing (PE10) held in place by the adhesive pad and placed slightly off center to avoid interference with the sensing device.

The modified probes are attached to the lower abdominal region of the rat, approximately 1–2 cm from the midline. Cutaneous blood flow is recorded continuously from 30 min prior to drug application and for 5 h post-dosing. Various doses of standard (0.015–0.75 mg/kg minoxidil) or test compound or control vehicle (propylene glycol 15 %, ethyl alcohol 65 %, water 20 %) are applied. At the end of the experiment, the animals are sacrificed.

Evaluation

Post-dosing values are expressed as % change from control and analyzed using Student's paired *t*-test.

Modifications of the Method

Knight et al. (1987) measured microvascular blood flow by a laser Doppler flow meter in **rabbit** epigastric island flaps made ischemic for various intervals of time.

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Measurement of Skin Microcirculation by Reflectance Spectroscopy

Purpose and Rationale

Kimura et al. (1988) and Kakizoe (1992) developed reflectance spectrophotometric measurement to analyze the microcirculation of the skin in real time. The light reflected from the skin tissue containing information about hemoglobin, the injected dye, and other data is continuously analyzed into the relative absorption spectrum, and the changes in relative absorption values at specific wavelengths are used as indices of oxyhemoglobin content and capillary permeation.

Procedure

Male Wistar rats weighing 300–400 g are anesthetized with pentobarbital (40 mg/kg i.p.). The skin of the back is shaved and the hair removed by a commercial hair remover. A polyethylene catheter (PE10) is inserted into the femoral artery to measure blood pressure on a polygraph. Another catheter is inserted into the femoral vein for injection of drugs or Evans blue.

To obtain the relative absorption spectra, a spectro-multichannel-photodetector system (MCPD 110; Otsuka Electronics Co., Ltd., Osaka, Japan) is used. White light (halogen lamp 150 W) is projected onto the skin through an optical fiber. The light reflected from the skin is transmitted to the detector system via another optical fiber. The positions of the optical fibers are fixed independently. Each fiber is attached to a steel arm on the stand, and the height of the arms is adjusted so that the top of the fibers can be in gentle contact with surface of the skin. The angles of the fibers are adjusted so that the relative absorbance spectrum with the best peaks of oxyhemoglobin can be obtained. The reflected light is passed through a slit and illuminated on the grating surface to obtain the spectrum. Following amplification by an image intensifier, the component wavelengths are sampled by photodiode array in a short time interval. The relative absorbance spectra from the skin against the spectra for the white light are obtained at wavelengths ranging from 450 to 643 nm, arranging the initial relative absorbance at 640 nm to zero. The sampling time is 50 ms. The average of 10 measurements is indicated at 1-s intervals on the output unit. The spectrum at a point of time and time-dependent changes in relative absorbance values at selected wavelength are shown on the display and registered by an X-Y recorder.

After achieving a steady state under anesthesia, the relative absorbance spectrum is obtained from the skin, and two peaks at a wavelength of about 540 and 577 nm, corresponding to those of oxyhemoglobin, are observed. The relative absorbance spectrum is measured at the posterior part of the back near the backbone because the shape is relatively flat in this region and it is easy to fix the optical fibers.

To standardize the changes in oxyhemoglobin content in skin tissue, graduated doses of noradrenaline are injected intravenously and the effect on relative absorbance is measured.

To measure the content of oxyhemoglobin and permeation of the capillaries at the same time, 1.5 ml/kg 0.05 % Evans blue solution is injected intravenously 10 min before measurement. Histamine (0.3–100 μg/50 μl/site) is injected intradermally into the skin of the back, and measurement is started 1 min after the injection. The changes in the relative absorbance values at 540 nm, an absorption peak in the oxyhemoglobin spectrum, and at 610 nm, an absorption peak in the Evans blue spectrum are measured for 15 min, and the absorption values at baseline and the point of maximum change are compared.

Evaluation

Results are expressed as mean ± SEM. Comparisons are made using the Student's *t*-test or Mann-Whitney's *U*-test.

Modifications of the Method

Hertel (1986, 1992) measured cutaneous microcirculation in the pinnal of conscious rats.

Erythrocyte flow velocities were measured by the "flying spot technique" (Tyml and Ellis 1982), and the diameters of the capillaries were measured from a monitor with a ruler.

Da Costa et al. (1992) measured the fluctuations in the diameter of selected arterioles in the cutaneous microcirculation of **Syrian golden hamster** dorsal skin flap chambers. These ranged in size between 10 and 70 μm at different branching order sites, before burn, at the same site after burn and after injection of drugs.

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Laser Doppler Velocimetry

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Isolated Perfused Skin Flap

Howard Maibach

Contents

Isolated Perfused Skin Flap

Purpose and Rationale

Riviere et al. (1986) and Riviere and Monteiro-Riviere (1991) described the isolated perfused porcine skin flap as an in vitro model for percutaneous absorption and cutaneous toxicology studies. This group has used the model for various purposes (Riviere et al. 1995; Vaden et al. 1996; Baynes et al. 1997; Inman et al. 2003; Monteiro–Riviere et al. 2003).

Procedure

Weanling female Yorkshire pigs weighing 20–30 kg are acclimated for 1 week prior to the study. The pigs are housed on elevated floors and provided water and 15 % protein pig and sow pellets ad libitum.

The isolated perfused porcine skin flap is a single, axial pattern tubed skin flap obtained from the ventral abdomen of female weanling swine. Two flaps per pig, each lateral of the ventral midline, may be obtained in a single surgical procedure. The surgery involves two steps: creation of the flap in stage I and harvest in stage II. Pigs weighing approximately 20–30 kg are premedicated with atropine sulfate and xylazine hydrochloride, induced with ketamine hydrochloride, and inhalation anesthesia is maintained with halothane. Each pig is prepared for routine

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surgery in the caudal abdominal and inguinal region, and a 4 × 12 cm area of skin is demarcated. Following incision and scalpel dissection of the subcutaneous tissue, the caudal incision is apposed and sutured and the tubed skin flap edges trimmed of fat and closed. Two days later, a second surgical procedure is used to cannulate the artery and harvest each of these skin flaps. The isolated perfused porcine skin flap is then transferred to a custom-designed temperature- and humidity-regulated perfusion chamber. A computer monitors perfusion pressure, flow, pH, and temperature. The flaps are perfused for 1 h prior to dosing with a modified Krebs/Ringer bicarbonate buffer (pH 7.4) containing bovine serum albumin, glucose, penicillin G amikacin, and heparan, during which 1.0-ml arterial and 3.0-ml venous samples are collected to assess glucose utilization. Once flap viability is confirmed, the perfusion is interrupted and each flap is removed from the chamber. Flap perfusion is resumed, with the venous perfusate sampled every 15 min for the first 2 h and every 30 min thereafter for the 8-h duration. Absorption is defined as the total amount of radioactivity detected in the perfusate over an 8-h perfusion period and expressed in units of % of applied radioactivity or mass absorbed. Penetration is defined as the sum of total radioactivity found in the perfusate (absorption), stratum corneum, fat, and dosed skin samples. Penetration is the maximum amount of test compound that could be absorbed into the systemic circulation if all test compounds within the skin ultimately were absorbed over a prolonged period of time. Penetration is also relevant to the direct toxic effect within the skin.

Evaluation

Total recovery is calculated as the sum of penetration plus the amount of radioactivity found in the washing samples. All data are expressed as mean ± standard error of the mean (SEM). The significant differences ($P < 0.05$) across each set of treatments are determined by ANOVA in PC SAS (SAS Institute, Cary, N.C., USA; version 8.01). Significant differences ($P < 0.05$) between each mean and paired nonaqueous counterpart are determined using a Student's *t*-test.

Modifications of the Method

Pickens et al. (1994) studied the effect of extended perioperative pentoxifylline on random skin flap survival in Yorkshire pigs.

Skin flap models have been described for several animal species, mostly in **rats** (Hahn et al. 1993; Stadelmann et al. 1998; Yenidunya et al. 1998; Cottler et al. 1999; Yang and Morris 1999; Jones et al. 2001; Oksar et al. 2001; Lay et al. 2003; Mittermayer et al. 2003; Ozkan et al. 2004; Agaoglu and Siemionow 2005; Ulusal et al. 2005; Hosnuter et al. 2006). Lineaweaver et al. (2004) described the influence of vascular endothelium growth factor and surgical delay on survival of the transverse rectus abdominis myocutaneous flap in rats. The model is based on the right abdominis rectus muscle as carrier and inferior epigastric vessels as vascular pedicle. Following induction of general anesthesia, the abdominal regions were shaved, and a rectangle measuring 3 × 8 cm was drawn onto the upper abdominal area of the rats. The borders of the skin paddle of the proposed transverse rectus abdominis myocutaneous flap were cut down to deep fascia. The branches of both superficial epigastric vessels joining the flap were ligated and cut. Both rectus abdominis muscles were divided at the superior border of the skin paddle. The superior deep epigastric vessels of both rectus abdominis muscles were divided. The skin incision was then closed using 4–0 nylon sutures.

Barker et al. (1989) and Minh et al. (2002) described skin flap models in **mice**.

Degner et al. (1996) described a medial saphenous fasciocutaneous free flap in **dogs**.

Teunissen et al. (2004) evaluated the primary critical ischemia time for the deep circumflex iliac cutaneous flap in **cats**.

Bristol et al. (1991) reported preparation and metabolic parameters of an isolated perfused skin flap in **horses**.

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Part XVIII

Functional Food

Overview of What Functional Foods Are

Bobbie Bradford

There is no globally accepted definition of what constitute functional foods, but in broad terms they can be described as foods that provide benefits beyond basic nutrition. The main drivers for the development of functional foods have included an increased focus on the role of certain foods and food ingredients in disease prevention and risk reduction, particularly for the developed world, along with a growing desire for "self-medication" with consumers much more likely to select foods based on their knowledge of healthy attributes ascribed to specific nutrients. Additional factors are the increase in the age of the population in the developed world and the propensity of obesity and associated so-called lifestyle diseases such as type 2 diabetes. A survey carried out on Functional Foods/Foods for Health in 2011 revealed that 87 % of consumers agreed that certain foods have health benefits that go beyond basic nutrition with 80 % agreeing that they can help to maintain health and wellness (IFIC 2011). Consumers agreed that functional foods could help to improve heart disease, circulation, bone health, and type 2 diabetes, while other health benefits were also linked with functional foods including immune, digestive, and eye health.

While in most regions functional foods are not specifically defined, in 1991 the Ministry of Health, Labour and Welfare (MHLW) in Japan was the first to use a more precise terminology for functional foods and define the requirements needed to obtain this classification whereby foods could bear a specific logo and claim (Fig. 1).

These were termed Food for Specified Health Use (FOSHU) and were classed as "foods composed of functional ingredients that affect the structure and/or function of the body and are used to maintain or regulate specific health conditions, such as gastrointestinal health, blood pressure, and blood cholesterol levels" (http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html). In other parts of the world, functional foods are considered more as a concept rather than as a specific food category. In 1998 the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) published the outcome of a consortium with a working definition of functional foods as "foods which have been satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Diplock et al. 1999; FUFOSE 1999). Included within this definition was a clear statement that "functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet; they are not pills or capsules, but part of a normal food pattern." This clearly differentiated functional foods from other

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Fig. 1 Symbol allowed in Japan for FOSHU foods

products, such as health supplements in tablet form. In contrast to this, the FOSHU classification does not discriminate in this way and therefore also encompasses products in the form of capsules and tablets. In 1999 the American Dietetic Society published a position statement describing functional foods as "including whole foods and fortified, enriched, or enhanced foods which have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels" (Position of The American Dietetic Association 2009). In China a provision for functional foods administration was circulated by the Ministry of Health in 1996 (Yang 2008) which simply defined functional foods as those which have special health functions. In 2005 the guideline of registration for functional foods was updated by the State

Table 1 Global definitions of functional foods

Definition	Reference
"Foods containing ingredient with functions for health and officially approved to claim its physiological effects on the human body" *FOSHU(Food for Specified Health Uses)* is intended to be consumed for the maintenance/promotion of health or special health uses by people who wish to control health conditions, including blood pressure or blood cholesterol	FOSHU: Japanese Ministry of Health, Labour, and Welfare
All foods are defined as *functional* at some physiological level because they provide nutrients or other substances that furnish energy, sustain growth, or maintain/repair vital processes. However functional foods move beyond necessity to provide additional health benefits that may reduce disease risk and/or promote optimal health	American Dietetic Association (ADA 2009)
Any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains	Institute of Medicine's Food and Nutrition Board (IOM/FNB, 1994)
Satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease Not pills or capsules, must remain foods, and demonstrate effects in amounts that can normally be expected to be consumed in the diet	The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE 1999)
"Functional foods" are foods or dietary components that may provide a health benefit beyond basic nutrition	International Food Information Council (IFIC 2011)
"Food similar in appearance to conventional food, which is consumed as part of the usual diet and has demonstrated physiological benefit and/or reduces the risk of chronic disease beyond basic nutritional functions"	Health Canada 1998
Foods "similar in appearance to conventional food and intended to be consumed as part of a normal diet, but modified to serve physiological roles beyond the provision of simple nutrient requirements"	Food Standards Australia and New Zealand (FSANZ)
Foods which are intended to be consumed as part of the normal diet and that contain biologically active components which offer the potential of enhanced health or reduced risk of disease	Subirade (2007)

Food and Drugs Administration (SFDA) in China, and the definition was extended as "Health (functional) foods means that a food has special health functions or is able to supply vitamins or minerals. It is suitable for consumption by special groups of people and has the function of regulating human body functions but it is not used for therapeutic purposes. And it will not cause any harm whether acute or subacute or chronic" (Yang 2008).

Thus it can be seen that there are a range of definitions, but typically they all state that there must be some proven health benefit above and beyond the nutritional benefits provided by food. Some of the more commonly used definitions are provided in Table 1, but even this is not complete and more extensive lists can be found elsewhere (Bigliardi and Galati 2013).

From the broad and varied definitions, it is clear that the types of foods that may be considered as functional foods can range from staple foodstuffs, such as carrots rich in the antioxidant beta-carotene, all the way through to isolated, purified preparations of active food ingredients (Fig. 2). In essence they can be classed into three broad categories:

- Unmodified or conventional foods which naturally contain bioactive components are the simplest form of functional foods. Many fruits and vegetables, grains, dairy products, fish, and meat will contain bioactive components that provide health benefits in addition to basic nutrition. Examples of these include lycopene in tomatoes, ellagic acid in raspberries, beta-glucans in oat bran cereal, antioxidant vitamins in citrus fruits, omega-3 fatty acids in oily fish, and calcium in dairy products.
- Modified foods containing enhanced, enriched levels or fortified with bioactive components. Examples of this include eggs containing increased levels of omega-3 fatty acids, calcium-fortified orange juice, tomatoes with

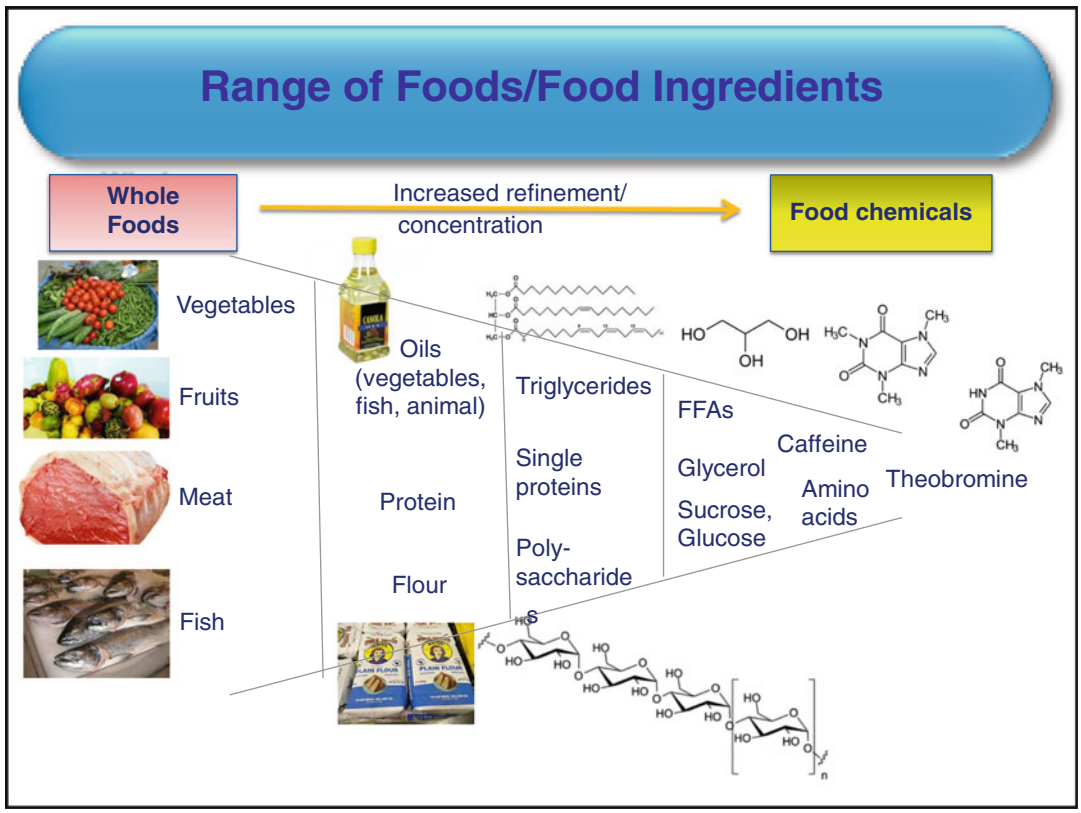

Fig. 2 Range of foods/food ingredients that may be included within functional food definition

high levels of lycopene, and bread made with flour enriched with folate.
- Isolated or purified food ingredients. Examples of these include isoflavones isolated from soy, phytostanols/sterols from vegetable oils, and omega-3 fatty acids extracted from oily fish.

Sitting outside of these definitions are other types of foods such as medical foods which are usually prescribed by a medical practitioner and are intended for the specific management of a disease or condition which requires distinct nutritional requirements. Examples include supplements free of phenylalanine (for patients with phenylketonuria) or sugar-free products for diabetics. When such foods are sold direct to the consumer, they are termed foods for special dietary use. Examples of these types of foods include infant formula or foods which do not contain specific ingredients such as gluten-free or lactose-free foods.

Health benefit claims for functional foods can range from those based on effects of nutrient content through to specific relationships between components in the diet and reduced risk of disease, supported by the weight of credible scientific evidence (see chapter "▶ Benefit Claims of Functional Food").

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Regulation of Functional Foods and Ingredients

Bobbie Bradford

Contents

Legislation usually does not consider functional foods as a specific category requiring targeted regulation, and the regulations covering typical food ingredients apply although there are some exceptions such as in Japan and China. A brief overview of the main legislation covering (functional) foods in different regions is provided in the following subsections.

Japan

In Japan the MHLW (Ministry of Health, Labour and Welfare) was the first regulatory agency to formally recognize functional foods as a food category. The Food for Specified Health Use (FOSHU) system was adopted to regulate functional foods in 1991, and the first foods, a hypoallergenic rice product and low-phosphorus milk for patients, were approved in 1993. In order to sell foods as FOSHU, they must be assessed for both safety and efficacy and be approved by the MHLW (http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html).

The requirements for FOSHU approval are as follows:

- Effectiveness on the human body is clearly proven (mechanism of the function itself or of the functions based on in vitro and/or human studies, amount of intake, scientific evidence for claiming the specific use)

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- Absence of any safety issues (animal toxicity tests, confirmation of effects in the cases of excess intake, history of safe use)
- Use of nutritionally appropriate ingredients (e.g., no excessive use of salt.)
- Guarantee of compatibility with product specifications by the time of consumption
- Established quality control methods, such as specifications of products and ingredients, processes, and methods of analysis (physicochemical properties of the functional component and analytical methods, stability tests)

These requirements are further categorized as qualified or standardized FOSHU:

1. Qualified FOSHU:
   Food with health function which is not substantiated on scientific evidence that meets the level of FOSHU or the food with certain effectiveness but without established mechanism of the effective element for the function will be approved as qualified FOSHU.
2. Standardized FOSHU:
   Standards and specifications are established for foods with sufficient FOSHU approvals and accumulation of scientific evidence. Standardized FOSHU is approved when it meets the standards and specifications.
   A third categorization was added in 2005:
3. Reduction of disease risk FOSHU
   Reduction of disease risk claim is permitted when reduction of disease risk is clinically and nutritionally established in an ingredient.

To obtain FOSHU approval, a dossier is put together by the marketing company which is then submitted to the Consumer Affairs Agency. The dossier is examined to approve the claims on effectiveness and assessment of safety, after which the MHLW makes a final check to ensure that the labeling and claims do not violate the Pharmaceutical Affairs Act before final approval is granted by the Consumer Affairs Agency.

Since 2005 FOSHU foods have been classified as Foods with Health Claims – the other group of foods within this classification are termed Foods for Nutrient Function Claims (FNFC). FNFC allows functional claims for 12 vitamins and five minerals based on international recognition of the scientific evidence relating to their function and benefits (Shimuzu 2003).

China

In China, a similar process occurs whereby the State Food and Drug Administration (SFDA) has overall responsibility for the regulation of functional foods. The "guideline for registration for functional foods" is the main directive document, and this includes the general principles, application, examination and approval, raw materials and supplementary materials, labels and specifications and testing, and reregistration and reexamination. Other areas included are hygiene requirements for functional foods and limits on contaminants such as heavy metals and microorganisms. Good manufacturing practice is specified to lay down the conditions for manufacturing and processing, raw materials, storage, transportation, management, etc. (Yang 2008). There are four main rules for functional food assessment in China (Table 1).

USA

In the USA a functional food can be regulated as a conventional food, a food additive, dietary supplement, drug, medical food, or food for special dietary use depending on how the manufacturer chooses to market the product and, in particular, the type of claims used on the package label or in labeling (Crowe and Francis 2013). These categories fall under the Federal Food, Drug and Cosmetic Act of 1938 (FDCA) which makes no provision for a definition of functional foods. The system of Generally Recognized As Safe (GRAS), for approval of food ingredients, was added to the FDCA in 1958. A substance is considered to be GRAS, if there is "agreement among experts qualified by scientific training and experience to evaluate its safety, as having been

Table 1 Regulations for functional foods in China (Adapted from Yang (2008))

Regulation	Description
The standard functional assessment procedures and methods of health foods	Basic requirements for test samples, standard procedures of assessment including animal and human tests, biomarkers, determinants, and judgment indicators for each method
The standard toxicological assessment procedures and methods of health foods	Basic requirements for test samples, standard procedures for safety assessment tests such as acute toxicity and mutagenicity (Ames test), 30- or 90-day feeding test, etc.
Regulation on nutrient supplements	Definition of nutrient supplements, the amount and composition of vitamins and minerals that can be used in nutrient supplements
The standard analytic methods for functional components	Basic requirements and components of herbs, foods, or extracts that must be tested if these are used in the product and the analytical methods for 100 plant substances

adequately shown through scientific procedures (or, in the case as a substance used in food prior to 1 January 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use." The GRAS expert panel examines the data relevant to assessing the safe use of a substance which includes test data, exposure (consumption) data, manufacturing information, and specifications. There are three types of GRAS.

Different Types of GRAS

- **GRAS affirmation by FDA**
  - Reviewed and approved by FDA
  - Listed in Federal Regulations
- **GRAS self-affirmation**
  - No review by FDA.
  - Qualified experts (panel) agree after review of the data.
  - Can be challenged by FDA.
- **GRAS notification**
  - Self-affirmation with notification to FDA.
  - FDA does not approve but can object to the self-affirmation conclusion.
  - Letter of no objection (90 days).

All GRAS notifications are published on the FDA website (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices) so that consumers and manufacturers can examine the approval letters and supporting dossiers. There are several elements of the GRAS evaluation (Fig. 1).

Europe

In Europe there is no legislation regarding safety of functional foods as such, these aspects are already covered by existing EU regulations (Magnuson et al 2013). However, foods with health claims must consider the overall dietary significance, including the amount and frequency of consumption, any potential interactions with other dietary constituents, any impact on metabolic pathways, and potential for adverse effects, including allergy and intolerance factors. Pre-approval or authorization is required for certain classes of foods or food ingredients, for example, chemical additives (Regulation EC 1333/2008;), flavorings (Regulation EC 1334/2004), genetically modified organisms (GMOs) for food and feed (Regulation EC 1829/2003), or novel foods. Typically, functional foods will fall under the legislation in place to regulate novel foods. Novel foods are considered by the European Commission (EC) as "foods and food ingredients that have not been used for human consumption to a significant degree in the EU before 15 May 1997" (http://ec.europa.eu/food/food/biotechnology/novelfood/index_en.htm). Novel foods must be "safe for consumers" and "properly labeled to not mislead consumers." In order to market a novel food or ingredient, companies must apply to an EU country authority for authorization, presenting the scientific information and safety assessment report. Following this the competent authority decides if additional assessment is necessary.

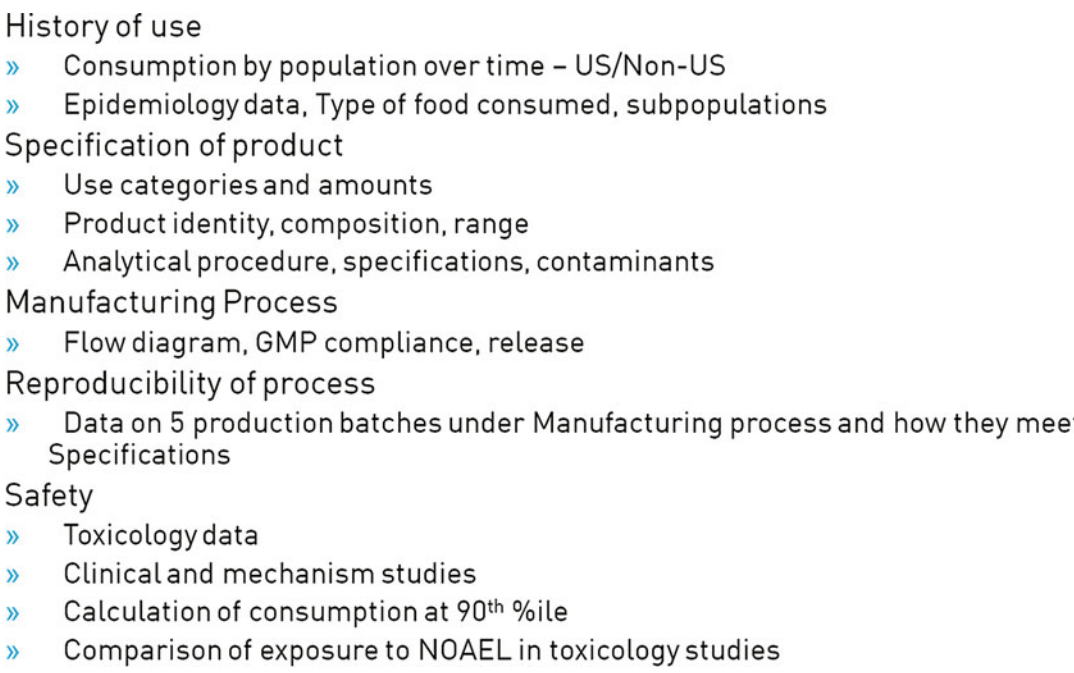

Fig. 1 Elements of the GRAS evaluation

If none is deemed necessary, then the product may be marketed if the EC and other EU countries do not object. Before approval is given, the EC asks the Standing Committee on Food Chain and Animal Health for an opinion. Authorization of marketing a novel food covers:

- Conditions of use
- Designation of novel food or novel food ingredient
- Specification and labeling requirements

The information required as part of the novel foods submission includes:

- The specification of the novel food
- Effects of any production process
- History of the organism used as the source of the novel food
- Anticipated intake or extent of use
- Information from previous human exposure to the novel food or its source
- Nutritional information
- Toxicological information
- Microbiological information

A novel food or ingredient may be marketed through a simplified procedure called "notification." The company notifies the EC about their marketing a novel food or ingredient based on the opinion of a food assessment body that has established "substantial equivalence." To show substantial equivalence, the food or ingredient is compared with regard to composition (such as the source organism and preparation method), nutritional value, metabolism, intended use (such as a food ingredient or supplement), and levels of undesirable substances (such as contaminants, mycotoxins, and allergens). Decisions which have been made on novel foods or ingredients which might affect public health are sent to the Scientific Committee for Food.

Labeling of novel foods and ingredients may be in addition to those laid down under the general legislation required by the EU. It may mention the characteristics of the food including the composition, nutritional value and intended use, materials included that might affect the health of some individuals, and materials that give rise to ethical concerns.

The safety of foods is reviewed on an ongoing basis, and if an EU country considers a novel food

or ingredient a risk to human or environmental health, it may suspend or temporarily restrict the marketing and use of this on their territory. The country must inform the EC which can either extend the suspension/restriction to all EU countries or ask that it is repealed in the country.

Other regions of the world have similar legislation covering foods and food ingredients. For example, in Canada the *Food and Drugs Act* is the primary legislation governing the safety and nutritional quality of food sold. Its scope includes food labeling, advertising, and claims; food standards and compositional requirements; fortification; foods for special dietary uses; food additives; chemical and microbial hazards; veterinary drug residues; packaging material; and pesticides (http://laws-lois.justice.gc.ca/eng/acts/F-27/index.html). In Australia and New Zealand, foods are governed by a Food Standards Code (http://www.foodstandards.gov.au/code/Pages/default.aspx). Russian legislation covers biologically active food supplements (BAFS) with manufacturers responsible for product quality, safety, and efficacy as well as for the truthfulness and completeness of information about their products under the Federal Law on Foods Quality and Safety (Tutelyan et al 2014).In addition the Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) have established "The Codex Alimentarius Commission" to be in charge of developing global food standards (http://www.codexalimentarius.org/). An excellent review of food regulations in a number of countries and jurisdictions is provided by Magnuson et al. (2013), and for more details, see *Nutraceutical and Functional Food Regulations in the United States and Around the World* (Bagchi 2014).

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Benefit Claims of Functional Food

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Consumers are increasingly aware and interested in the health benefits claimed by functional foods and functional food ingredients. It is therefore imperative to ensure that functional food claims are not misleading consumers and that the underlying scientific evidence should establish the efficacy of these materials at levels that are physiologically achievable in the normal dietary regime. In an attempt to achieve this, there are regulatory systems laid down by a number of regulatory authorities which describe the health claims that can be made and how to go about obtaining a health claim for a functional food product. Examples of regulatory bodies that have defined procedures include the European Authority of Food Safety (EFSA) in Europe, the Food and Drug Administration (FDA) in the USA, and the MHLW in Japan.

The FOSHU system has been described previously with the Qualified, Standardised and Reduction of Disease risk categories (Shimizu 2003; MHLW http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html).

In the USA, health claims are categorized into three different groups: nutrient content claims, structure/function claims, and health claims (Ellwood et al. 2010; Hasler 2002).

- **Nutrient content** claims make a direct statement about the level (increased or decreased/absent) of a nutrient in the food which implies that being present at this level may be useful in maintaining healthy dietary practice. An example would be "high in fiber" linked to good digestive health or "low in fat" linked to body weight or cardiovascular health.
- **Structure/function** claims describe the effect on the structure or function of the body.
- **Health claim** means any claim (including statements, symbols, etc.), made on the label that characterizes the relationship of any substance to a disease or health-related condition.

A list of allowed health claims for particular foods/ food ingredients in the USA is provided in Table 1.

Originally health claims in the USA had to be based on substantive scientific evidence which was evaluated by the FDA. However, after a legal challenge was made to this, in 1999, the "*Qualified Health Claims"* were introduced (Table 2). These claims are for relationships between ingredients and diseases/risk of disease for which there is a lower standard of evidence. In making these claims, there has to be included qualifying statements that identify the limits of the scientific evidence available to support the relationship between the ingredient and the health benefit claimed.

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Table 1 FDA-approved health claims

Ingredient	Associated disease
Calcium, vitamin D	Osteoporosis
Dietary lipids (fat)	Cancer
Dietary saturated fat and cholesterol	Risk of coronary heart disease
Dietary noncarcinogenic carbohydrate sweeteners	Dental caries
Fiber-containing grain products, fruits and vegetables	Cancer
Folic acid	Neural tube defects
Fruits and vegetables	Cancer
Fruits, vegetables, and grain products that contain fiber, particularly soluble fiber	Risk of coronary heart disease
Sodium	Hypertension
Soluble fiber from certain foods (whole oat, barley, psyllium seed husk)	Risk of coronary heart disease
Soy protein	Risk of coronary heart disease
Stanols/sterols	Risk of coronary heart disease

The new European Regulation on nutrition and health claims came into force in January 2007. This followed a concerted action project supported by the European Commission titled "Process for the Assessment of Scientific Support for Claims on Foods" (PASSCLAIM). This project reviewed the scientific state of the art in diet and health areas regarded as most likely for health claims with the aim of defining a set of generally applicable criteria for the scientific substantiation of health claims. The guidance can be used to help to meet the requirements for health claims under the European legislation (http://www.ilsi.org/Europe/Pages/PASSCLAIM_Pubs.aspx).

The European legislation distinguishes two types of claims. The first of these are "Nutrition" claims which state, suggest, or imply that a food has beneficial nutritional properties due to the energy it provides or the nutrients it contains (EU regulation 1924/2006 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:012:0003:0018:EN:PDF). Examples of these are low energy or low fat claims whereby the food making the claim must conform to certain levels of the

Table 2 Qualified health claims allowed in the USA (http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ucm073992.htm)

Ingredient(s)	Associated disease
Tomatoes and/or tomato sauce	Prostate, ovarian, gastric, and pancreatic cancer risk
Calcium	Colon/rectal cancer and recurrent colon/rectal polyps risk
Green tea	Cancer risk
Selenium	Cancer risk
Antioxidant vitamins	Cancer risk
Nuts/walnuts	Heart disease
Omega-3 fatty acids	Coronary heart disease
B vitamins	Vascular disease
Monounsaturated fatty acids from olive oil	Coronary heart disease
Unsaturated fatty acids from canola oil	Coronary heart disease
Corn oil	Heart disease
Phosphatidylserine	Cognitive dysfunction and dementia
Psyllium husk	Diabetes
Chromium picolinate	Diabetes
Calcium	Hypertension, pregnancy-induced hypertension, and preeclampsia
Folic acid	Neural tube birth defects
100 % whey protein partially hydrolyzed infant formula	Atopic dermatitis

Table 3 Health claims within the European Regulation

Article 13: Health claims other than those referring to the reduction of disease risk and to children's development and health
13.1 Health claims describing or referring to: (a) The role of a nutrient or other substances in growth, development, and the functions of the body (b) Physiological or behavioral functions (c) Without prejudice to Directive 96/8/EC, slimming or weight control or a reduction in the sense of hunger or an increase in the sense of satiety or the reduction of available energy in the diet 13.5 Claims based on newly developed scientific evidence and/or that include protection of proprietary data
Article 14: Reduction of disease risk claims and claims referring to children's development and health

Table 4 Example of EU register authorized health claims

Nutrient, substance, food or food category	Claim	Conditions of use of the claim/ restrictions of use	Health relationship	EFSA opinion reference/ journal reference
Alpha-linolenic acid (ALA)	ALA contributes to the maintenance of normal blood cholesterol levels	The claim may be used only for food which is at least a source of ALA as referred to in the claim SOURCE OF OMEGA-3 FATTY ACIDS as listed in the Annex to Regulation (EC) No 1924/2006. Information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 2 g of ALA	Maintenance of normal blood cholesterol concentrations	2009;7(9):1252, 2011;9(6):2203
Beta-glucans	Beta-glucans contribute to the maintenance of normal blood cholesterol levels	The claim may be used only for food which contains at least 1 g of beta-glucans from oats, oat bran, barley, barley bran, or from mixtures of these sources per quantified portion. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 3 g of beta-glucans from oats, oat bran, barley, barley bran, or from mixtures of these beta-glucans	Maintenance of normal blood cholesterol concentrations	2009;7(9):1254, 2011;9(6):2207
Calcium	Calcium contributes to normal neurotransmission	The claim may be used only for food which is at least a source of calcium as referred to in the claim SOURCE OF [NAME OF VITAMIN/S] AND/OR [NAME OF MINERAL/S] as listed in the Annex to Regulation (EC) No 1924/2006	Muscle function and neurotransmission	2009;7(9):1210
Creatine	Creatine increases physical performance in successive bursts of short-term, high-intensity exercise	The claim may be used only for food which provides a daily intake of 3 g of creatine. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 3 g of creatine The claim may be used only for foods targeting adults performing high-intensity exercise	Increase in physical performance during short-term, high-intensity, repeated exercise bouts	2011;9(7):2303
Eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA)	EPA and DHA contribute to the normal function of the heart	The claim may be used only for food which is at least a source of EPA and DHA as referred to in the claim SOURCE OF OMEGA-3 FATTY ACIDS as listed in the Annex to Regulation (EC) No 1924/2006. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 250 mg of EPA and DHA	Maintenance of normal cardiac function	2010;8 (10):1796, 2011;9(4):2078

(*continued*)

Table 4 (continued)

Nutrient, substance, food or food category	Claim	Conditions of use of the claim/ restrictions of use	Health relationship	EFSA opinion reference/ journal reference
Guar gum	Guar gum contributes to the maintenance of normal blood cholesterol levels	The claim may be used only for food which provides a daily intake of 10 g of guar gum. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 10 g of guar gum Warning of choking to be given for people with swallowing difficulties or when ingesting with inadequate fluid intake – advice on taking with plenty of water to ensure substance reaches stomach	Maintenance of normal blood cholesterol concentrations	2010;8(2):1464
Melatonin	Melatonin contributes to the reduction of time taken to fall asleep	The claim may be used only for food which contains 1 mg of melatonin per quantified portion. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained by consuming 1 mg of melatonin close to bedtime	Reduction of sleep onset latency	2011;9(6):2241
Monascus purpureus (red yeast rice)	Monacolin K from red yeast rice contributes to the maintenance of normal blood cholesterol levels	The claim may be used only for food which provides a daily intake of 10 mg of monacolin K from red yeast rice. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 10 mg of monacolin K from fermented red yeast rice preparations	Maintenance of normal blood LDL-cholesterol concentrations	2011;9(7):2304
Olive oil polyphenols	Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress	The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil	Protection of LDL particles from oxidative damage	2011;9(4):2033
Plant sterols and plant stanols	Plant sterols/ stanols contribute to the maintenance of normal blood cholesterol levels	In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of at least 0.8 g of plant sterols/stanols	Maintenance of normal blood cholesterol concentrations	2010;8 (10):1813, 2011;9(6):2203

nutrient to make the claim. For example, to make a low fat claim, the product must contain "no more than 3 g of fat per 100 g for solids or 1.5 g of fat per 100 ml for liquids (1.8 g of fat per 100 ml for semiskimmed milk)." Other claims which fall under these include "source of" claims such as "source of fiber" or "source of omega-3 fatty acids" or "high in," for example, "high in polyunsaturated fatty acids". Again there are stipulated levels of each nutrient which the product must contain to make the claim.

The second type of claims are "Health" claims which are defined as "any claims that state, suggest, or imply that a relationship exists between a food category, a food, or one of its constituents and health." A *reduction of disease risk* claim is defined as "any health claim that states, suggests, or implies that the consumption of a food category, a food, or one of its constituents significantly reduces a risk factor in the development of a human disease." Reduction of disease risk claims and claims referring to children's development and health are addressed in Article 14 of the EU regulations, whereas other health claims are dealt with in Article 13 (Table 3).

The health claims under Article 13 need to be based on "generally accepted scientific evidence" and "well understood by the average consumer." The European Food and Safety Authority (EFSA) analyzes the evidence for the health claim, although the final decision on whether to accept or reject it lies with the European Commission. EFSA finalized the evaluation of "general function" health claims prioritized by the Commission in June 2011 and has published 341 opinions providing scientific advice on 2,758 "general function" health claims (http://www.efsa.europa.eu/en/topics/topic/article13.htm). Some examples of claims authorized under Article 13 are listed in Table 4.

Despite the regulations around food claims and the increased scrutiny under which these have come, it is still likely that certain foods will be associated with particular functions by consumers despite there being a lack of robust evidence according to an authority.

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Safety Assessment of Functional Food

Bobbie Bradford

Contents

The safety assessment of functional foods follows the same paradigm established for conventional foods consisting of an assessment of the intrinsic hazards associated with the food/food ingredient combined with knowledge about the expected exposure that will occur. Addressing safety concerns on single components is relatively straightforward. However, functional foods can be whole foods and/or may consist of multiple components with increasing complexity (Fig. 1) which results in a different strategy often being required depending on the type of food/food ingredient. Traditionally whole foods have been considered safe because no evidence has accumulated on adverse effects and/or any intrinsic hazards have been addressed by adequate processing/preparation techniques. For example, red kidney beans need to be soaked and boiled at high temperatures to ensure destruction of phytohemagglutinin associated with gastrointestinal adverse effects (http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ucm071092.htm). However, if these whole foods are exotic compared with the region where they are now to be marketed, then it was recognized that there needed to be governance and guidance in how to determine the safety of such food. These now fall into the category of novel foods which are legislated for in Canada, Australia and New Zealand, and Europe, all of which require a premarket assessment. Detailed information on the origin, production, composition, nutritional characteristics, and prior exposure to the novel food is

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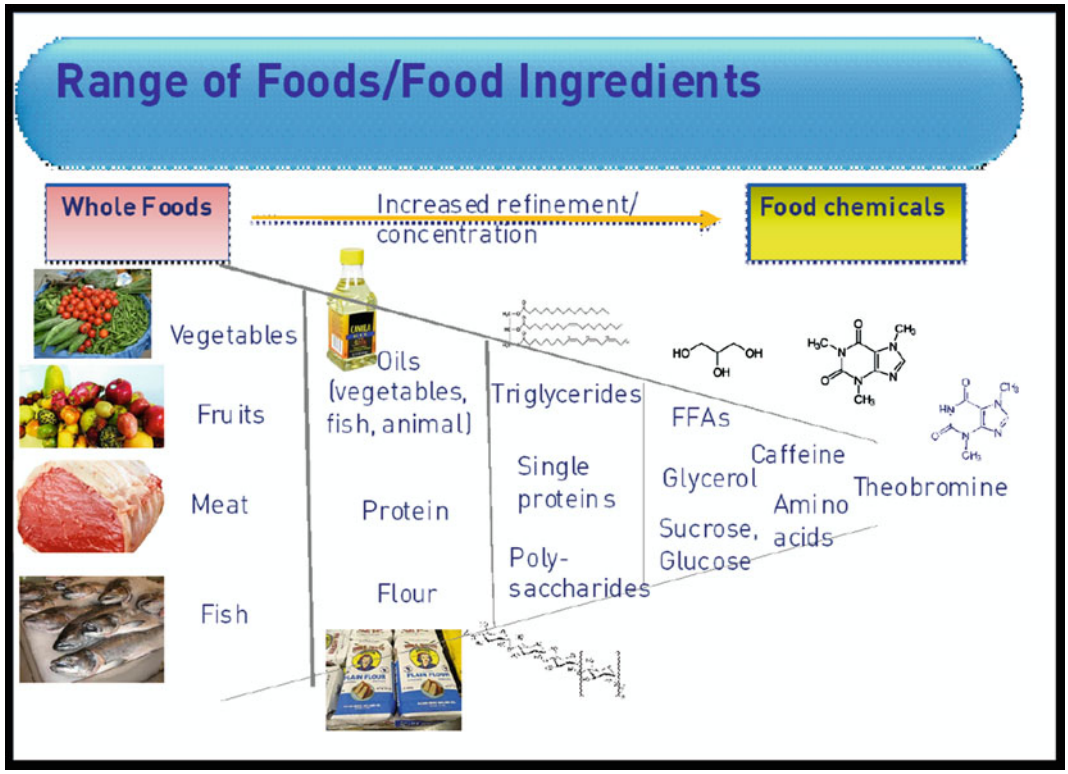

Fig. 1 Range of foods/food ingredients that may be included within Functional Food definition

required (Edwards 2005). The European Novel Foods legislation in May 1997 (http://ec.europa.eu/food/food/biotechnology/novelfood/initiatives_en.htm) established the need for determining safety of exotic fruits and vegetables introduced to Europe after this date.

Despite this range of foods/food ingredients that need to be assessed, the same basic four-step approach is followed (Fig. 2), although the data used at each step may vary:

- Hazard identification
- Hazard characterization
- Exposure assessment
- Risk characterization

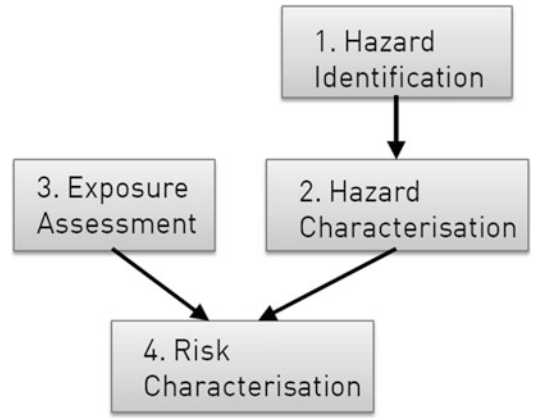

Fig. 2 Four-step approach for foods/food ingredient risk assessment

Hazard Identification

The first step in the hazard identification is to gather information on what is known about the food/ingredient already. Detailed knowledge is required on the proposed material including a precise biological identification (taxonomy, phenotype, and genotype) if it is naturally occurring. The origin of plant-based foods/ingredients as well as their geographical distribution must be

described as well as analysis of variability in composition (Constable et al. 2007). The importance of making an accurate assessment of the biological identification has been demonstrated by problems such as intoxication caused by using teas made with Japanese star anise (Illicium anisatum L.) which contains a number of neurotoxins, rather than the traditionally used spice Chinese anise (Illicium verum Hook.f.) in a herbal tea in the Netherlands (Johanns et al. 2002). Components such as moisture, protein, ash, amino acid and fatty acids, vitamins, and minerals should be identified. Any chemical hazards such as toxicants, antinutrients, allergens, mycotoxins, and heavy metals should be determined. Of importance for functional foods is the presence of the relevant bioactive components. For purified ingredients, emphasis is placed on identifying chemicals present and any potential impurities arising from the manufacturing process, for example, extraction solvents.

The information gathered on the food/ingredient may be in the form of data information sheets from the supplier of an ingredient, from previous regulatory approvals, literature data on published studies, media stories, or uses of the chemical other than in food. For some ingredients, expert toxicological evaluations will already have been published, for example, in Europe by the EFSA (www.efsa.europa.eu) or the predecessor to EFSA, the Scientific Committee for Food (http://ec.europa.eu/food/fs/sc/scf/index_en.html), as well as individual member states Food Safety organizations; in the United States the Food and Drug Administration (FDA) Everything Added to Food (EAFUS) list as well as the GRAS inventory list (www.fda.gov); and from Australia and New Zealand the Food Standards (www.foodstandards.gov.au). Other sources of information include the Flavors and Extracts Manufacturing Association (FEMA) or associations outside of foods use (e.g., Cosmetic Ingredient Review http://www.cir-safety.org/ or the European Chemicals Agency http://echa.europa.eu/web/guest). Global organizations such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) provide a searchable database which contains summaries of all the evaluations of flavors, food additives, contaminants, toxicants, and veterinary drugs that JECFA has performed (http://apps.who.int/ipsc/database/evaluations/search.aspx). Each JECFA summary contains basic chemical information, acceptable daily intake (ADI)/tolerable daily intake (TDIs) levels, links to the most recent reports and monographs as well as to the specification database, and a history of JECFA evaluations.

Wherever possible, existing data for ingredients are used in safety assessments, and what is available is scrutinized for quality and robustness.

For single chemicals (or mixtures of discrete known chemical ingredients), quantitative structure-activity relationships (QSAR) can be evaluated to both predict potential hazards and determine if a read across can be made to a similar ingredient/chemical. QSARs are mathematical models that use the physical characteristics of the structure of chemicals (known as molecular descriptors) to predict measures of toxicity (http://www.epa.gov/nrmrl/std/qsar/qsar.html). In addition, other considerations such as history of safe use or human clinical data can be used in a weight of evidence approach. The history of safe use approach is discussed further in section "Human Studies."

A further aspect that must be considered when assessing the hazard potential for a functional food/food ingredient is an evaluation of the nutritional aspects. The impact of the food on the human diet must be considered to ensure that it will not cause adverse effects through nutritional inadequacy or excess (Edwards 2005).

If data do not exist, or are considered inadequate, toxicological testing may be conducted to identify and characterize the toxicological hazard. The nutritional impact of the functional food must also be taken into consideration when designing in vivo toxicity studies.

Hazard Characterization

In cases where biological testing is required, these must be undertaken according to good laboratory practice and following relevant guidelines (e.g., OECD http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788).

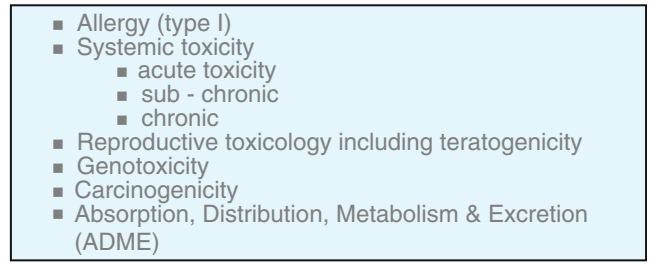

Fig. 3 Relevant safety endpoints for hazard characterization of (functional) foods

Testing methods need to be adapted on a case-by-case basis to identify the levels of intake that will be without an adverse effect and therefore be used in the safety risk assessment. Although the studies required will vary, the relevant safety endpoints that are considered are listed in Fig. 3.

Genotoxicity studies will usually only be possible for defined chemicals or simple mixtures; it is not usually appropriate to perform such studies on whole foods (Howlett et al. 2003). However, analysis of the whole food or knowledge about existence of certain components may trigger studies on isolated chemicals. Indications of the absence of genotoxic potential may be obtained from examination of the bone marrow or peripheral lymphocytes from rodent feeding studies undertaken with complex foods. Botanical food materials often contain components that may test positive in genotoxicity assays in vitro but are often negative in in vivo assays. An example of this is quercetin, a naturally occurring flavanol which has a long history of consumption in the human diet, and while in vitro studies consistently demonstrate quercetin-related mutagenicity, there is an absence of carcinogenicity when it has been tested in vivo (Harwood et al. 2007). Unless there is an understanding of the mechanisms behind positive results in vitro attributed to an identified component, they will need to be followed up with in vivo studies (Knudsen et al. 2008).

Where the food ingredients consist of discrete chemical substances that do not have nutritional effects, then standard design experimental studies in rodents can be performed; typically, a 90-day feeding study will be employed to assess systemic toxicity. The parameters examined in the study will be those typically employed such as clinical observations, body weights and food consumption, hematology and blood chemistry, macroscopic and microscopic examinations, and organ weights. A range of doses, several times the magnitude of the human dose, will be administered to identify potential adverse effects in humans and to achieve a no observed adverse effect level (NOAEL).

The need for additional studies, such as long-term or reproductive studies, is decided on a case-by-case basis depending on the results of the 90-day toxicity studies, any alerts related to chemical structure or in vitro studies or from the literature reviews. Examples of this could be where there is structural similarity to estrogenic materials which might highlight the need for reproductive toxicity assays.

Exposure Assessment

The exposure assessment for the introduction of a new food or food ingredient involves two aspects, the level of the ingredient in the food product and the amount of the food or food product that is consumed. Information on the amounts of food consumed can be obtained from national dietary or nutritional surveys. Examples of these include the National Diet and Nutrition Survey (NDNS) in the UK and the National Health and Nutrition Examination Survey (NHANES) in the USA. The NDNS (https://www.gov.uk/) is jointly funded by Public Health England (PHE) and the UK Food Standards Agency (FSA) and is designed to assess the diet, nutrient intake, and nutritional status of the general population aged 1.5 years and over living in private households in the UK. The NDNS provides nationally representative data on the types and quantities of foods

consumed by individuals, from which estimates of nutrient intake for the population are derived. The NHANES (http://www.cdc.gov/nchs/nhanes.htm) is conducted by the National Center for Health Statistics (NCHS) to assess the health and nutritional status of adults and children and to track changes over time. The interview part of the survey includes demographic, socioeconomic, and dietary and health-related questions.

The three principle methods for measuring food intake are food records (or diaries), 24-h recalls, and food frequency questionnaires (FFQ). Food records require respondents to record all foods and beverages consumed over a specified period of time, generally between 3 and 7 days, while the 24-h recalls are interviewer led and ask the respondent to remember and record all foods consumed in the preceding 24-h period. FFQs are also retrospective assessment tools and require respondents to report the frequency of consumption of a predefined list of foods over a prolonged period of time, typically the previous 6 or 12 months (Ellwood et al. 2014).

In recent years, the European Food Safety Authority (EFSA) has called for harmonization of dietary surveys across Europe (http://www.efsa.europa.eu/en/efsajournal/pub/1435.htm) since many countries carry out national dietary surveys, yielding information that is valuable for shaping national policies on nutrition and for monitoring dietary patterns. The methodology undertaken can vary from recalling intake from the previous day (24-h recall) to keeping a record of the consumption of food and drinks over several days (dietary record). In addition the detail and the quality of the data collected are also uneven, affected by differences in the survey design, the tools used to collect and measure the data, the clustering of age groups, and the food description and categorization systems. In 2009, EFSA issued guidelines on how food intake surveys should be undertaken: "It is recommended that surveys cover two non-consecutive days and use the dietary record method for infants and children and the 24-h recall method for adults. It is further recommended to use a food propensity questionnaire and that supplementary information, in particular on brand name, physical characteristics of the packaging, cooking procedures and other specific information, such as fortification should be collected. Detailed information on the use of nutritional supplements by respondents should also be covered as well as physical measures of the survey participants (weight and height) along with an estimate of their physical activity level" (EFSA 2009).

As well as the limits with regard to consistency, there are other issues with available surveys, for example, they may not cover all food products and functionality may alter the normal patterns of consumption including specific groups of consumers and new product formats.

When analyzing food intakes, two approaches can be taken, the deterministic approach which uses the highest intakes (eg 95%ile) and adds all intakes together or the probabilistic approach which models intake across the full distribution of intakes. This is usually used when considering intake from multiple food sources. There are software packages which can determine the intakes using a probabilistic approach, such as Crème (http://www.cremeglobal.com/home). There is a lack of general dietary intake data outside of the EU and the USA with some data available from China; therefore, other sources are required.

Data can also be obtained following marketing of the new food/food product by monitoring sales or by specific human studies. These can be combined with evaluation of the efficacy and confirmation of safety although the data obtained may not be an accurate picture of intakes following marketing to the general population.

Based on the above information, estimations of intakes can be made across the population to give consumer exposure levels (CEL). These are usually given as mg or g per day converted into mg/kg/day based on average bodyweights for different age groups and genders.

Risk Characterization and Risk Assessment

The NOAEL derived from toxicology studies is used to calculate the safe level of intake or acceptable daily intake (ADI). The ADI is defined as the

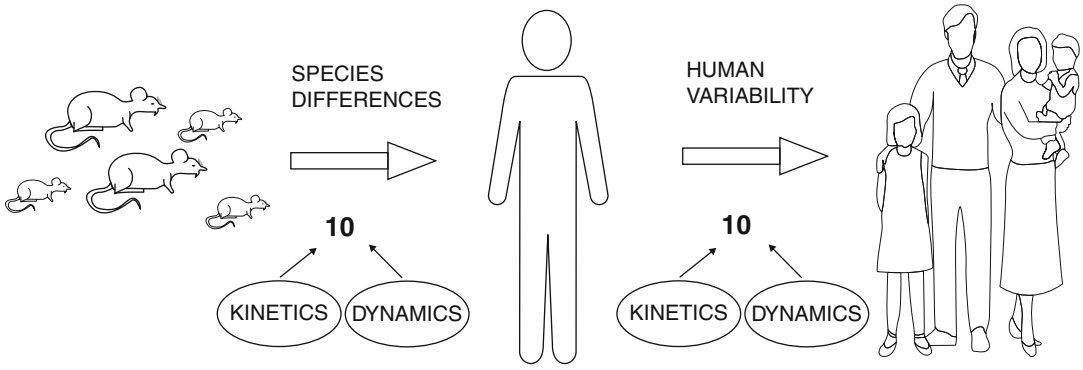

Fig. 4 Determination of uncertainty (safety) factors

estimated amount of a substance, expressed on a body mass basis (usually mg/kg/day), to which a human may be exposed over lifetime without appreciable health risks. It is obtained by dividing the NOAEL with an uncertainty (or safety) factor, usually of 100. This large margin of safety is applied to reduce any risk to health to a minimum in all groups of consumers. The safety factor of 100 is made up of a factor 10 to account for uncertainties as a consequence of extrapolating from animals to humans (interspecies variation) and a factor 10 to account for differences between individuals (intraspecies variation); each factor of 10 is further made up of a combination of differences in kinetics and dynamics (Fig. 4).

This large default safety factor of 100 has been used for over 40 years and provides a clear, transparent tool to control the uncertainties associated with using data from studies in animals to derive a safe intake level for humans. However, for many foods and food ingredients that may be considered as functional, it is unlikely that large margins between test levels and the expected (or required for efficacy) levels of consumption are achievable. For these foods, maximum test levels should be set at the highest amount that can be included in the feeding study without causing nutritional imbalance or exaggerated effects related to functionality. In these cases, a safety factor of lower than 100 may be appropriate for extrapolation between animal toxicity studies and humans and it may then be important to confirm safety in human studies.

The risk characterization determines whether intake of a food or food ingredient is safe for consumers. This is determined by comparing the acceptable daily intake (ADI) usually from the toxicity studies and given as mg/kg/day compared with the consumer exposure level (CEL) typically considered as the worst case (95%ile). If the intake is lower than the ADI, then the risk is considered acceptable. Occasional excursions above the ADI are usually unlikely to pose safety concerns unless the ingredient is known to be acutely toxic.

Human Studies

Human studies may be carried out for confirmation of nutritional quality and the absence of adverse effects (Edwards 2005). In particular where the margin of safety between animal toxicity studies and the expected human exposure may be low. Human studies may also be useful to confirm absence of reactions such as intolerance (eg nausea) or identify subjective responses which may not be predicted in animal studies. They may be useful to demonstrate the nutritional quality of the food where it may make up a significant portion of the diet, confirm a food's suitability and safety in specific population subgroups or be designed for the study of specific end points suggested by other studies or considerations. An example of the usefulness of human studies was the long-term safety study undertaken with plant

sterol esters (Hendriks et al. 2003) which reported on the effects of consuming a low-fat spread providing 1.6 g of plant sterols daily for a year in healthy men and women. A variety of variables were measured including efficacy markers such as total and LDL cholesterol, a large range of safety parameters, and monitoring of adverse events. The only potential safety effects seen in this study were related to carotenoid concentrations which changed over time with reductions greater in subjects consuming the spreads containing sterols compared to control spread. However, the decreases were small and well within the seasonal ranges observed and were therefore not considered to be biologically significant. The study confirmed that daily consumption of 1.6 g of plant sterol in the long-term consistently lowered blood cholesterol levels and did not appear to have any adverse health effects (Hendriks et al. 2003).

For whole or complex foods, it is not always feasible or required to undertake toxicology studies, and in these cases, a comparative approach has been developed for their safety assessment using history of safe use. This approach can, of course, only be undertaken if the pattern and level of consumption are within those of a traditional comparator material which has itself a significant history of use. This is further described in section "Human Studies."

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History of Safe Use

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Most foods that are consumed have not been subject to systematic toxicological and nutritional assessment but are generally regarded as safe to eat because of their long history of consumption and knowledge around preparation. This "history of safe use" of traditionally consumed foods can serve as a benchmark for comparative safety assessment of more novel foods and ingredients. It should be remembered that although traditionally consumed foods are considered safe, they may contain components such as antinutrients, toxins, and/or allergens. For example, potatoes are known to contain the glycoalkaloid solanine and the health risks of eating old or green potatoes have been understood for over a century (BMJ 1979; American Journal of Public Health 1917). Thus complete freedom from risks is an unattainable goal, safety and wholesomeness are related to a level of risk that society regards as reasonable in the context, and in comparison with other risks in everyday life (FAO 1997), and this needs to be considered when introducing new foods/ingredients.

The starting point for using the history of safe use approach is identifying an appropriate comparator material which has been consumed for a reasonable period and for which there is adequate information on how the food has been prepared both traditionally and in the current preparation, composition, and characterization of the food as well as the results of any toxicology studies or insight from human exposures. To determine that a food has history of safe use, either the food itself or the relevant comparator should have a significant human consumption, for example, over several generations and in a large, genetically diverse population, and there should be adequate toxicological and allergenicity data to provide reasonable certainty that no harm will result from the consumption of the food. Some foods will have a history of consumption in different parts of the world while unknown in others, while for other cases it is possible to benchmark specific safety issues with similar food types; an example of this is ngali nuts versus European nuts for assessing allergenicity risk (Constable et al. 2007)

Databases are available which can help to establish whether a particular food has a history of safe use such as national food survey reports and global, regional, and national surveys of plants with food uses (Constable et al. 2007)

In the majority of cases, the history of safe use is applied to plant or herbal materials, and the first step in assessing the safety of a novel food using the history of use should be to determine what (if any) existing food(s)/plants could be used as a comparator (or material of reference). A systematic review of the history of use approach is described by Neely et al. (2011). In order to identify an appropriate comparator material, one needs to first be clear on the origin, composition, and

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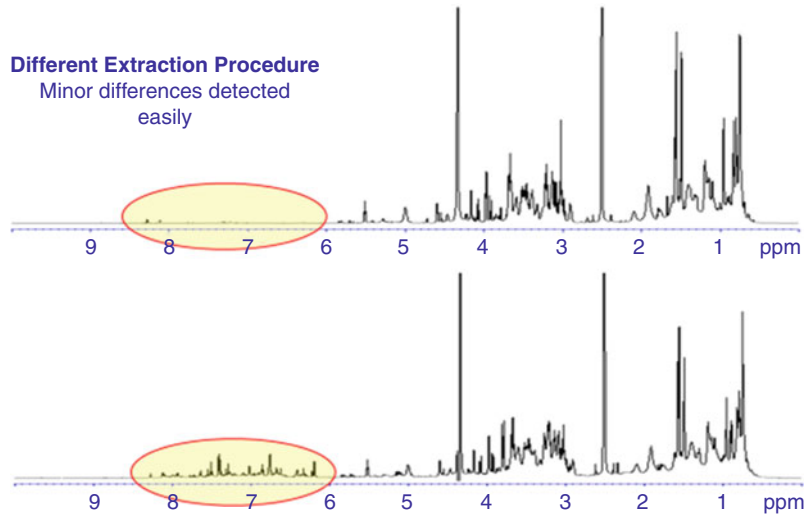

Fig. 1 Comparative analysis using chemical fingerprinting

method of production of both the novel food and the comparator. Details of origin of the plant ingredient including the botanical description, part, or portion of the plant being consumed are required to ensure that the same food as traditionally consumed is being compared. It is important, if possible, to assess the similarity of specification or active components of the proposed substance to the traditional comparator material. As plant materials are typically composed of multiple different chemicals, it is not usually feasible to identify every component. A more logical approach is to define an analytical fingerprint which, for the purpose of history of safe use, is defined as "a unique pattern representing the presence of known and/or unknown characteristic chemical components" (Neely et al. 2011; Goodarzi et al. 2013). An effective fingerprint needs highly sensitive detection systems with the ability to identify/give a response to as many components as possible. The most commonly used techniques are high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR) spectroscopy. Using this approach differences and similarities between the material and comparator can be easily made (Fig. 1).

Any differences could be related to the material itself or differences in extraction/preparation methods, and therefore aligned with the information on specification are details on preparation and processing, highlighting disparity between local/home traditional preparations of the comparator and the intended manufacture, likely in an industrial setting of the proposed substance. Special attention needs to be paid to processing and preparation methods as these may increase the toxicity potential of certain foods/food ingredients. For example, extraction methods may lead to concentration of contaminants such as pesticides or heavy metals or introduce residual solvents that must then be assessed for safety.

Once it is established that the proposed material is similar enough in composition to the comparator, the next step would be to assess details on the amount and similarity of exposure to the comparator material. This includes factors such as whether the populations exposed previously are comparable considering ethnicity, age, gender, and diseased/healthy populations. For the comparator, information on the numbers of people currently exposed and duration of exposure are important as well as the typical frequencies and patterns of consumption, for example, eaten daily or on occasions. Of importance is the estimated daily intake of the proposed food/ingredient compared with that of the traditional food. These considerations ensure that the comparator has an

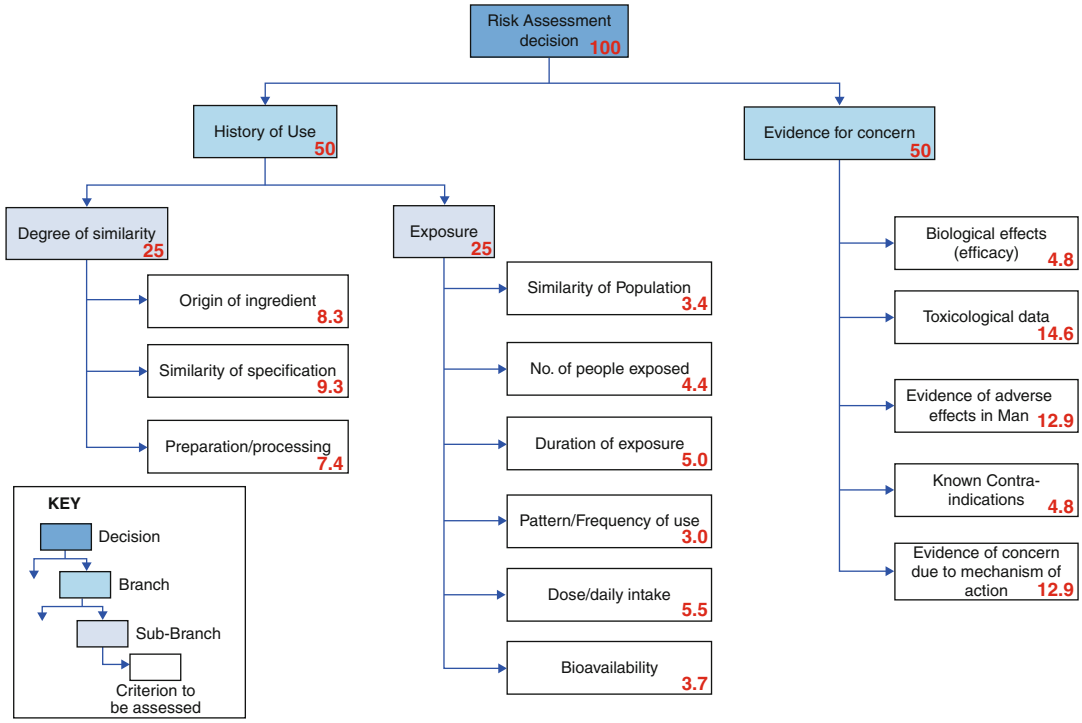

Fig. 2 Multi-criterion decision analysis for making history of safe use decisions

adequate history of use on which to base the assessment of the novel food.

Evidence must then be gathered on safety or health concerns around either the novel food or the comparator. Adverse data may be obtained from the region where the comparator food has been traditionally consumed or from clinical study reports, for example, if the comparator has been used as a traditional medicine (Constable et al. 2007). It is possible that the proposed use will increase the bioavailability of some components of the food compared with traditional/comparator use and thus give rise to more evidence of concern, and therefore any information on this should be gathered. Key data to be considered to assess any safety concerns include toxicology data and nutritional data including knowledge of antinutritional factors, allergenicity, potentially toxic contaminants (natural, such as mycotoxins, or residual solvents, heavy metals, pesticides), bioactive components (e.g., phytoestrogens/androgens), and any metabolic or gastrointestinal effects in humans (Constable et al. 2007).

Once all of the information has been gathered on both the history of use and any evidence for concern, then this can be weighted to provide a decision on whether there is enough evidence to make a risk assessment decision. An example of this approach using multi-criterion decision analysis is shown in Fig. 2 and described in detail by Neely et al. (2011).

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Post Launch Monitoring

Bobbie Bradford

Post-marketing surveillance (PMS) is the system in place for monitoring of the known and unknown side effects of pharmaceuticals with the main sources for this information being physicians or pharmacists. This is a mandatory system that is the responsibility of the marketing company to administer but requires that the results be reported regularly to the regulatory agencies. Access to medicines is carefully controlled through the issue of prescriptions and availability through pharmacies or hospitals. In contrast, foods are widely available without prescription through retail outlets which means that the PMS system for drugs and medicines cannot be applied for food products. The main feedback on health concerns will come directly from consumers, often into company carelines, and therefore a different system of surveillance is required. To differentiate between PMS and the approach for foods, the term post-launch monitoring has been coined.

Post-launch monitoring (PLM) has been defined as "a hypothesis driven, scientific methodology for obtaining information through investigations relevant to the safety of a (novel) food after market launch" (Hepburn et al. 2008). It can be used as a tool for confirming that product use is as predicted by the pre-market assessment providing assurance that the consumer exposures used for the risk assessment were appropriate. This may include both the extent to which the product is consumed by the target group and estimation of exposure in other population groups (EC 2000). It can also provide reassurance that any effects which were observed as part of the pre-market assessments occur with no greater frequency or severity once used by a more extensive population. Finally it can give an opportunity to investigate the significance of any adverse effects reported by consumers after market launch. It should be recognized that PLM is more limited in precision compared to PMS both because of the reliance on direct consumer reporting of health concerns and as the consumption of the particular food of interest will be in conjunction with other foods (potentially also with prescribed pharmaceuticals) making direct association with a health effect difficult to determine.

There is no mandatory requirement for PLM in most regions; however, in the EU it was required as a condition for the approval of yellow fat spreads containing added phytosterol esters (EC 2000). The European Food Safety Authority has recommended that it should be performed, where appropriate for foods derived from genetically modified (GM) sources (EFSA 2011). In Australia and New Zealand, the FSANZ guidelines suggest that post-market monitoring "will provide additional reassurance regarding long-term safety of products, as well as their impact on the food supply" (FSANZ 2007).

While not a standard regulatory requirement, food manufacturers may consider the use of PLM

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as a complement to pre-market safety assessment. A number of triggers have been defined which might suggest that PLM would be worthwhile (Hepburn et al. 2008):

- If the estimated daily intake (EDI) is close to the acceptable daily intake (ADI): to monitor the real consumption patterns.
- Where an original application was for one product, the exposure to a novel ingredient was limited. Further applications leading to several different products being marketed will have the potential to change the original exposure estimate. This may have consequences for the previously established acceptable margin of exposure which need to be monitored.
- If a product was intended for use in foods for certain target populations, for example, cholesterol-lowering foods aimed at older, adult consumers, it may be desirable to monitor the extent of use by the target population and/or by other groups in the population.
- To monitor potential misuse of a product (overconsumption or use in applications not originally intended).
- Any food (traditional or novel) may have known potential to exert side effects. Effects which are severe in nature and are likely to reach a level of significance for health would preclude the marketing of the food, but other effects (e.g., laxation, slight nausea), while observable, may be of lesser health significance, but undesirable in the target consumer. Pre-market assessments address the possibility of these occurring, but reassurance that any occurrence in the general population is of low frequency and intensity via PLM would be important.

An example of a functional food for which PLM was requested and applied was for the novel foods approval of yellow fat spreads containing added phytosterol esters (EC 2000). The EC required a PLM program alongside marketing of the product specifically to address questions related to how much is consumed and whether the product was being used by the target consumers. The scheme devised by the marketing company, Unilever, was a more detailed analysis which consisted of three components: (A) Is the use as predicted/recommended? (B) Are the known effects and side effects as predicted? (C) Does the product induce unknown side effects? (Lea and Hepburn 2006). The results of the PLM scheme established that the products were being bought by the target population with intakes lower than the original assumption made in the novel foods submission indicating no concerns around overconsumption, long-term use results in slight reduction in the serum levels of the most lipophilic carotenoids, but this was less than either the individual or seasonal variation, and there was no evidence of occurrence of adverse effects. PLM on sterol-containing product has continued since the product range was extended with similar results (Willems et al. 2013).

It is important to emphasize that PLM should be used to complement the pre-market safety assessment with hypothesis-driven objectives solely related to confirming the conclusions made prior to marketing. It is not appropriate to use it as a replacement of any steps in pre-market assessment, safety should be assured prior to launch.

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Impact of Functionality on Safety Risk Assessment

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The functionality of a food/food ingredient may affect both the hazard characterization and the consumer exposure to the food/food product. The amount, frequency of intake, and pattern of exposure may be altered compared with that historically observed for similar foods, or the functional food may be delivered using a novel product type. These aspects must all be considered as they may impact upon the safety risk assessment.

For a functional food or food ingredient it is important to have both an understanding of the mode of action of the bioactive and to know how potent is the functional effect. While it is unlikely that a functional food will have a highly potent pharmacological action, as this would then move it into a pharmaceutical area which would not be appropriate, the potential health effects of the bioactivity need to be considered and measures included to assess these. For example, phytoestrogens, such as isoflavones from soy or red clover and lignans from flaxseed, whole grains, fruits, and vegetable, are used as functional foods for relief of menopausal symptoms and lowered risk of osteoporosis but could potentially have effects on reproductive health or hormone-dependent cancers due to their interactions with hormone receptors (Chen et al. 2014a). In order to assess such risks, the levels of exposure and the potency need to be put into context by comparison with endogenous estrogens and estrogenic drugs such as the contraceptive pill (Safford et al. 2003). A useful approach would be to conduct in vitro receptor binding assays to determine potency and then benchmark this alongside potency of endogenous hormones and/or drugs to determine the level of risk for consumers.

For functional foods with cardiovascular effects such as control of blood pressure or lowering of cholesterol, it is important to understand the mode of action in order to design appropriate safety investigations to highlight any safety concerns. For example, peptides present in fermented milk products are purported to inhibit the angiotensin-converting enzyme (ACE), and therefore functional foods have been developed to help to control blood pressure (Chen et al. 2014b). A known side effect of ACE inhibiting drugs is fetopathy characterized by fetal hypotension, disruption in the development of the fetal kidney, and subsequent reduction in the production of amniotic fluid; this health risk is needed to be considered in the development of the functional food products despite the potency being considerably lower. Reproductive toxicity studies were designed to assess whether similar or milder forms of fetopathy would be induced by a specific milk peptide, lactotripeptide (Dent et al. 2007), which confirmed that this would not be a risk for consumers using such products.

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Cholesterol-lowering functional foods also have differing modes/mechanisms of action which could result in risks for human health. For example, phytosterols/stanols compete with cholesterol for absorption across the gut wall, while monacolins, such as monacolin K/lovastatin, present in red yeast rice decrease cholesterol production by the liver with a similar mechanism to statin drugs of inhibition of HMG convertase. In the latter case, the side effects related to inhibition of HMG convertase, such as myopathy, are well documented and therefore need to be considered in safety appraisal of similarly acting functional foods (Gordon and Becker 2011).

In vitro assays to determine potency or metabolic interactions should be considered, and design of toxicity studies with additional end points and/or human studies with safety biomarkers may be considered to address any concerns.

As well as adverse effects related to functionality, consumer exposure may also be affected as a consequence of a product being marketed as a functional food. There may be increases in consumption of traditional foods as consumers assume that by increasing intake a more desired health benefit will be achieved or patterns of consumption may change. New product formats might be introduced for which there is little information on consumer intakes. An example of this is the mini drink product format containing probiotic bacteria, lactobacillus, for gastrointestinal health benefits. Such drinks were originally developed in Japan and were some of the first functional foods receiving FOSHU (Food for Specified Health Uses) status but are now sold globally with the market for probiotics predicted to be worth $15.3bn globally in 2013. China is one of the biggest markets for probiotics due to the use of traditional medicines which has enabled acceptance; in 2008 it was reported that there was a daily average of 660,000 bottles of Yakult consumed in China (Tallon 2009). This was an increase of 57 % from the previous year and clearly demonstrates the impact of introduction of a novel functional food on consumption.

A further impact of functionality on exposure is that food ingredients which have traditionally been consumed as part of traditional medicines/supplements for specific purposes may now be consumed more widely and by a diverse population. This is one of the important considerations as part of using the history of safe use approach which may result in other testing or approaches being required to assess safety across the consumer population.

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Part XIX

Miscellaneous

Orphan Drugs

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Introduction

Pharmacological research and biotechnology sciences have always been focused at developing new therapeutic strategies capable of modifying diseases' natural history and improving patients' quality of life all around the world.

In the last decades, in particular, thanks to the huge advances in molecular biology, genomics, and genetic animal models, drug development has made enormous progresses bringing to the market thousands of new drugs.

Notwithstanding such enormous advances in drug discovery and development, there are diseases that still represent a major challenge for the therapeutic area since they lack a satisfactory medical treatment, as in the case of rare diseases. Developing a new medicine is, in fact, extremely challenging due to the related high costs and low rate of success. Accordingly, pharma companies have always been quite reluctant to invest large amounts of money in drug development if profit on their investment is not guaranteed. To mostly stimulate the research toward the clinical development of drugs capable to meet medical needs of rare and orphan diseases, special orphan drug legislations have been introduced across the globe. Firstly in 1983, in the USA, Orphan Drug Act and its subsequent amendments were introduced. Later similar acts were introduced in 2000 in the EU, in 1991 in Singapore, in 1993 in Japan, and in 1997 in Australia, further stimulating research globally (Development 2010; Meekings et al. 2012). Although there are some differences among countries (Table 1), these acts share a common aim consisting in making the development of orphan drugs profitable and therefore motivating the pharmaceutical industry to develop and bring to the market appropriate drugs for rare, or so-called "orphan," diseases. Notably, what these legal frames take particularly into account is the fact that patients suffering from rare conditions should be entitled to the same quality of treatment as other patients. Specifically they are meant to encourage the development of drugs that otherwise might be uneconomic under normal market conditions, by guaranteeing the developer of such products several years of market exclusivity following their orphan drug designation.

EU Orphan Drug Designation

The term orphan drug (from the Greek word orphanos, meaning a child who has lost one parent or both) refers to a medicinal product that has been granted orphan status by a regulatory agency. Orphan designation is reserved for medicines that target diseases with prevalence below the threshold set for rare diseases and may have additional factors such as the lack of availability of alternative treatments (Pryde and Palmer 2014). There is no universal definition of orphan disease. In the USA, it relates a condition affecting fewer than 200,000 individuals, while Japan and Australia set the limit to 50,000 and 2,000, respectively. In the European Union, the definition refers to those medicinal products intended for diagnosis, prevention, or treatment of life-threatening or debilitating rare diseases affecting not more than 5 in 10,000 EU patients (Lavandeira 2002).

To qualify for orphan designation, a medicine must meet the following criteria:

- It must be intended for the treatment, prevention, or diagnosis of a disease or condition that is life threatening or chronically debilitating.
- The prevalence of the condition in the EU must not be more than 5 in 10,000 or it must be unlikely that marketing of the medicine would generate sufficient returns to justify the investment needed for its development.
- No satisfactory method of diagnosis, prevention, or treatment of the condition concerned can be authorized, or, if such a method exists, the medicine must be of significant benefit to those affected by the condition.

Applications for orphan designation are examined by the European Medicines Agency's Committee for Orphan Medicinal Products (COMP), using the network of experts that the Committee has built up. The evaluation process takes a

Table 1 Features of orphan drug incentive systems in the USA, EU, Japan, and Australia

Country	USA	EU	Japan	Australia
Year	1983	2000	1991	1997
Program established	1983 – the Orphan Drug Act modified the Federal Food, Drug and Cosmetic Act	2000 – Orphan, Medicinal Products Regulation	1991 amendment to drug regulatory and taxation laws	1997 Australian orphan drugs policy
Orphan disease criteria	<200,000 patients in the USA (or prevalence <7.5 : 10,000)	Life-threatening or chronically debilitating disorder that affects <5: 10,000 in the EU	<50,000 patients on the Japanese territory (2.5 cases per 10,000)	<2,000 patients in the Australian population
Requirements for orphan drug designation	Rare disease or research and development costs cannot be recovered in 7 years	Rare disease or product unlikely to be developed without incentives or new product that will be of significant benefit	Disease for which use of the drug is claimed must be incurable. There must be no possible alternative treatment; or the efficacy and expected safety of the drug must be excellent in comparison with other available drugs	Rare disease or drug is not commercially available, when used in the patient population it is indicated for
Products eligible for orphan drug designation	Drugs and biologicals (including vaccines and in vivo diagnostics)	Drugs and biologicals (including vaccines and in vivo diagnostics)		
Market exclusivity	7 years, prevents same product being approved for the same indication unless clinical superiority is shown	10 years, can be reduced to 6 years if orphan drug criteria are no longer met	Up to 10 years	5-year exclusivity
Other benefits	Regulatory fee waivers, 50 % tax credit on clinical research after designation; grants for clinical research (pharmaceutical companies and academia eligible); protocol assistance; faster review if indication warrants; research grants for medical devices and medical food	Regulatory fees can be reduced or waived; access to centralized procedure; protocol assistance. Individual member states have to implement measures to stimulate the development of orphan medicinal products	Financial assistance to cover a proportion of the expenditure devoted to research and development of orphan drugs. Reimburse up to 50 % of the development costs. A 6 % tax reduction for research and development expenses is granted, other than those coming from funding grants and within the limit of 10 % of company tax	A legal framework for orphan drug designation; waiver of application and evaluation and no annual registration fees

maximum of 90 days from validation. The European Commission's (EC's) decision then follows in a maximum of 30 days.

However, getting designation as an orphan medicinal product does not mean having approval for the use of the drug in the orphan condition. Orphan designation by itself in fact does not indicate that the product has the amount of data regarding efficacy, safety, and quality required for marketing authorization. Similar to the

requirements for any other medicinal product, these criteria can only be assessed once the application for marketing authorization has been submitted (Dear et al. 2006).

Rare Diseases and Orphan Drugs

The term "rare disorder" generally refers to diseases characterized by low prevalence and, often times, no therapy. As reported in Table 1, there is no single definition for what constitutes a rare (also called orphan) disease. This, together with the fact that getting a definitive diagnosis is often very difficult and there are limitations in systems for reporting and tracking such diagnoses, makes the counting and the assessment of the epidemiology of rare diseases a not easy task. As a result, rare disease epidemiology, including the determination of prevalence (the number of people affected at any one time), incidence (the number of new cases in a given year), and patterns of disease (e.g., age distribution) in the population, is inexact (Development 2010). Today are anyway recognized 5,000–8,000 rare diseases, with about 250 new rare diseases described each year, affecting more than 55 million individuals in the EU and USA (Stolk et al. 2006). Data from the European Organization for Rare Diseases (EURORDIS) further highlights the huge societal impact of these frequently progressive, disabling, and life threatening in nature diseases. It has in fact been estimated that rare diseases together affect around 10 % of individuals worldwide, 80 % of these disorders have identified genetic origins, 50 % of rare diseases affect children, and 30 % of patients with rare diseases die before the age of 5.

Rare genetic conditions are often inherited, but they may also arise as result of sporadic or chance mutations. Many if not most are caused by defects in a single gene, but often multiple different mutations in that single gene produce many different clinical presentations with a great variability in symptom severity and disease progression (Development 2010). This, together with the scarcity of information about the diseases' natural history and related pathophysiological mechanisms, poses tough drug development challenges.

Things are further complicated by the fact that the majority of rare diseases have a prevalence of less than 10 patients per one million (less than 5,000 patients in the EU) and related rarity is responsible of the paucity of patients available for clinical studies and consequent difficulties in defining specific endpoints and outcome measures. These challenges raise the uncertainty that a research program will lead to a new therapy, resulting in historically less investment into these therapies (Pryde and Palmer 2014).

Orphan Drug Categories

The introduction of specific orphan designation in the USA, EU, and several other jurisdictions has successfully stimulated the development of products for rare diseases. In the first 25 years of the Orphan Drug Act in the USA, 1892 products have been designated as orphan, and 326 products have been approved (Braun et al. 2010). Similar success has been shown in the EU, where, although the orphan drug designation was introduced later, in the first decade more than 850 orphan drug designations have been granted by the European Commission and more than 60 orphan drugs have received marketing authorization (Westermark et al. 2011). Although such important achievements, orphan applications are associated with the rare disease prevalence and level of available scientific knowledge on the proof of concept, linking possible drug candidates to the disease of interest (Putzeist et al. 2013). According to FDA data, to date there are about 200 rare diseases targeted by orphan drugs (Franco 2013), but for many rare diseases, there are yet no available therapies.

Orphan Diseases and Rare Diseases

Rare diseases exist in all disease categories and range from exceptionally rare diseases affecting only few patients worldwide to more prevalent but still rare ones (Bali et al. 2013).

Table 2 EU Orphan designations by therapeutic area

	2000–2010	2011	2012	2013
	EMA/279601/2010	*2012 Report on the State of the Art of Rare Disease Activities in Europe*	*2013 Report on the State of the Art of Rare Disease Activities in Europe*	*2014 Report on the State of the Art of Rare Disease Activities in Europe*
Applications received	1,113	166	197	201
Applications which received positive opinions on orphan designations	760	111	139	136
Number of application which received marketing authorization per year	/	5	10	7
Total number of application which received marketing authorization	63	68	78	85
Oncology	45.2 %	41 %	39&	40 %
Musculoskeletal and nervous system	12.4 %	12 %	11 %	8 %
Immunology	9.7 %	7 %	6 %	3 %
Metabolism	9.7 %	12 %	10 %	20 %
Cardiovascular and respiratory	9.4 %	8 %	9 %	13 %
Anti-infectious	3.3 %	4 %	6 %	/
Hematology	/	3 %	7 %	9 %
Other	10.3 %	13 %	12 %	7 %

Orphan diseases include many different rare conditions such as neurological conditions, infectious diseases, rare cancers, autoimmune disorders, respiratory disorders, muscle disorders, blood disorders, and a wide range of inherited genetic disorders (Pryde and Palmer 2014). In the USA, among these categories, oncology therapeutics is the dominant one, accounting for 33 % of the marketing authorizations from 2006 to 2011. Following then are inborn errors of metabolism and gastrointestinal disorders (17 %), neurological conditions (17 %), hematological and immunological diseases (8 %), and rheumatology disorders (8 %) (Melnikova 2012). A similar trend is observed also in the EU where EMA data EMA/279601/2010) shows that in the decade 2000–2010, the largest group of orphan medicines for which the Agency's Committee for Orphan Medicinal Products has adopted a positive opinion was for oncology treatments (Table 2).

It is clear that in the EU there is a steady increase in the number of the orphan drug designations as seen in the USA (Hernberg-Stahl and Reljanovic 2013).

With the constant supremacy of the oncological category, it is interesting to note that the attention on rare metabolic diseases is constantly growing and today the two largest groups of orphan drug designations are for rare forms of cancer and metabolic disorder (Hollak et al. 2011; Wastfelt et al. 2006).

In fact, it has been shown that although efforts in translating rare disease research into an orphan drug development program are more likely focused on more prevalent rare diseases than the less prevalent ones (Heemstra et al. 2008), orphan drugs approved for low prevalence rare diseases are certainly not uncommon (Westermark et al. 2011). Above all it has been shown that preclinical proof of concept of a potential medicinal product constitutes the major knowledge-related determinant associated with an orphan designation application (Putzeist et al. 2013).

Drug Development for Rare Inherited Metabolic Diseases, in Particular LSDs

Among metabolic disorders, all the ones due to genetic defects may be considered to be orphan diseases, as their incidence in the population is less than 1/5,000 (Martinez-Pardo 2001). Inherited metabolic diseases (iMDs) are a broad group of more than 600 orphan genetic diseases caused by mutations/defects of single metabolic genes. Individually these disorders affect very small numbers of individuals, but collectively they have a tremendous global impact since the entire population of patients affected by iMDs is large (Haffner et al. 2008). iMDs represent some of the rarest but most life-threatening, unmet, medical needs. Data confirmed that orphan drug designation constitutes a successful stimulus in producing new therapies that are desperately needed by iMDs patients and that drug development has been particularly successful for some iMDs categories, in particular lysosomal storage disorders (LSDs) (Talele et al. 2010).

LSDs are a group of about 70 inherited metabolic and neurodegenerative disorders (see Table 3) due to deficiency of a specific protein responsible for lysosomal function, such as enzymes or lysosomal components, or to errors in enzyme trafficking/targeting and defective function of nonenzymatic lysosomal proteins, all preventing the complete degradation and recycling of macromolecules (Bellettato and Scarpa 2010). Lysosomes are ubiquitous organelles present in all body cells, with the exception of the red blood cells. This is responsible for a multiorgan deficiency, although inside the same disorder various degrees of severity of the disease are observed in a sort of phenotypic continuum (for a review, see Cox and Cachon-Gonzalez 2012; Gieselmann 1995; Klein and Futerman 2013; Platt et al. 2012). About 70 % of LSDs affect the CNS. The fact that neurodegeneration is a common hallmark of many other more common age-related conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), spinal cerebellar ataxias, and spinal muscular atrophy and

Table 3 Main classification of the lysosomal storage diseases

Acid phosphatase deficiency
Disorder of lysosomal amino acid transport
Cystinosis
Free sialic acid storage disease
Disorder of sialic acid metabolism
Sialuria
Salla disease
Glycoproteinosis
Mucolipidosis
Mucolipidosis type 2
Mucolipidosis type 3
Mucolipidosis type 4
Oligosaccharidosis
Alpha-mannosidosis
Alpha-*N*-acetylgalactosaminidase deficiency
Aspartylglucosaminuria
Beta-mannosidosis
Fucosidosis
Galactosialidosis
Sialidosis
Lysosomal glycogen storage disease
Glycogen storage disease due to acid maltase deficiency
Mucopolysaccharidosis
Mucopolysaccharidosis type 1
Mucopolysaccharidosis type 2
Mucopolysaccharidosis type 3
Mucopolysaccharidosis type 4
Mucopolysaccharidosis type 6
Mucopolysaccharidosis type 7
Mucopolysaccharidosis type 9
Neuronal ceroid lipofuscinosis (NCL)
NCL1
NCL2
NCL3
NCL4
NCL5
NCL6
NCL7
NCL8
NCL9
NCL10
Pycnodysostosis
Sphingolipidosis
Autosomal-recessive cerebellar ataxia with late-onset spasticity
Encephalopathy due to prosaposin deficiency
Fabry disease

(*continued*)

Table 3 (continued)

Farber lipogranulomatosis
Gangliosidosis
GM1 gangliosidosis
GM2 gangliosidosis
Gaucher disease
Krabbe disease
Lipid storage disease
Lysosomal acid lipase deficiency
Cholesteryl ester storage disease
Wolman disease
Niemann–Pick disease type C
Metachromatic leukodystrophy
Multiple sulfatase deficiency
Niemann–Pick disease type A
Niemann–Pick disease type B
Niemann–Pick disease type E

that growing evidence implicates lysosomal dysfunction in the pathogenesis of all these disorders (Appelqvist et al. 2013) has probably contributed to such growing interest and attention on LSDs. Such possible sharing of common biochemical pathway could in fact make the development of an orphan drug beneficial for more than one disease.

LSDs and Orphan Drug

The first breakthrough therapy in the LSDs commercial treatment occurred in 1991 (Gaucher diseases enzyme replacement therapy, ERT) as a result of collaborative efforts between the National Institutes of Health and the biotechnology company Genzyme, fostered by the Orphan Drug Act (ODA 1983) (Pastores and Gupta 2013). It represents an important milestone in the treatment of this disorder as it signed the passage from a simple symptomatic management to proper therapeutic interventions that address the underlying metabolic defect. ERT, in fact, consists in the substitution of the dysfunctional enzyme by exogenous administration of an in vitro synthesized functional one. It does not constitute a cure for these disorders yet, but, although many limits, it can greatly modify or attenuate the phenotype (the signs and symptoms and severity of the condition) and disease progression (Kirkegaard 2013). Since 1991, many other therapies have been introduced for other LSDs. Follow a list of the main enzymatic therapies and other treatment options available today for many LSDs (Table 4).

Orphan Drug for Gaucher Disease

Gaucher disease (GD) is a very rare disorder (prevalence about 1 in 70,000) caused by a deficiency of the lysosomal enzyme glucocerebrosidase (or acid β-glucosidase, EC 3.2.1.45) (van Dussen et al. 2014) which induces storage of glycolipids in lysosomes, typically inside the cells of the macrophage–monocyte system. Such accumulation is responsible of the characteristic manifestations including hepatosplenomegaly, severely debilitating bone and hematological disease, and, in the more severe cases, central nervous system involvement. Three different phenotypes of GD are identified. Type I GD (GDI), the most common one, differs from the more severe types II and III GD because neurologic manifestations are absent (Biegstraaten et al. 2008). This unique feature has rendered GDI an ideal target for ERT.

GDI is the first disorder for which purified enzyme, administered intravenously, has shown to be effective in reversing most of the manifestations. To date, three recombinant enzymes are available (imiglucerase, Cerezyme®, Genzyme Corporation, Cambridge, MA, USA; velaglucerase alfa, VPRIV®, Shire Human Genetic Therapies, MA, USA; and taliglucerase alfa, Elelyso, Protalix Biotherapeutics, Karmiel, Israel) of which the last one is approved in the USA only. An alternative second choice treatment is represented by the substrate reduction therapy (SRT) (miglustat, Zavesca, Actelion Pharmaceutical, Switzerland) which is only indicated for the treatment of mildly to moderately affected GDI patients for whom ERT is unsuitable (van Dussen et al. 2014) or when the disease is stable. It has to be noted that Zavesca is also used to reduce the progression of clinically relevant neurological symptoms in adult and pediatric patients affected by Niemann–Pick

Table 4 LSDs for which orphan drugs are available (MPS excluded)

Disorder	Enzyme deficiency or altered gene	Drug		Company
Cystinosis	CTNS gene	SDT	Cystagon (cysteamine) immediate-release capsule	Mylan Pharmaceuticals Inc
		SDT	Cystaran (cysteamine) ophthalmic solution	Sigma-Tau Pharmaceuticals
		SDT	Procysbi (cysteamine bitartrate) delayed-release capsule	Raptor Pharmaceutical Corp
Gaucher disease	Glucocerebrosidase	ERT	Cerezyme (imiglucerase alfa)	Genzyme
		ERT	VPRIV (velaglucerase alfa)	Shire HGT
		ERT	Elelyso (taliglucerase)	Protalix
		SRT	Zavesca (miglustat)	Actelion
Fabry disease	Alpha-galactosidase	ERT	Fabrazyme (agalsidase beta)	Genzyme
		ERT	Replagal (agalsidase alfa)	Shire HGT
Pompe disease	Alpha-glucosidase	ERT	Myozyme (alglucosidase alfa)	Genzyme
		ERT	Lumizyme (alglucosidase alfa)	Genzyme
Niemann–Pick type C disease	NPC1/NPC2 gene	SRT	Zavesca (miglustat)	Actelion

NB: Clinical trials are ongoing also for metachromatic leukodystrophy, neuronal ceroid-lipofuscinosis type 2, acid lipase deficiency, alpha-mannosidosis deficiency, GM2 gangliosidosis, acid sphingomyelinase deficiency
SDT substrate depletion therapy

disease type C for which it was approved in Europe in 2009 (Lyseng-Williamson 2014b).

Orphan Drug for Fabry Disease

The exceptional clinical results of ERT in GD disease led to high expectations when, almost a decade later, Fabry disease (FD) attempts were made. Not just because after GD, FD is the second most common LSD but also because both diseases are inherited glycosphingolipidoses caused by deficiencies in the lysosomal glycosidases (glucocerebrosidase and alpha-galactosidase respectively). Nevertheless, so far ERT approaches with the two different α-galactosidase enzymes developed, one made by recombinant technology in CHO cells (Fabrazyme, agalsidase-β produced by Genzyme) and the other by overexpression of the native gene in a cultured human cell line (Replagal, agalsidase-α produced by Shire), have not achieved the expected results (Platt and Lachmann 2009). Recent reports in fact showed that, although cells and tissues of GD and FD patients are uniformly deficient in enzyme activity, cell types showing lysosomal accumulation of the glycosphingolipid substrates are considerably different and consequently induced pathologies and related mechanisms are entirely dissimilar (Ferraz et al. 2014). Early evidence suggests that, while ERT induces extensive specific clearance of endothelial cell storage of the glycosphingolipid Gb3, widespread pathological storage of glycosphingolipids persists in numerous sites and organs throughout the body (Cox 2006). Outcomes from two systematic reviews indicate that ERT response is variable and no robust evidence for use of either agalsidase alpha or beta is provided (Alegra et al. 2012; El Dib et al. 2013). These results are further confirmed by a recent systematic review and meta-analysis showing that long-term ERT generally does not prevent disease progression. Nevertheless, efficacy in reducing left ventricular hypertrophy was reported, and the risk of developing a first or second complication declined with increasing treatment duration (Rombach et al. 2013, 2014).

Although previously no evidence of ERT-related benefits in patients with advanced FD had been reported, recent data indicate that ERT may reduce the progression of vascular disease, even in advanced FD patients, suggesting that early treatment may stabilize white matter lesion progression and stroke risk (Fellgiebel et al. 2014).

Taking into account that ERT so far available might not be capable of effectively completely reversing some of the FD pathologies, another new enzyme for the therapy of FD has recently been developed. The new drug (PRX-102, developed by Protalix BioTherapeutics) is a chemically modified version of the human α-galactosidase-A enzyme expressed in a BY2 tobacco cell culture. Outcomes from a recent study confirm that PRX-102 is equivalent in functionality to the current ERTs available, but has superior stability and prolonged circulatory half-life. PRX-102 therefore constitutes a promising alternative for treatment of FD (Kizhner et al. 2015).

Besides ERT must be highlighted the promising role of the oral therapy with migalastat hydrochloride (GR181413A), an investigational pharmacological chaperone, developed by Amicus Therapeutics in collaboration with GlaxoSmithKline, which selectively binds, stabilizes, and increases cellular levels of the lysosomal enzyme α-galactosidase. Oral administration of migalastat has showed to reduce tissue GL-3 in FD transgenic mice, and in urine and kidneys of some FD patients (Germain et al. 2012; Young-Gqamana et al. 2013).

Orphan Drug for Mucopolysaccharidosis

Mucopolysaccharidosis (MPS) are a heterogeneous group of 7 inherited metabolic disorders caused by a deficit in one of 11 lysosomal enzymes involved in the degradation of mucopolysaccharides, also called glycosaminoglycans (GAGs). They share many clinical features, but a wide heterogeneity in their severity is present, and also each distinct type of MPS is characterized by a wide spectrum of clinical manifestations from attenuated (slowly progressing) to very severe (rapidly progressing) forms of the disease (Lampe et al. 2013).

Up to date two are the main treatment options for patients affected by MPS, both directed at the underlying pathophysiology: hematopoietic stem cell transplantation (HSCT), which is useful for selected patients, and recombinant i.v. ERT, which is available for MPS I, II, IV, and VI (Table 5).

ERT for MPS I

Mucopolysaccharidosis type I (MPS I) is due to a deficiency of the lysosomal hydrolase a-L-iduronidase leading to accumulation of the GAGs, dermatan sulfate, and heparan sulfate. It comprises a wide spectrum of symptoms that differ from patient to patient with regards to age of onset and severity. In particular MPS I disease spectrum includes a form with severe involvement and CNS disease (Hurler disease – HPS I H), a chronic form of the disease without CNS disease (Scheie disease – HPS I S5), and an intermediate form of the disease (Hurler/Scheie – HPS I HIS) (Wraith and Jones 2014).

ERT with Aldurazyme (laronidase, recombinant human α-L-iduronidase produced by Genzyme Corporation, Cambridge, MA, and BioMarin Pharmaceutical, Inc., Novato, CA, USA) has been available in the USA and Europe since 2003. Several clinical trials, including one randomized controlled trial, have demonstrated ERT safety and efficacy for the treatment of non-neurological symptoms of the disease at long term with amelioration of the growth rate, stability of the cardiac involvement, improvement of the joint mobility, and reduction of the organomegaly. In addition a multidisciplinary consensus procedure yielded agreement on the fact that treatment with laronidase should be started at diagnosis in all symptomatic MPS I patients and laronidase may also improve the pre- and peri-HSCT (de Ru et al. 2011; Jameson et al. 2013).

Table 5 Mucopolysaccharidosis: enzyme defects and availability of orphan drugs

Disorder	Alias	Enzyme deficiency		Drug	Company
MPS I	Hurler/Scheie	Alpha-L-iduronidase (IDST)	✓	Aldurazyme (laronidase)	Genzyme
MPS II	Hunter	Iduronate 2-sulfatase (IDNS)	✓	Elaprase (idursulfase)	Shire HGT
MPS III A	Sanfilippo A	Heparan *N*-sulfatase	✗		
MPS III B	Sanfilippo B	*N*-acetyl-alpha-D-glucosaminidase (ANAT, ANAS)	✗		
MPS III C	Sanfilippo C	Acetyl-CoA/alpha-glucosaminide *N*-acetyltransferase	✗		
MPS III D	Sanfilippo D	*N*-acetylglucosamine-6-sulfatase	✗		
MPS IV A	Morquio A	Galactosamine-6-sulfatase (G6ST)	✓	Vimizim (elosulfase alfa)	Biomarin
MPS IV B	Morquio B	Beta-galactosidase (BGAT, BGA)	✗		
MPS VI	Maroteaux–Lamy	Arylsulfatase B (ARSB)	✓	Naglazyme (galsulfase)	Biomarin
MPS VII	Sly	Beta-glucuronidase (BGLR)	✗		
MPS IX	Hyaluronidase deficiency	Hyaluronidase	✗		

N.B. Clinical trials are ongoing for MPSIII A, B, and MPSVII

ERT for MPS II

Mucopolysaccharidosis type II (MPS II) or Hunter syndrome is the only mucopolysaccharidosis inherited as X-linked disease. It is due to the deficiency of the lysosomal enzyme iduronate-2-sulfatase, responsible for the related accumulation of GAGs leading to pathological changes in multiple body systems. Age at onset, signs and symptoms, disease severity, and disease progression vary significantly. Severely affected patients have profound neurological involvement associated with progressive airway and cardiac disease, usually resulting in death in the first or second decade of life (Scarpa et al. 2011). In 2006, the FDA approved ERT for MPS II with idursulfase (Elaprase, Shire), a purified form of human lysosomal enzyme iduronate-2-sulfatase.

As stated in a recent review, clinical safety and efficacy of ERT have been extensively assessed by several clinical studies. Since 2005, in fact, the Hunter Outcome Survey collects data on Hunter patients to delineate disease natural history and to monitor ERT safety and effectiveness. Many other studies have been performed, clearly showing that ERT with idursulfase has positive effects on functional capacity (distance walked in 6 min and forced vital capacity), liver and spleen volumes, and urine GAGs excretion (da Silva et al. 2014; Tomanin et al. 2014). Recently, a 3.5-year independent study determined that long-term use of ERT is safe and its efficacy is similar in both young (1.6 to 12 years at the start of ERT) and older patients (12–27 years at the start of ERT). Further evidences confirming the efficacy of ERT in improving somatic signs and symptoms of the disease for all patients, including infants under 1 year of age and patients with severe MPS II phenotype, are provided by two recent studies (Lampe et al. 2014a, b). It was anyway observed that ERT efficacy was extremely subjective, despite a widely accepted common protocol (same dose/kg of body weight, time schedule, and velocity of infusion). Therefore, dose and

frequency regimens of administration must be carefully tailored to every single patient, according to his clinical picture (Tomanin et al. 2014). More studies are still necessary to obtain additional information on the long-term effectiveness and safety of ERT. Unfortunately, options for alleviating the neurological manifestations of MPS II remain limited. In fact, intravenously administered idursulfase does not pass through the blood–brain barrier (BBB). In order to overcome this problem, Shire is sponsoring a phase I/II clinical trial examining the use of intrathecal iduronate-2-sulfatase in young patients with MPS II with central nervous system involvement (https://clinicaltrials.gov/ct2/show/NCT02055118).

ERT for MPS IVA

Mucopolysaccharidosis type IVA (MPS IVA) or Morquio A syndrome is caused by the deficiency of *N*-acetylgalactosamine-6-sulfate sulfatase that leads to impaired catabolism and consequent accumulation of two GAGs: chondroitin-6-sulfate and keratan sulfate. The disease is characterized by a wide spectrum of clinical presentation, and disease progression rages from a severe "classical" phenotype to a slower progressive phenotype. Predominantly common manifestation includes short stature and skeletal dysplasia (dysostosis multiplex), including atlantoaxial instability and cervical cord compression. However, abnormalities in the visual, auditory, cardiovascular, and respiratory systems can also affect individuals with MPS IVA (Hendriksz et al. 2013). ERT for MPS IVA with Vimizim (Elosulfase alfa, a recombinant form of the human lysosomal enzyme GALNS, produced by BioMarin) was recently approved by the US FDA on 14 February 2014 for the treatment of MPS IVA (Sanford and Lo 2014). In clinical studies, ERT with elosulfase alfa in fact significantly improved physical endurance, respiratory function, growth, and quality of life in Morquio patients. Treatment increased clearance of GAGs and induced gene expression consistent with improved chondrocyte function (Haddley 2014). Patient's safety was also evaluated, and results showed that elosulfase alfa had an acceptable safety profile (Hendriksz et al. 2014).

Elosulfase alfa is today approved for the treatment of MPS IVA also in Europe (Haddley 2014) and constitutes the first, and currently only, disease-specific treatment option for this very rare, progressively degenerative, autosomal-recessive LSD (Lyseng-Williamson 2014a).

ERT for MPS VI

Mucopolysaccharidosis VI (MPS VI) or Maroteaux–Lamy syndrome is due to a deficiency in the arylsulfatase B (ASB) enzyme. This deficiency impairs the stepwise degradation of GAGs resulting in the accumulation of partially degraded GAGs in tissues and organs throughout the body which can lead to progressive tissue and organ dysfunction and premature death in most instances (Valayannopoulos et al. 2010). The gold standard therapy for MPS VI is ERT with Naglazyme® (galsulfase, a human recombinant arylsulfatase B produced by Biomarin). Unfortunately, due to the presence of the BBB or to a poor circulation, the drug is not able to reach the cornea and the articular cartilages which are very impaired in these patients. Extensive preclinical and human clinical trial studies have been conducted showing patients' improvements in walking and stair-climbing capacity, increments in endurance, improvements in pulmonary function, and reductions (but not normalization) of urinary GAGs excretion (Harmatz et al. 2005, 2006). Such important outcomes led to galsulfase market approval in 2005 in the USA and in January 2006 in the EU (Harmatz et al. 2005; Valayannopoulos et al. 2010). It has also been demonstrated that long-term ERT with recombinant human arylsulfatase B is effective in reducing intraventricular septal hypertrophy and preventing progression of cardiac valve abnormalities when administered to patients <12 years of age (Braunlin et al. 2013). Furthermore, it significantly increases patient's height and growth rate particularly in those aged below 16 years, suggesting the advantage of initiating

ERT as early as possible to maximize its potential benefits also in effectively treating delayed puberty which is common in MPS VI patients (Decker et al. 2010).

Although such treatment received marketing authorization in the USA in 2005 and in the European Union in January 2006, patients' access to this treatment varies geographically due to differences between national reimbursement schemes for orphan drugs (Schlander and Beck 2009).

Orphan Drug for Pompe Disease

Pompe disease (PD) is a rare inherited metabolic myopathy caused by deficiency of the acid alpha-glucosidase (GAA) enzyme in lysosomal cells. As a result, glycogen storage occurs in muscles, and patients present a wide clinical spectrum, ranging from early onset (infantile) severe cardiomyopathy (with death occurring by years 1 of age) to adult onset forms. No therapies were available for this disease until 2006, when Myozyme (alglucosidase alfa, recombinant human GAA produced by Genzyme) became the first ERT for PD. Afterward Lumizyme (alglucosidase alfa, Genzyme) was approved by the FDA in 2010 as orphan drugs to treat patients with late-onset PD.

Important outcomes on the efficacy of ERT are provided by the IPA/Erasmus MC survey, an international longitudinal prospective survey established to collect information on PD natural and its burden on patients. It involved 408 patients between 2002 and 2013, and the analysis of the cumulative data confirmed the positive effects of ERT on patients' quality of life, fatigue, and participation in daily life (van der Meijden et al. 2014). It also provides the first evidence that survival is reduced in adult Pompe disease and improved by ERT. It has been proved that the sooner ERT begins, the better are the results. However, ERT still presents some limitations, and therefore a decrease in immune responses to ERT, a higher dosage, a better uptake formulation together with adjunctive and alternative therapies are being explored and tested (Chien et al. 2013; Kishnani and Beckemeyer 2014).

Limits of the ERT and New Treatment Prospects

ERT represents the most important advancement and a major breakthrough in the treatment of many LSDs. Its successful use in the treatment of several LSDs has stimulated investigators and companies to develop new and improved recombinant enzymes to treat other LSDs including MPS VII (Sly disease), MPS IIIA (Sanfilippo disease A and B), metachromatic leukodystrophy, and acid lipase deficiency for which today, new studies to test enzyme efficacy are ongoing (Parenti et al. 2013). Besides, clinical experience with ERT has revealed many related limitations of this approach, mostly associated to enzyme bioavailability. Indeed ERT is a lifelong therapy, and disease progression is still observed in treated patients. In addition, factors such as immune reactions against the infused enzyme, miss-targeting of recombinant enzymes, and difficult delivery to crucial tissues (i.e., brain and bone) represent further obstacles to successful ERT (Ortolano et al. 2014). To restore health, or to ameliorate patients' quality of life, ERT should, in fact, be directed toward correction of pathology and function in all affected tissues. Unfortunately to date enzyme biodistribution still represents a crucial problem as recombinant enzymes are large molecules that do not freely diffuse across membranes and depend on mannose-6-phosphate receptors (M6PR) pathways for delivery to lysosomes where they can be activated and hydrolyze specific substrates (Wraith 2006). Of even greater clinical relevance is the presence of the BBB, a selectively permeable barrier between the capillaries and the brain that acts as a filter, preventing many substances from entering the CNS. So far, in fact, none of the therapeutic enzymes have demonstrated to be effectively capable of reaching the brain in significant amounts to treat the neurological manifestation and degeneration. The main cause seems to be the enzymes' high molecular weight and the lack of expression of a functional M6PR at the level of the BBB. Research attempts to overcome this major obstacle, the BBB, have never been so intensive and promising as in the

last few years. In this setting, some new therapeutic approaches (chemical, biological, or technological) for the achievement of effective concentrations of drugs in the CNS are under development (Scarpa et al. published ahead-of-print; Calias 2012; Calias et al. 2014). In particular, many efforts are focused at developing therapeutic drugs of low molecular weight and with a high degree of lipid solubility. Several approaches take advantage of the receptors/transporters expressed in the luminal or abluminal membranes of the BBB or of the temporary breakdown of the tight junctions. Other more invasive procedures consist in the direct intracerebral injection or use of intracerebral implants such as erodible diffusional polymeric systems, microchip, or catheter-based infusion system. These attempts have been evaluated in preclinical studies for several lysosomal disorders and have been translated into human therapy for MPS I. Other approaches consist in the enzyme manipulation, as done with the β-glucuronidase, where chemical modification abolished mannose and M6PR-mediated uptake of the enzyme and resulted in sustained enzyme plasma levels and increased delivery to brain cells by an unknown pathway (Grubb et al. 2008, 2010). Other direct manipulation of the enzyme can consist in the use of peptide-based targeted delivery system to form chimeric proteins (wherein a non-transportable drug is conjugated to a BBB transport vector) and carrier peptides (genetically engineered molecular "Trojan horses" or nanoparticles for drug delivery across BBB). Pharmacological chaperone therapy (PCT) in particular holds great promise as potential therapeutic since it is based on small-molecule compounds that can enter the brain through the BBB, enhance enzyme activity, reduce substrate storage, and improve neurological deterioration. After proof-of-concept studies, PCT is now being translated into clinical applications for Fabry, Gaucher, and Pompe disease. Moreover the recent demonstration of a synergistic effect of chaperones and ERT expands the applications of PCT (Parenti 2009; Parenti et al. 2014). Substrate reduction therapy (SRT) is another promising and well-developed method that aims to reduce the amount of accumulated stored material by reducing the level of the substrate to a point where residual degradative activity is sufficient to prevent substrate accumulation. Gene therapy constitutes another important viable alternative or adjunctive therapy to current management strategies. It aims at restoring enzyme activity by delivering the wild-type copy of the defective gene into the recipient's cells and thus represents a promising solution for some more general problems including the fact that typically the neuropathology in LSDs is spread throughout the entire brain and is not limited to specific areas (Scarpa et al. published ahead-of-print). To date, several phase 1/2 clinical studies have been initiated for gene therapy-based treatments for LSDs, and the field is constantly growing (Byrne et al. 2012). In addition, to more efficiently improve clinical outcome of LSDs patients, various therapies have been combined. One promising strategy consists in providing a persistent source of the deficient enzyme (gene therapy, stem cell transplantation) while targeting a secondary consequence of disease with a more transient approach (substrate reduction, anti-inflammatories, pharmacological mimetic, etc.) (Hawkins-Salsbury et al. 2011). The idea of combination protocols in LSDs has also been proposed for ERT and SRT. This strategy was used for GD disease, to obtain therapeutic effects in tissues and organs (such as bone and cartilage) unresponsive to ERT alone, or in the brain in the neuronopathic forms of the disease (Cox-Brinkman et al. 2008).

Conclusion

Although different therapeutic options have been introduced over the past two decades, it is now becoming clear that these approaches have limitations and important issues remain unsolved. LSDs are very complex disorders characterized by a wide diversity of pathology, clinical phenotypes, and age of onset. In particular, the complexity of related pathophysiology and the fact that they might affect all organs makes therapeutic interventions particularly challenging. Although mutations responsible for most LSDs are largely noted, the molecular mechanisms through which

the storage material causes cellular and organ pathology are indeed still largely unknown (Gieselmann 2006). Treating LSDs affecting the CNS is particularly challenging and is an almost insurmountable hurdle for most available and future potentially effective drugs (after peripheral administration). Therefore, research efforts have recently focused on the development of new alternative strategies to enhance drug delivery across the BBB. The discovery and development of novel drug delivery systems for the treatment of CNS diseases is a major challenge for both the academic and pharmaceutical community. Significant advancements in the field have already been made and different approaches have been developed, raising significant hopes for affected patients. Although further investigations are necessary for a better comprehension of the mechanisms, which drive the drug delivery process, and for assessing related efficacy and safety, it seems quite reasonable to think that BBB is no longer an impenetrable barrier. The pilot studies show that ERT, SRT, PCT, gene therapy, bone marrow transplantation, the use of stem cells, and the application of nanotechnology constitute important therapeutic strategies potentially capable of delivering the drug to the brain. Clinical application of these therapeutic options is limited by the associated costs. Therapies are in fact expensive, limiting access to patients from those countries that are not able to afford expensive health care treatments. The initial investments in research and the costs related to the production process are the main factors that contribute to their high prices. In the case of ERT, for example, the treatment of a single LSD patient may cost several hundred thousand euros (or dollars) per year (Parenti et al. 2013). However, some of these strategies have the potential to broaden the therapeutic horizons for all patients suffering from neurodegenerative diseases, which represent one of the major public health problems.

Thanks to the introduction of legislation that provides financial and regulatory incentives, activity in the pharmaceutical industry continues at a high level as witnessed by the new drug approvals and launches made in 2014 (Graul et al. 2015). The development and commercialization of drugs for rare diseases have become more attractive, and new developments are making their way through the regulatory approval processes. Unfortunately, delays in availability of new drugs for treating rare disease continue to persist (Feltmate et al. 2015). It is therefore clear that besides the necessity to increase the development of new orphan drugs, there is also an imperative need to improve a timely access to safe and effective treatment. This last issue has been recently recognized by regulatory agencies around the world which have implemented legislation to support market access modifying the approval timelines for access to new treatments (Feltmate et al. 2015). The optimization of new criteria for speeding up the process of orphan drug development, facilitating the translational research, and optimizing drugs price levels and use is fundamental to ameliorate the conditions of millions of orphan patients in developing and developed countries.

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Zebrafish

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Contents

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General Considerations

Zebrafish as a Model System for Biological Questions

The zebrafish (*Danio rerio*) is a small freshwater fish native to the Ganges River valley and a popular species for amateur aquaria and scientific investigators alike due to its bright striped coloration, low cost, and easy maintenance. Researchers have also been attracted to the zebrafish because of numerous useful biological features. A single mating pair can generate hundreds of fertilized embryos in a single clutch, producing thousands of offspring over their breeding lifetime. As fertilization occurs externally and the embryos are optically transparent, all of the major developmental processes leading to a fully patterned animal are easy to observe. Moreover, development happens rapidly. At 28 °C, a single fertilized zebrafish cell becomes fully patterned from head to tail within 24 h, complete with a brain, major organs such as a liver and heart, and a functioning circulatory system (Kimmel et al. 1995). By 3 days post fertilization (dpf), the zebrafish eye is functional; by 5 dpf, larvae have inflated their swim bladders and are capable of spontaneous swimming, feeding, and other complex behaviors including sleep, hunting, and learning. The genetics of zebrafish are also well developed, with a fully sequenced genome (Howe et al. 2013), large collections of mutants (Kettleborough et al. 2013), and transgenic lines

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to label or manipulate many different tissue types (Kawakami et al. 2010; Kondrychyn et al. 2011). Modern genome-editing technologies, such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (the CRISPR/Cas system) allow for targeted mutagenesis and the generation of genetic disease models (Auer and del Bene 2014; Bedell and Ekker 2015). Finally, the zebrafish is the only cost-effective vertebrate model system for large-scale genetic and pharmacological screens. For example, the Tübingen and Boston forward genetic screens of the mid-1990s identified nearly 1,500 zebrafish mutants in developmental and simple behavioral processes, effectively launching the zebrafish as a model genetic system (Nüsslein-Volhard 2012).

Pharmacology and the Zebrafish

Unlike most other model organisms, which normally require pharmacological agents to be directly injected or fed, zebrafish larvae and adults can uptake compounds directly from the water through their gills, skin, and mouth. Depending on the developmental stage, zebrafish are also tolerant to low concentrations of dimethyl sulfoxide (DMSO), methanol, or ethanol, meaning that even relatively water-insoluble compounds can still be directly administered by this method. As embryonic and larval zebrafish fit easily into 96-well plates, exposure by bath application provides a simple means to perform image- and behavioral-based screens. Embryos can be plated quickly by hand, but there are now many robotic systems capable of collecting and sorting embryos. These automated systems can sort zebrafish embryos based on expression of fluorescent markers and then dispense single or small pools of embryos into 96-well plates (Pfriem et al. 2012) or advanced microfluidics devices (Pardo-Martin et al. 2010). Drug dispensation can similarly be handled manually with a multichannel or repeat pipette or through robotic delivery of specific volumes. Once fish and drugs are combined in the plates, numerous endpoints can easily be measured, including acute toxicity; developmental processes such as patterning events, blood vessel formation, or hematopoietic stem cell production; disease processes such as cancer biology; genetic disease modeling; regeneration of tissues including the fin, heart, brain, and spinal cord; organ function such as heart rhythmicity or gut function; and behaviors such as visual acuity, anxiety, sleep, or seizure activity. The effects of small molecules can be assessed by manual scoring, advanced video analysis of behavioral responses, high-content imaging, high-throughput in situ hybridization or antibody detection, electrophysiology, and many other methods.

While direct addition to the water is by far the most common route of exposure, both larval and adult zebrafish can also be exposed to compounds via intraperitoneal injection, delivery into the bloodstream through the heart or the retro-orbital vein (Pugach et al. 2009), or direct injection into the organs such as the brain ventricle, heart, or liver (Chang et al. 2014). Rapid advances in automation have made these delivery methods suitable for screening efforts. For example, small antisense oligonucleotides can be rapidly injected into the yolk at the single cell stage, as can infectious agents for immunological studies (Wang et al. 2007; Spaink et al. 2013). These methods expand the utility of zebrafish as a pharmacological model to include water-insoluble compounds, tissues that may be less accessible to compounds, or to isolate compound function onto specific tissues or organs without confounding effects on other systems.

Throughout this chapter, we focus specifically on biological questions for which the zebrafish is especially suited. Given the flexibility of the zebrafish system, this perhaps unsurprisingly covers most current questions in development, neuroscience, and disease. We have paid particular attention to zebrafish as the only vertebrate model for medium and high-throughput small-molecule screening and indicate those assays especially suited for this purpose (Peterson and Fishman 2011; Rennekamp and Peterson 2015). To date, zebrafish chemical screens have not only provided mechanistic insight into a diverse range

of processes but have also been successful in identifying novel drugs, including those that have great promise in the clinic (Tan and Zon 2011; Tamplin et al. 2012).

Limitations and Considerations

Pharmacological screening in zebrafish is still in its infancy, and there are many limitations that must be kept in mind. One primary concern is that while small-molecule application directly into the water makes delivery quick and easy, not all compounds will be equally taken up into the fish and bioavailable at all developmental stages through adulthood. In the context of screening, systematic testing of drug concentrations in tissues is impossible, meaning that for any given screen, many false negatives will be likely. For most compounds tested in the zebrafish to date, issues of absorption, distribution, metabolism, excretion, and toxicity (ADMET) have been under-explored, and how bioavailability of compounds delivered in the water translates into bioavailability for traditional delivery methods is not well understood, although a few reports are promising (Parng 2005; Radi et al. 2012). How these parameters will translate into expected bioavailability in mammalian systems is unknown and may be impossible to predict. For example, the role of the blood-brain barrier for compound uptake in the zebrafish brain is poorly understood. In zebrafish the endothelial cells that compose the blood-brain barrier (BBB) begin to form as early as 3 dpf, but smaller molecules still appear capable of entering the brain up through 9–10 dpf (Jeong et al. 2008; Xie et al. 2010; Eliceiri et al. 2011; Fleming et al. 2013).

Another issue is the assumption of conservation of drug targets from fish to mammals. Zebrafish have clear orthologs for greater than 70 % of all human genes (80 % of disease genes) (Howe et al. 2013; Kettleborough et al. 2013). However, in most cases, how the zebrafish protein targets respond to small molecules, and how similar this is to known drug interactions on human receptors, is often unknown. In vitro binding efforts do show a good conservation of small-molecule receptor pharmacology, for example, on opioid, nociceptin, M2 muscarinic, melanocortin, androgen, GABA-A and GABA-B, histamine, and adrenergic receptors (Gonzalez-Nuñez et al. 2007; Rivas-Boyero et al. 2011; Hsieh and Liao 2002; Ringholm et al. 2002; Hossain et al. 2008; Dunér et al. 2002; Williams and Messer 2004; Renier et al. 2007; Peitsaro et al. 2007). However, differences between mammalian and zebrafish receptor subtype classes, drug specificity, and expression patterns may have dramatic – and thus far unknown – consequences. For example, there are five mammalian dopamine receptors but eight in zebrafish; how dopamine agonists/antagonists map onto the zebrafish dopamine receptor subtypes, and whether they will have similar functions, is still unclear. Furthermore, when novel compounds with interesting bioactivity are discovered, working out the in vivo targets of these molecules is not trivial, although many clever methods to match compounds to protein targets now exist (Rennekamp and Peterson 2015).

While the small size of the zebrafish makes it ideally suited for whole-animal studies, in typical assays, all tissues and organs are simultaneously exposed. On the one hand, while whole-animal testing for drug discovery may be an advantage when searching for efficacious molecules with low toxicity and side effects, indirect effects in other organ systems may affect the experimental outcomes. For example, toxicity in the heart may preclude studying a drug's effect on the brain.

Finally, many methodologies are not uniformly applied from one zebrafish laboratory to another. Some potential lab-to-lab confounds include the great variability in animal feeding and husbandry, the strain that is used for testing, the raising of larvae at different temperatures (from 25 to 29 °C), in different light/dark regimes, and different embryo water compositions. Throughout this chapter, we defer to The Zebrafish Book (Westerfield 2000) on animal husbandry, unless specifically noted. Otherwise, this chapter highlights the diversity of methodologies and makes suggestions for best practice where possible. Community efforts such as the Zebrafish Model

Organism Database (Sprague et al. 2008) or the Neurophenome Project (Kyzar et al. 2012), to name just a few, will continue to be valuable in maintaining lab-to-lab uniformity in experimental practice.

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Normal Development and Disease

Developmental Signaling, Cell Proliferation, and Cancer

Introduction

Since the two large forward mutagenesis screens of the mid-1990s described nearly 1,500 zebrafish mutants in more than 400 developmental and other genes (Driever et al. 1996; Haffter

et al. 1996), the zebrafish has become an established model for studying the signaling, cell proliferation, and tissue remodeling events that transform a single fertilized cell into a fully patterned, growing animal. Zebrafish are also well suited to the study of how these processes go awry in diseases such as cancer. Such studies are facilitated by both the large collection of mutants that perturb developmental processes as well as transgenic lines to visualize or manipulate specific cell populations. There are also well-established methods to label gene and protein expression by in situ hybridization (Thisse and Thisse 2008) and immunohistochemistry. As the zebrafish develops externally, probing developmental processes with pharmacology is relatively straightforward and can be scaled up easily to small-molecule screening of hundreds to thousands of compounds (Peterson et al. 2000). To comprehensively cover the techniques for examining the myriad of developmental processes that have been pharmacologically investigated in the zebrafish would require a full textbook. Instead, we highlight the use of small molecules to study developmental signaling events, cell proliferation, and cancer biology, with an emphasis on drug screening.

Developmental Signaling

Small-molecule inhibition of signaling pathways has long been used to investigate embryonic development in zebrafish. Compounds are simply added to the fish water during embryogenesis, and the developmental consequences are examined at later endpoints. Classically, single inhibitors are used to target specific pathways, for example, cyclopamine to block hedgehog signaling (Neumann et al. 1999); gamma-secretase inhibitors to block the Notch pathway (Geling et al. 2002); lithium, which upregulates the Wnt pathway via inhibition of GSK-3beta (Klein and Melton 1996; Stachel et al. 1993); and retinoic acid signaling (Holder and Hill 1991; Papalopulu et al. 1991), to name just a few prominent examples. In developmental studies, 1-phenyl 2-thiourea (PTU), an inhibitor of tyrosinase, is routinely used to prevent pigmentation and allow easier visualization of later stages of development (Dryja et al. 1978).

Recently, several groups have leveraged a combination of developmental phenotypes and reporter transgenes to screen for novel inhibitors of specific signaling pathways. For example, the first small-molecule regulator of BMP, dorsomorphin, as well as the novel Wnt inhibitor, windorphin, were discovered by screening for compounds with dorsalizing effects in zebrafish embryos (Yu et al. 2008; Hao et al. 2013). Similarly, the novel retinoic acid receptor agonist, DTAB, was identified by effects on anterior-posterior patterning (Sachidanandan et al. 2008), and other small-molecule screens in zebrafish have found additional retinoids (Das et al. 2010). Gebruers and colleagues (2013) uncovered a modulator of noncanonical Wnt signaling based on the development of ectopic tails in treated embryos, and the copper-chelating activity of kalihinol F was noted for phenocopying a zebrafish copper deficiency mutant (Sandoval et al. 2013). Other screens have used transgenic reporter lines to identify new pathway regulators, for example, a screen to identify novel FGF signaling regulators (Molina et al. 2009; Saydmohammed et al. 2011) and estrogen mimetics (Brion et al. 2012).

Cell Proliferation and Differentiation

Using transgenic markers of specific cell types to express fluorescent labels such as GFP allows for facile, imaged-based, and cell-counting methods to identify small molecules that regulate the differentiation and growth of tissues. Slower, but also reliable, are in situ hybridization and immunohistochemical staining of tissues of interest. Both approaches have been used to find compounds that modulate cell growth and differentiation. These screens can be for global effects on cell cycle inhibition (Murphey et al. 2006) or apoptosis (Langheinrich et al. 2002). Other studies have employed in situ hybridization to focus on the expansion or elimination of specific cell types, for example, to find small-molecule regulators of the hematopoietic stem cell progenitor pools (North et al. 2007, 2009), primitive hematopoiesis in a cdx4 mutant background (Paik et al. 2010), and expansion of renal tissue (de Groh et al. 2010). For alterations in

pigmentation (Jung et al. 2005) or melanocyte toxicity (Zhou et al. 2012), visual inspection is sufficient to observe phenotypic changes. In other small-molecule screens, transgenes expressing GFP have been used as visual markers for changes in differentiation, for example, the pax6b:EGFP line to identify regulators of pancreatic beta cell differentiation (Rovira et al. 2011) or the fli1:EGFP, flk1:GFP, or VEGFR2:GRCFP lines to screen for regulators of angiogenesis in the eye (Kitambi et al. 2009; Alvarez et al. 2009) or other tissues (Zhang et al. 2014; Wang et al. 2010; Tran et al. 2007). These screens need not even involve whole animals; Xu and co-workers used a zebrafish culture system of a double transgenic line (myf5-GFP; mylz2-mCherry) that labeled muscle in different developmental states (Xu et al. 2013). Other screens have used the transgenic fluorescent ubiquitylation-based cell cycle indicator (FUCCI) zebrafish, which allows for the visualization of cell cycle progression (Sugiyama et al. 2009), to discover molecular regulators of proliferation in the pancreas (Tsuji et al. 2014) and the heart (Choi et al. 2013). Naturally, the relevance of these developmental processes extends beyond maturation into at least two other areas much studied in the zebrafish: regeneration (Mathew et al. 2007) and cancer (Terriente and Pujades 2013). Due to the diversity of techniques, we discuss regeneration elsewhere in this chapter and now turn to the use of zebrafish in cancer pharmacology.

Cancer Biology

In addition to studying cancer-relevant developmental events such as proliferation or angiogenesis, several groups have used mis-expression of oncogenes in zebrafish tissues to generate models for a number of cancers, which can then be subjected to small-molecule screening. In one leukemia model, the AML1-ETO oncogene is transgenically mis-expressed in the hematopoietic linage to produce a model of acute myeloid leukemia (AML). A small-molecule screen that used expression of a marker of the erythroid lineage (gata1) found that some cyclooxygenase inhibitors could ameliorate the oncogenic phenotype in the AML model (Yeh et al. 2009). Another study screened an FDA-approved library on a model of T cell acute lymphoblastic leukemia (T-ALL), in which the human c-MYC oncogene is selectively expressed in thymocytes (Langenau et al. 2003), and discovered that phenothiazines can selectively induce apoptosis of the c-myc-expressing thymocytes (Gutierrez et al. 2014). Similarly, Ridges and co-workers screened for molecules that eliminate immature T cells in zebrafish, showing that one hit, Lenaldekar, also prevented cancer growth in the T-ALL zebrafish model (Ridges et al. 2012).

Melanoma can be modeled in zebrafish through the transgenic expression of oncogenic BRAF (Patton et al. 2005), NRAS (Dovey et al. 2009), or HRAS (Michailidou et al. 2009; Anelli et al. 2009; Santoriello et al. 2010) under a melanocyte-specific promoter such as *mitfa* (Patton et al. 2005; Michailidou et al. 2009; Dovey et al. 2009) or *kita* (Anelli et al. 2009; Santoriello et al. 2010). Overexpression of oncogenic BRAF (V600E) specifically in the zebrafish neural crest lineage prevents terminal differentiation into melanocytes and predisposes the animals to develop melanomas as adults (White et al. 2011). Small-molecule screening for suppression of the neural crest lineage uncovered DHODH as a modulator of neural crest and, subsequently, melanoma (White et al. 2011).

Collectively, these studies reinforce the relevance of zebrafish as a model organism for cancer pharmacology, especially for whole-animal screening protocols that can identify clinically relevant molecules.

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Regeneration

Introduction

One advantage of zebrafish as a model system is that tissues continue to grow throughout the lifetime of the animal, and many of the tissues and organs are capable of regenerating in response to damage. Thus, the zebrafish is an excellent system in which to study how tissues and organs regenerate, especially those that normally do not regrow in mammals (Gemberling et al. 2013).

Fin Regeneration

Purpose and Rationale

After cutting the caudal or pectoral fins, the tissue will regenerate. Regeneration of the fin is particularly suitable for studies of tissue damage and regrowth because of its experimental accessibility, ease of small-molecule exposure, and rapid regrowth including angiogenesis and reinnervation (Goessling and North 2014). Following amputation, the larval caudal fin will regenerate within a few days (Kawakami et al. 2004), while the adult caudal fin requires approximately 2 weeks.

Procedure

Larval zebrafish are anesthetized in tricaine, placed on an agar plate, and the tail is cut just caudal to the notochord with a surgical blade (Mathew et al. 2007). The larvae are then exposed

to small molecules in embryo water in 96-well plates. For adult fin regeneration, the animals are anesthetized in tricaine, and a portion of the tail fin is amputated with a razorblade (Johnson and Weston 1995; Oppedal and Goldsmith 2010). The fish are then transferred back to system water and exposed to small molecules or vehicle for 72 h. The fin is imaged immediately after amputation and at subsequent time points.

Evaluation

To quantify the amount of fin regeneration, the distance grown from the cut site to the end of the tail is measured. The effects of small molecules can be assessed by statistically comparing the growth distance to vehicle-treated controls.

Critical Assessment

This very simple growth assay has been successfully implemented to uncover numerous genetic and pharmacological regulators of regeneration, including the role of the aryl hydrocarbon receptor (Mathew et al. 2006; O'Donnell et al. 2010), BMP signaling (Smith et al. 2006), Wnt signaling (Stoick-Cooper et al. 2007), FGF signaling (Poss et al. 2000; Whitehead et al. 2005), TGFbeta signaling (Jaźwińska et al. 2007), hydrogen peroxide signaling (Niethammer et al. 2009), and the role of VEGF receptor in regeneration of blood vessels into the tail (Bayliss et al. 2006), to name just a few.

Modifications

Instead of bath application, small molecules and toxicants can be delivered by intraperitoneal injection in adult zebrafish prior to the fin amputation (Zodrow and Tanguay 2003; Andreasen et al. 2006, 2007). The use of fluorescent probes or transgenic fish enables detailed study of angiogenesis (Bayliss et al. 2006), nerve regeneration (Rieger and Sagasti 2011), cell proliferation (Moon et al. 2013), and other remodeling processes.

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Heart Regeneration

Purpose and Rationale

Unlike the mammalian heart, the adult zebrafish heart is capable of extensive regeneration in response to damage (Poss et al. 2002). Thus, understanding the signaling events and tissue rearrangements that occur during zebrafish heart regeneration is a promising avenue with potential implications for the treatment of human heart tissue following ischemic tissue damage.

Procedure

Following Dickover et al. (2013), an adult is anesthetized with tricaine and placed ventral side up into a slot in a damp sponge. An incision is made in the skin just above the heart, and the pericardial sac is opened with forceps. Blunt tweezers are used to expose the ventricle, which is then cut with scissors to redact approximately 20 % of the ventricle at the apex. After wound clotting, the fish is returned to a tank and allowed to recover. The chest incision should heal within 48 h. Test molecules can be added to the fish tank during the recovery period and replaced daily to maintain the desired test concentrations.

Evaluation

The heart will fully recover within 30–60 days and can be removed from the fish for histological analysis at a desired endpoint (e.g., early regeneration phase, late recovery, etc.).

Critical Assessment

Although low throughput, the adult heart regeneration assay has been used to dissect numerous pharmacological and genetic regulators of this process, including polo kinase (Jopling et al. 2010), FGF (Kikuchi et al. 2010), PDGF (Kim et al. 2010), TGFbeta (Chablais and Jaźwińska 2012a), Cxcr4 (Itou et al. 2012), IGF (Haung et al. 2013a), Notch (Zhang et al. 2013; Zhao et al. 2014), and glucocorticoid signaling (Haung et al. 2013b). Direct small-molecule screening on adult heart regeneration will be difficult. However, Choi and colleagues (2013) demonstrated that by prescreening small molecules for enhancing cardiomyocyte differentiation during larval development, one can identify compounds that facilitate adult heart regeneration.

Modifications

Instead of direct resection of the heart's ventricular apex, other methods of heart damage have been developed, including cardiac puncture (Itou et al. 2014), cryoinjury (González-Rosa and

Mercader 2012; Chablais and Jaźwińska 2012b), and genetic cardiomyocyte depletion (Wang et al. 2011). Regeneration can be examined in vitro in explanted epicardial tissue (Kim et al. 2012).

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Hair Cell Damage and Regeneration

Purpose and Rationale

Zebrafish have hair cells in the inner ear but also have experimentally accessible hair cells in the lateral line, an organ used to detect water vibrations.

These cells are sensitive to similar ototoxic compounds as mammals. However, unlike mammals, zebrafish hair cells have the potential to regenerate in response to damage. Thus, the zebrafish hair cell is an excellent system to study both ototoxicity and the subsequent regrowth of damaged cells (Esterberg et al. 2013).

Procedure

Ototoxicity is induced in 5-day postfertilization zebrafish larvae by exposure to 400 μM neomycin for 1 h, 10 mM copper (II) sulfate for 2 h (Hernández et al. 2006), or 1 mM cisplatin for 6 h (Ou et al. 2007), rinsed three times, then allowed to recover in fish water at 28.5 °C (Murakami et al. 2003; Santos et al. 2006). If protective compounds are sought, the larvae are preexposed to small-molecule libraries and then co-exposed with the ototoxic compound in 96-well plates (Owens et al. 2008; Ton and Parng 2005; Ou et al. 2007). For discovery of small molecules affecting hair cell regeneration, the larvae are exposed to the compounds following induction of hair cell death (Ma et al. 2008; Moon et al. 2011; Namdaran et al. 2012). The regeneration recovery rate depends to some extent on the ototoxin used (Mackenzie and Raible 2012). Following neomycin exposure, hair cells recover by 72 h.

Evaluation

At the appropriate endpoint (e.g., immediately before ototoxic exposure to examine acute toxicity or afterward to assess regeneration), the hair cells can be visualized by immersing larvae with the dye FM 1-43FX (3 μM, 30 s), to label hair cells with functional mechanotransduction (Seiler and Nicolson 1999); Yo-Pro-1 (3 μM for 1 h) to label hair cell nuclei; or DASPEI (0.005 %, 15 min) to label hair cell cytoplasm (Owens et al. 2008). Experiments can be conducted in transgenic lines, such as the pou4f3:gap43-GFP or brn3c:GFP lines, which selectively label the zebrafish hair cells (Namdaran et al. 2012; Esterberg et al. 2013). Larvae are then anesthetized and imaged with a fluorescent microscope. Intact hair cell counts can then be statistically compared to controls.

Critical Assessment

The experimental accessibility of the zebrafish lateral line makes compound exposure, dye labeling, and imaging relatively straightforward and scalable to medium- to high-throughput screening. Small-molecule screens have been successful to identify known and novel ototoxic drugs (Chiu et al. 2008; Hirose et al. 2011), compounds that protect against ototoxicity (Ton and Parng 2005; Ou et al. 2007; Owens et al. 2008; Thomas et al. 2015), and compounds that modulate hair cell regeneration (Ma et al. 2008; Moon et al. 2011; Namdaran et al. 2012; He et al. 2014).

Modifications

Hair cell toxicity and regeneration can also be performed in adult zebrafish using 0.004 % gentamicin exposure for 24 h at 28 °C (Pisano et al. 2014). Adult neuromasts can be labeled for imaging with 0.08 % DASPEI for 1 h (Pisano et al. 2014).

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Retinal Regeneration

Purpose and Rationale

In response to damage, the zebrafish retina can regenerate all major cell types, including neurons, from the Müller glia (Goldman 2014). This regenerative capacity is unlike in mammals, in which the damaged retina undergoes gliosis without producing new neurons. Thus, the regenerating zebrafish retina is a good vertebrate system to examine factors that permit newly differentiated neurons to be made in response to tissue injury.

Procedure

Photoreceptors

There are two common procedures for inducing photoreceptor neuron damage with light (Taylor 2012). Following Vihtelic and Hyde (2000), *Albino* zebrafish adults are dark adapted for 14 days and then placed in a tank illuminated at 20,000 lx for 16 h for up to 4 days (at 30–33 °C). This method can also be used to lesion pigmented wild-type zebrafish retinas (Rajaram et al. 2014a). Alternatively 1 week or older zebrafish are exposed to intensely bright light (120,000 lx) for 30 min (Bernardos et al. 2007).

Inner Retinal Layers

To selectively damage the inner retinal layers with minimal damage to the photoreceptor layer, low-dose ouabain can be delivered by intravitreal injection into an anesthetized adult zebrafish (Fimbel et al. 2007). A small incision is made into the cornea adjacent to the lens and 0.2–0.5 μL of ouabain (the final concentration, as estimated from eye size, should be 2.0 μM) with a 33-gauge needle. The contralateral control eye is injected with 0.65 % saline. Higher concentrations of ouabain will damage all retinal layers (Raymond et al. 1988).

Alternatively, the retinal layers can be mechanically damaged by inserting a 30-gauge needle into the sclera of an anesthetized zebrafish (Senut et al. 2004) or by microsurgical removal of a small patch of retina, as adapted from goldfish (Cameron et al. 2000; Hitchcock et al. 1992).

Small-Molecule Exposure

Prior, during, or following retinal lesioning, the zebrafish retina can be exposed to small molecules either dissolved in the bath water or by intravitreal injection to the desired concentration.

Evaluation

The impact of small molecules on retinal regeneration can be evaluated by histological assessment (Cameron 2000; Vihtelic and Hyde 2000). Alternatively, comparisons between manual (Bernardos et al. 2007) or automated (Rajaram et al. 2014b) cell counts or fluorescence intensity (Senut et al. 2004) are readily possible using transgenic markers of retinal cell types.

Critical Assessment

Similar to screening methods in heart regeneration (Choi et al. 2013), it may be possible to increase screening throughput by first searching for modulators of Müller cell proliferation and differentiation in larval stages and then testing candidate molecules in the adult retinal lesioning assay (Meyers et al. 2012). Nevertheless, the adult assay has been used to investigate the pharmacological properties of retinal regeneration, for example, to identify roles for Wnt (Ramachandran et al. 2011), TGFbeta (Lenkowski et al. 2013), leptin (Zhao et al. 2014), and various kinase signaling pathways (Wan et al. 2014).

Modifications

Conditional ablation of specific retinal neuronal subtypes including rods (Montgomery et al. 2010), cones (Fraser et al. 2013), and bipolar cells (Ariga et al. 2010) is possible through the use of transgenic fish that express conditionally active cellular toxins, such as the *Escherichia coli* nitroreductase enzyme.

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Spinal Cord Regeneration

Purpose and Rationale

Unlike mammals, zebrafish have the ability to extensively regrow axons across damaged or severed spinal cord (Becker et al. 1997). Thus, the study of zebrafish spinal cord transection promises to identify factors that permit regrowth of nerve tissue following injury.

Procedure

Following Becker et al. (1997), an adult zebrafish is anesthetized and a longitudinal incision is made at the level of the spinal cord, about halfway between the dorsal fin and operculum (approximately 4 mm from the junction between the brain stem and spinal cord, at the eighth vertebra). The spinal cord is exposed by moving the muscles and then cut with micro-scissors. The incision is sealed with a drop of histoacryl, and the zebrafish is allowed to recover. To assess the effects of small molecules on regeneration, compounds can be delivered by intraperitoneal injection once prior and once a day following the lesion. Full recovery of the spinal cord takes about 5 weeks (Goldshmit et al. 2012).

Evaluation

At the desired endpoint, the adult zebrafish is sacrificed and prepared following standard protocols for histology, immunohistochemistry, or in situ hybridization. To examine the extent of new connections, axon tracing with dye crystals can be used. A crystal of Fluoro-Ruby (Goldshmit et al. 2012) or biocytin-soaked gel-foam (Becker et al. 1997) can be placed on the exposed spinal cord 3 mm anterior or posterior to the lesion. The animal is sacrificed for histology 24 h after dye exposure.

Critical Assessment

The throughput and time course of the spinal cord regeneration assay will make screening efforts difficult, although prescreening small molecules that alter motor neuron production during development may be a promising way to identify candidate compounds for testing in adults (Reimer et al. 2013). Nevertheless, this assay has been used successfully to investigate the role of

sonic hedgehog (Reimer et al. 2009), notch (Dias et al. 2012), FGF (Goldshmit et al. 2012), and dopamine (Reimer et al. 2013) signaling in adult spinal cord regeneration.

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Brain Puncture Injury and Regeneration

Purpose and Rationale

Compared to mammals, the adult zebrafish brain retains an extensive ability to regenerate damaged tissue (Kizil et al. 2012a). Thus, a zebrafish brain injury and regeneration model may help to uncover processes that can facilitate human recovery from brain injury.

Procedure

An anesthetized adult zebrafish (0.5–1 years old) is placed dorsal side up into a slit of damp foam, and then a 30-gauge syringe is pushed through the medial side of one brain hemisphere to a depth of 2 mm (Schmidt et al. 2014; März et al. 2011). Alternatively, the syringe can be inserted through the nostril until the telencephalon is pierced (Kroehne et al. 2011). The zebrafish is then placed in a water-filled tank and allowed to recover.

Evaluation

At desired endpoints post-injury, the zebrafish is sacrificed and prepped with standard protocols for histology, immunohistochemistry, and in situ hybridization. The contralateral hemisphere serves as a negative control.

Critical Assessment

Although one study investigated the role of inflammation in this process (Kyritsis et al. 2012), relatively few pharmacological studies have been performed using the adult brain regeneration assay. However, injections of the antisense knockdown technology, vivo morpholinos (Morcos et al. 2008), directly into the ventricle have been successfully used to study molecular pathways involved in brain regeneration (Kizil et al. 2012a, b, c; Kroehne et al. 2011).

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Other Developmental Diseases

Muscular Development and Dystrophies

Introduction

The term muscular dystrophy encompasses a group of genetic conditions that cause progressive skeletal muscle weakening and degeneration. To date many common muscular dystrophies have been modeled in zebrafish including facioscapulohumeral (Mitsuhashi et al. 2013; Wallace et al. 2011) and Duchenne muscular dystrophy (Bassett et al. 2003; Berger et al. 2012; Guyon et al. 2003, 2009); see Gibbs et al. (2013) for a comprehensive list. The zebrafish's amenability to genetic manipulation and optical transparency have facilitated mechanistic insights and the identification of chemical suppressors, as detailed in Maves (2014).

Birefringence

Purpose and Rationale

The regular, repeating pattern of muscular fibers in zebrafish larvae appears bright when illuminated with polarized light. Disruptions in this brightness, which are specific to the modeled muscular disorder, are readily apparent (Smith et al. 2013). The ease of this assessment has facilitated both targeted (Johnson et al. 2013; Winder et al. 2011) and large-scale (Kawahara et al. 2011, 2014) screening for small molecules that rescue muscle development in genetically modeled muscular dystrophy.

Procedure

An anesthetized larva (4 dpf) is placed on a glass-polarizing filter and covered with a second polarizing filter (Kawahara et al. 2010; Smith et al. 2013). While observing through an underlit dissecting microscope, the top polarizing filter is twisted until only the light refracting through the striated muscle is visible. Because the degree of birefringence, the way the muscle refracts polarized light, is affected by the orientation of the fish, the fish is manually oscillated to account for slight differences in positioning (Kawahara et al. 2010). Screening compounds can be applied by bath immersion prior to the assessment of muscle integrity (Johnson et al. 2013; Kawahara et al. 2011; Winder et al. 2011).

Evaluation

If muscular fiber is disorganized, damaged, or detached, dark patches will appear in the normally bright birefringence. Birefringence can be quantified by measuring pixel brightness in images (Smith et al. 2013) or by performing Fourier analysis across the entire animal (Winder et al. 2011). Follow-up examination of muscle integrity can be carried out by histological and immunohistochemical labeling, such as staining with Evans Blue or phalloidin (Bassett et al. 2003; Hall et al. 2007; Kawahara and Kunkel 2013; Johnston et al. 2013; Goody et al. 2012). Muscle function can also be assessed in vitro with force measurements (Li et al. 2014).

Critical Assessment

The simplicity of the birefringence method has made it highly amenable to pharmacological screening. To date, several screens have identified suppressors of genetic models of muscular dystrophy in zebrafish, including proteasomal (Winder et al. 2011) histone deacetylase (Johnson et al. 2013), and nonselective phosphodiesterase inhibitors (Kawahara et al. 2011), as well as compounds that increase heme oxygenase signaling (Kawahara et al. 2014).

Modifications

An automated Abrio polarizing light microscope can be used to quantify birefringence (Berger et al. 2012). Xu and colleagues (2013) describe a high-throughput imaged-based screen using cultured zebrafish blastomere cells to identify chemicals that promote myogenesis. In the future, such a screen with mutant cells might enable higher-throughput identification of chemical suppressors of muscular dystrophy.

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Fetal Alcohol Syndrome

Purpose and Rationale

Consumption of alcohol during pregnancy can lead to fetal alcohol syndrome, a congenital disease characterized by a spectrum of developmental abnormalities. Fetal alcohol syndrome can be recapitulated in zebrafish, leveraging their *ex utero* development and optical transparency to gain mechanistic insight into the abnormalities that result from ethanol exposure during early development.

Procedure

Zebrafish embryos are collected from a mating cross into Petri dishes and then exposed to ethanol by bath application at the desired time points. The dose and timing vary considerably in the literature, with ethanol concentrations as low as 0.25 % (volume percent) (Buske and Gerlai 2011; Fernandes and Gerlai 2009; Li et al. 2007; Mahabir et al. 2014) to as high as 10 % (Ali et al. 2011a), while the timing of exposure ranges from 2 (Sarmah et al. 2013) to 60 h post fertilization (Bilotta et al. 2002). The length of exposure can vary from brief 1–3 h "binges" (Buske and Gerlai 2011a; Fernandes and Gerlai 2009; Flentke et al. 2014; Mahabir et al. 2014; Zhang et al. 2014) to six continuous days (Carvan et al. 2004). During and after the ethanol exposure, ethanol evaporation is minimized by covering eggs during incubation (Dlugos and Rabin 2010) or sealing the Petri dishes with parafilm (Sarmah et al. 2013).

Evaluation

To date, a wide range of ethanol's effects have been described, including developmental abnormalities (Sarmah et al. 2013; Zhang et al. 2014), visual problems (Arenzana et al. 2006; Bilotta et al. 2002a), and social impediment in adulthood (Buske and Gerlai 2011; Fernandes and Gerlai 2009).

Critical Assessment

The main challenges to zebrafish models of fetal alcohol syndrome include the unclear nature of ethanol pharmacodynamics in zebrafish, the actual levels of ethanol experienced by the embryos in these paradigms, and the relevance of these levels and dosing schedules to the human fetal experience. In light of these challenges, Flentke and colleagues (2014) quantified embryonic ethanol levels and equilibration times, suggesting that short exposures to 1 % ethanol for chorionated embryos and 0.5 % for dechorionated embryos compare best with established rodent exposures. Genetic screening has identified ethanol-sensitive loci (McCarthy et al. 2013; Swartz et al. 2014). Though to date no large-scale pharmacological testing has been performed, Marrs and colleagues (2010) demonstrate that nontoxic doses of retinoic acid supplementation can rescue ethanol-induced developmental defects.

Modifications

Methods for quantifying the amount of ethanol absorbed by embryos are described in Lockwood et al. (2004), Fernandes and Gerlai (2009), and Flentke et al. (2014).

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Systems and Organs

Cardiac Function and Disease

Introduction

The zebrafish two-chambered heart begins to contract and drive blood flow as early as 24 h post fertilization. By 48 h post fertilization, the zebrafish's cardiac electrophysiology is more akin to the electrical properties of the adult human heart than the mouse or other small mammals (Sabeh et al. 2012). These properties predispose the zebrafish for modeling a plethora of cardiac functions and abnormalities, including developmental defects, conduction problems, and hematological disorders (Santoriello and Zon 2012).

Heart Rate

Purpose and Rationale

The optical transparency of the zebrafish larva allows for the simple assessment of pharmacological manipulations on heart rate.

Procedure

The zebrafish heart begins to contract around 24 h post fertilization, enabling heart rate analysis in early embryos or later in larval stages (Freeman et al. 2014; Yozzo et al. 2013). For imaging heart rate, embryos are directly placed into the wells of

a microplate (Freeman et al. 2014; Yozzo et al. 2013), while larval stages are first immobilized with a low concentration of tricaine (25–175 mg/L), taking care to avoid significant changes in heart rate by higher doses (Craig et al. 2006). Larvae can be placed into a well (Berghmans et al. 2008; Miller et al. 2014; Mittelstadt et al. 2008), transferred onto a glass slide along with at least 100 μL of liquid (Rana et al. 2010), or fixed in mounting medium such as methylcellulose (Berghmans et al. 2008) or low-melting temperature agarose (Chan et al. 2009; Parker et al. 2014). Small molecules can be applied at any time point for embryonic stages, while in larval stages, compounds are applied prior to (Berghmans et al. 2008; Parker et al. 2014) or in conjunction with anesthesia (Langheinrich et al. 2003; Miller et al. 2014; Mittelstadt et al. 2008; Rana et al. 2010).

Evaluation

Heart rate can be assessed by observing the fish under a light microscope and counting the number of heart beats over a small interval, e.g., 10–15 s, and multiplying to obtain beats per minute (Berghmans et al. 2008; Freeman et al. 2014; Langheinrich et al. 2003; Miller et al. 2014; Mittelstadt et al. 2008; Rana et al. 2010). Video analysis software can be employed to detect changes in pixel density, which can then be converted into heart rate (Milan et al. 2003; Parker et al. 2014).

Critical Assessment

A number of chemical screens have demonstrated the conserved effects of cardiac drugs on larval heart rate (Berghmans et al. 2008; Lai et al. 2014; Parker et al. 2014; Yozzo et al. 2013). A particular focus has been the validation of the larval sensitivity to QT prolonging drugs, as these compounds can lead to life-threatening arrhythmia (Burns et al. 2005; Langheinrich et al. 2003; Milan et al. 2003; Mittelstadt et al. 2008). Genetic long QT syndrome is a congenital disorder which can lead to cardiac arrest and sudden death. Peal and colleagues (2011) carried out a high-throughput small-molecule screen using a zebrafish model of long QT syndrome and identified two compounds that rescue the mutant phenotype, demonstrating the power and potential clinical impact of this screening method.

Modifications

If fish are plated, they can be continuously perfused with anesthetic and/or drugs using filtered or mesh-bottomed microplates and a pump or gravity-fed system to generate flow (Miller et al. 2014). The assay throughput can be increased by the use of automated image acquisition and analysis procedures in conjunction with transgenic zebrafish that express fluorescent transgenes in cardiac myocytes (Burns et al. 2005; Mickoleit et al. 2014), the vascular endothelium (Yozzo et al. 2013), or endocardial, endothelial, and red blood cells (De Luca et al. 2014). Heart rate can be determined computationally by video analysis of peripheral blood circulation (Chan et al. 2009).

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Circulation and Blood Vessel Formation

Purpose and Rationale

The transparency of zebrafish larvae makes them well suited as a model to evaluate the impact of small molecules on vascular parameters related to blood circulation and angiogenesis.

Procedure

Embryos or larval zebrafish are aliquoted either individually or as groups into the wells of a microplate filled with embryo water (Han et al. 2012; Peterson et al. 2004). Dissolved small molecules can be pipetted into the wells of the plate at any time point and for any duration. For example, Peterson et al. (2004) applied drugs at 50 % epiboly and then incubated the fish at 28.5 °C for 24 h, while Parker et al. (2014) waited until the fourth-day post fertilization and exposed the fish for an hour prior to testing. If hatched larvae are used, they should be anesthetized with a low concentration of anesthetic, fixed in low-melting temperature agarose, and then mounted on a microscope slide prior to imaging (Parker et al. 2014; Watson et al. 2013). Alternatively, microplates can be centrifuged post anesthesia to orientate the larvae for imaging (Leet et al. 2014; Yozzo et al. 2013).

Evaluation

Through the use of microscopes fitted with high-speed video cameras, blood flow is determined by

filming regions of interest, such as the dorsal aorta, and then calculating changes in pixel density in relation to vessel diameter (Parker et al. 2014). Alternatively, transgenic zebrafish such as *fli*:GFP, which express green fluorescent protein in the blood vessels (Lawson and Weinstein 2002), can be used to employ automated algorithms to determine circulation (Leet et al. 2014; Yozzo et al. 2013) or even the trajectory of individual erythrocytes in real time (Watkins et al. 2012). Pharmacological effects on angiogenesis can be determined by the use of a stereomicroscope and suitable stain (Han et al. 2012) or fluorescence microscopy and transgenic fish (Tran et al. 2007; Watson et al. 2013).

Critical Assessment

To date, this simple assay has been leveraged by high-throughput studies to identify antiangiogenic compounds (Tran et al. 2007) and a herbicide that abolishes hemoglobin production (Leet et al. 2014). Most notably, this assay was used to identify a class of compounds that suppress the gridlock mutant phenotype, which has disrupted aortic blood flow, without directly targeting the gene affected (Peterson et al. 2004). These results demonstrate the potential to identify adverse effects and potential new therapeutics with a range of applications on vascular function.

Modifications

An alternative method to the use of transgenes to highlight the vasculature is to inject embryos with small diameter (0.01 μM) fluorescent latex beads prior to confocal imaging (Weinstein et al. 1995).

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Larval Electrocardiogram

Purpose and Rationale

The effect of pharmacological manipulations on the electrical properties of the heart can be noninvasively examined in larval zebrafish.

Procedure

Larval zebrafish are sedated with 0.02 % tricaine (Arnaout et al. 2007; Yu et al. 2010) and then positioned ventral side up by embedding in agarose (Huttner et al. 2013; Yu et al. 2010). Alternatively, the larva can be placed into a groove in a Petri dish with a paraffin wax surface and covered with fish water (Dhillon et al. 2013). Under a microscope, a micromanipulator is used to position a reference electrode in the surrounding solution 2 mm from the fish and a recording electrode filled with Danieau solution on the ventral surface of the larva above the contracting heart (Dhillon et al. 2013; Huttner et al. 2013; Yu et al. 2010), taking care to apply enough pressure that the recording electrode retains contact without mechanically disturbing the heart. Recording equipment should be housed on an air table within a Faraday cage to reduce background noise (Dhillon et al. 2013). Compounds for testing can be added to the larvae by bath application at any time point during recording.

Evaluation

Output signals from the amplifier can be digitalized and then analyzed by software programs (Dhillon et al. 2013; Huttner et al. 2013; Yu et al. 2010). In order to evaluate the impact of test compounds on the heart's electrical properties, the duration of and intervals between P waves, QRS complexes, and T waves are compared to baseline values (Dhillon et al. 2013; Huttner et al. 2013; Yu et al. 2010).

Critical Assessment of the Method

This technique is suitable for follow-up studies on compounds that have been identified by higher-throughput methods, such as by effects on heart rate or circulation.

Modifications

It is possible to perform the same measurements invasively by using a micromanipulator to puncture the skin over the heart with the recording electrode (Dhillon et al. 2013).

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Adult Electrocardiogram

Purpose and Rationale

The effect of pharmacological manipulations on the electrical properties of the heart can be directly examined in adult zebrafish.

Procedure

Adult zebrafish are anesthetized by immersion in 0.02 % tricaine and then immobilized by intraperitoneal injection with either 60 ng per 500 mg of pancuronium or 1.2 nmol/g of μ-conotoxin GIIIB and placed onto a damp sponge (Arnaout et al. 2007; Chaudhari et al. 2013; Milan et al. 2006). A 1 mm perfusion needle is placed into the oral cavity to allow perfusion with preoxygenated 10 mM HEPES in embryo water at a rate of 6–8 ml/min (Chaudhari et al. 2013). Dissolved compounds can be administered to the fish through this route (Arnaout et al. 2007). Two 29-gauge needle electrodes are inserted to a depth of 1 mm in the ventral midline, with one electrode placed between the pectoral fins and the other two-thirds of the body length from the head,

near the anal fin (Arnaout et al. 2007; Milan et al. 2006). The electrocardiogram signal is amplified, filtered, and digitalized using standard electrophysiological methods. The signal-to-noise ratio can be increased by processing the signal with a wavelet transform and thresholding algorithm (Sun et al. 2009). The recording chamber should be situated inside a Faraday cage to reduce electromagnetic interference (Yu et al. 2010).

Evaluation

The effect of compounds are evaluated by comparing effects on parameters including the duration of and intervals between P waves, QRS complexes, and T waves to baseline recordings and vehicle-treated controls (Milan et al. 2006; Yu et al. 2010b). Arrhythmias can also be scored qualitatively (Chaudhari et al. 2013). If QT intervals are examined, they should be corrected for heart rate (Arnaout et al. 2007; Chaudhari et al. 2013; Sun et al. 2009; Yu et al. 2010).

Critical Assessment

This assay has been employed to demonstrate the conserved effect of QT prolonging drugs in adult zebrafish (Chaudhari et al. 2013; Milan et al. 2006). This method is best employed to characterize conduction deficits in disease models (Arnaout et al. 2007) and to assess the functionality of regenerated cardiac tissue (Sun et al. 2009; Yu et al. 2010).

Modifications

An alternative to oral drug delivery is intraperitoneal injection (Chaudhari et al. 2013). This method may be preferable when compounds are unstable in water.

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Action Potential Recording

Purpose and Rationale

The electrical properties of the embryonic or adult zebrafish heart can be examined ex vivo.

Procedure

Zebrafish embryos are dechorionated and anesthetized with 0.02 % tricaine prior to heart dissection with fine forceps (Arnaout et al. 2007; Jou et al. 2010). Alternatively, cardiac tissue can be isolated enzymatically, as in Alday et al. (2014). Spontaneously beating hearts are transferred to a chamber perfused with recording solution (Arnaout et al. 2007; Jou et al. 2010). The transmembrane potential can be measured by positioning a suction pipette adjacent to the heart and applying a minimal level of suction to form a seal. After recording baseline action potentials by standard electrophysiological methods, the tissue is perfused with test compounds.

Adult zebrafish are anesthetized by immersion in 0.02 % tricaine and then decapitated and pithed. Explanted hearts are placed under a microscope and washed extensively in oxygenated Tyrode's solution to remove anesthetic from the tissue (Nemtsas et al. 2010). The hearts are continuously perfused with oxygenated Tyrode's solution (Nemtsas et al. 2010), and then blebbistatin

(10 μM), an excitation-contraction uncoupler, is bath applied to enable microelectrode positioning and reduce movement artifacts (Kovács et al. 2004). The bulbus arteriosus is pinned to the floor of the chamber, the preparation is allowed to equilibrate, and intracellular action potentials are recorded using a glass pipette filled with 2.5 M KCL solution (Nemtsas et al. 2010).

Evaluation

Numerous measures including the heart rate, QT interval (Tsai et al. 2011), resting membrane potential, action potential amplitude, maximum upstroke velocity (Nemtsas et al. 2010), and action potential duration (Arnaout et al. 2007; Jou et al. 2010; Nemtsas et al. 2010) can be calculated from a series of five or more action potentials. Bazett's formula can be used to correct observed QT interval measurements for variations in heart rate (Bazett 1920). Effective refractory period can be determined by pacing the heart with injected pulses of depolarizing current (Arnaout et al. 2007; Jou et al. 2010).

Critical Assessment of the Method

Studies have demonstrated that the electrical properties of these in vitro preparations closely resemble both those of humans (Arnaout et al. 2007; Brette et al. 2008) and zebrafish in vivo (Tsai et al. 2011). This assay is best used to verify findings from other primary assays such as heart rate or circulation or used to characterize conduction abnormalities in disease models (Arnaout et al. 2007).

Modifications

Brette et al. (2008) describe an enzymatic method to isolate individual cardiac myocytes, for electrophysiology, from the adult zebrafish ventricle.

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Optical Mapping

Purpose and Rationale

The impact of small molecules on global electrical activity can be studied in explanted zebrafish hearts.

Procedure

Adult zebrafish are euthanized and the hearts are harvested and placed into calcium-Tyrode's solution (Lin et al. 2014; Sedmera et al. 2003). To reduce motion artifacts, the isolated hearts are incubated with bath applied 10 μM blebbistatin for at least 60 min and then secured in place with a pin through the bulbous arteriosus (Kovács et al. 2004). Prior to imaging, the hearts are incubated in a potentiometric dye (Lin et al. 2014). During imaging the dye is excited using a 540 nm laser and a 50 % beam splitter to stimulate both

sides of the specimen simultaneously, and then fluorescent emission is measured to obtain a voltage-dependent signal that varies with membrane potential (Lin et al. 2014; Sedmera et al. 2003).

Evaluation

By defining regions of interest, such as the atrium and ventricle, changes in average fluorescence intensity across space and time can be determined to plot action potential peaks (Lin et al. 2014). Each action potential cycle can then be separated to calculate the heart rate, the time taken to 50 % repolarization, and atrioventricular delay, which is the difference in time between corresponding atrial and ventricular peaks (Lin et al. 2014). It is also possible to superimpose changes in fluorescence level per pixel per unit of time over images of the hearts to build contour maps of activation (Lin et al. 2014; Sedmera et al. 2003).

Critical Assessment

This method has been employed as a secondary assay to confirm the results of a primary screen for compounds that rescue a genetic long QT phenotype (Peal et al. 2011).

Modifications

An alternative to the voltage-sensitive dye is using the cmlc2:mermaid transgenic zebrafish, which expresses a genetically encoded probe for transmembrane potential specifically in the heart (Tsutsui et al. 2010). Optical mapping of the embryonic heart can be achieved using modifications to the technique described above (Peal et al. 2011; Samson et al. 2013; Tsutsui et al. 2010).

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Flow Cytometry of Red Blood Cells

Purpose and Rationale

Flow cytometry provides a platform for the high-throughput screening of compounds that alter red blood cell number in zebrafish models of anemia. These models recapitulate human hematological conditions, facilitating high-throughput drug screening to identify potential therapeutics.

Procedure

Anemic zebrafish models, either morphants (Danilova et al. 2008; Payne et al. 2012; Uechi et al. 2008) or mutants (Brownlie et al. 1998; Dooley et al. 2008; van Rooijen et al. 2009; Shafizadeh et al. 2002; Taylor et al. 2012b), are generated in a transgenic background expressing a fluorescent protein under an erythroid-specific promoter, such as *gata1* (Long et al. 1997). The larval fish are then incubated in test compounds according to a dosing regimen. For example, Payne and colleagues (2012) exposed 20–40 embryos per microplate well at 24 h post fertilization and analyzed blood cell counts at 3 days post fertilization. At the desired endpoint, cold phosphate-buffered saline (PBS) is used to rinse each embryo prior to dissociation, filtrated

through a 40 μM filter, and a second wash with PBS containing 5 % fetal bovine serum (Payne et al. 2012). Dissociation can be achieved by an hours' agitation at 30 °C in PBS with bovine serum, 1 mg/mL collagenase, 10 mg/mL trypsin, and 10 units/mL of DNAse1 (Danilova et al. 2008). The addition of 1 μg/mL of DAPI (4′6-diamidino-2′-phenylindole dihydrochloride) permits the identification and exclusion of dead cells (Danilova et al. 2008). Finally, the addition of flow-check fluorospheres (2.5×10^{-5} in a final volume of 300 μL) enables determination of absolute cell number by calculating the fluorescent protein events compared with the number of beads (Payne et al. 2012).

Evaluation

Red blood cell counts in treated embryos are compared to background-matched control fish by two-way analysis of variance (Payne et al. 2012).

Critical Assessment

While no high-throughput drug screen has yet utilized this assay, a targeted drug screen in a model of Diamond-Blackfan anemia demonstrated a striking improvement, in terms of both hemoglobinization and erthyroid cell numbers, in response to L-leucine (Payne et al. 2012). Another large-scale screen used *o*-dianisidine staining to identify effectors of hematopoietic development, uncovering a novel anemia-inducing compound (Shafizadeh et al. 2004). Together these studies demonstrate the feasibility of performing high-throughput screening in anemic zebrafish models using flow cytometry.

Modifications

Erythroid hemoglobin content is examinable by *o*-dianisidine staining (Detrich et al. 1995; Shafizadeh et al. 2004) and blood flow can be assayed as described in this chapter's section on "Circulation and Blood Vessel Formation." Blood for smear preparation or calculation of hematological parameters, such as mean corpuscular volume or mean corpuscular hemoglobin content, can be obtained from either larval or adult fish by tail amputation or cardiac puncture, respectively (Brownlie et al. 1998; Shafizadeh et al. 2002, 2004).

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Gastrointestinal Diseases

Introduction

Zebrafish are well suited to the study of gastrointestinal disease. The zebrafish and human gastrointestinal tract are anatomically and cellularly homologous. The early development of spontaneous gut contractions (at 3–4 days post fertilization) and larval transparency permits the visualization of these contractions across the entirety of the gastrointestinal tract in vivo (Burzynski et al. 2009; Rich 2009).

Video Analysis of Gut Motility

Purpose and Rationale

As larval zebrafish are optically transparent, changes in gut motility patterns in response to pharmacological agents can be easily visualized and quantified.

Procedure

Larval zebrafish older than 5 days post fertilization are anesthetized with tricaine (100 mg/L) and then mounted on their side in either agarose (Holmberg et al. 2004, 2006, 2007) or methylcellulose (Berghmans et al. 2008; Kuhlman and Eisen 2007) and covered with a solution of fish water and tricaine in order to maintain anesthesia. After the larva has habituated for 5 min, the gastrointestinal tract is filmed under a microscope (Berghmans et al. 2008; Holmberg et al. 2004; Kuhlman and Eisen 2007). Test compounds can be bath applied prior to mounting (Berghmans et al. 2008) or during imaging either by application next to the abdomen or by intraperitoneal injection using a micromanipulator (Holmberg et al. 2004, 2006).

Evaluation

The number of antero- and retrograde gut contractions (Berghmans et al. 2008) as well as periodicity (i.e., the time between contractile waves as they pass a fixed point in the image frame) can be counted by eye from the video (Kuhlman and Eisen 2007). Alternatively, the average frequency of contractions per minute can be calculated from video stills (Holmberg et al. 2004, 2006). Spatiotemporal maps of the movement of luminal content and gut walls can be made by inferring contractions from changes in average pixel density (Holmberg et al. 2007). These maps allow visualization of contraction initiation sites and enable the calculation of a number of parameters, including the frequency, the velocity, the distance traveled, and the coordination of contraction (Holmberg et al. 2007; Rich et al. 2013; Roach et al. 2013). Control larvae are imaged in parallel to account for variation in developmental timing or feeding experience.

Critical Assessment

This assay has been employed to probe the development of the excitatory and inhibitory innervation of the gut (Holmberg et al. 2004, 2006, 2007; Rich et al. 2013) as well as to screen for mutations affecting the development and function of the enteric nervous system (Kuhlman and Eisen 2007). A small panel of test drugs displayed significant and expected effects on motility (Berghmans et al. 2008), indicating the potential of this assay to make discoveries that will translate to the clinic.

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Intestinal Transit Assay

Purpose and Rationale

The intestinal transit assay is a method to visualize changes in gut motility.

Procedure

Larvae are fed either paramecia in embryo media containing 2.5 μl/mL fluorescent latex beads for 2 h (Abrams et al. 2012) or with a fluorescent tracer mixture (Field et al. 2009). The tracer mixture is prepared by forming a paste from 100 mg of powdered larval food, 150 μl of 2 μm polystyrene microspheres, and 50 μl of deionized water. A thin layer of this paste is spread onto a watch glass, left to dry in a dark room at room temperature, and then crushed into a powder (Field et al. 2009). After feeding, the larvae are placed in media containing only paramecia, and bead expulsion is monitored under a fluorescence microscope (Davuluri et al. 2010). Alternatively, larvae are anesthetized with 0.2 % tricaine and sorted under a fluorescence microscope; larvae with a single, fluorescent bolus in the intestinal bulb or anterior intestine are analyzed (Abrams et al. 2012; Field et al. 2009). Test larvae are transferred to a clean Petri dish or microplate well filled with embryo medium, re-anesthetized, and imaged at set time points after feeding (Abrams et al. 2012; Field et al. 2009). After each time point, larvae are allowed to recover from anesthesia in clean embryo water (Field et al. 2009).

Evaluation

Intestinal motility can be scored based on the location of the florescent bolus at each time point (Abrams et al. 2012; Field et al. 2009).

Critical Assessment of the Method

Zhou and colleagues (2014), using a small drug panel, demonstrated the potential of this assay for high-throughput screening, especially in conjunction with a quantitative assessment of fluorescence. However, while free feeding is a natural and stress free process, it is difficult to accurately monitor and control ingestion. Furthermore, there is high variability in individual consumption of material during these exposure periods. These issues can be addressed by larval microgavage (Cocchiaro and Rawls 2013).

Modifications

A detailed procedure for the microgavage of larval zebrafish can be found in Cocchiaro and

Rawls (2013). Briefly, 6–7 day posfertilization zebrafish larvae are anesthetized and mounted in agarose, and a microinjector apparatus is used to slowly (23 nl/s) deliver a small volume (4.6 nl) of material through the mouth of the fish directly into anterior bulb of the intestine.

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Renal Function

Purpose and Rationale

The larval pronephros is composed of two pronephric tubules connecting a midline pair of fused glomeruli to bilateral pronephric ducts that join to expel blood filtrate at the cloaca. Though structurally and functionally simple, this system develops in a similar sequence to that of the mammalian kidney and is composed of cell types typical of higher vertebrate kidneys (Drummond 2005; Swanhart et al. 2011). This similarity has enabled successful modeling of a number of ciliopathies, as cilia are readily examined by electron microscopy and high-speed video microscopy as in Drummond et al. (1998) and Kramer-Zucker et al. (2005). Furthermore, the zebrafish is able to generate new nephrons throughout its lifespan, making it an exciting model in which to study acute kidney injury by microinjection of nephrotoxic agents, such as gentamicin or cisplatin (Hentschel et al. 2005; Cianciolo Cosentino et al. 2010).

Procedure

Embryos (72 hpf) are collected, anesthetized with 0.2 mg/mL of tricaine, and then injected intravenously with 25–50 ng of tetramethylrhodamine-labeled 10 kDa dextran (Drummond et al. 1998; Hentschel et al. 2005; Kramer-Zucker et al. 2005). The impact of test compounds on renal filtration can be assessed by either by bath application (Tobin and Beales 2008) or injection of the drug into the cardiac venous sinus (Hentschel et al. 2005).

Evaluation

The efficacy of renal filtration is determined by measuring the intensity of cardiac fluorescence, which normally decreases as the dextran is filtered and excreted by the kidney, at specific time points post injection (Hentschel et al. 2005; Tobin and Beales 2008). Videography of urine, and hence dye excretion, can also be made under fluorescent microscopy (Kramer-Zucker et al. 2005). Alternatively, glomerular function can be ascertained by fixing, embedding, and sectioning embryos 5 min post injection (Drummond et al. 1998). If the glomerulus is functioning, dextran should pass through the glomerular basement membrane and appear in the lumen of the pronephric duct.

Critical Assessment

This assay, employed in conjunction with targeted small-molecule testing, has identified a compound that ameliorates cisplatin-induced renal failure (Hentschel et al. 2005) and two drugs that restore renal function in a range of ciliopathy models (Tobin and Beales 2008). The protective effect of the former compound, UCF-101, is replicable in a mammalian model, making it a potential candidate for the prevention of nephrotoxicity in cancer patients receiving cisplatin (Hentschel et al. 2005). The possible translatability of these findings underscore the use of this assay,

particularly as many mouse models of ciliopathy suffer from embryonic lethality and variable temporal cyst development (Hentschel and Bonventre 2005).

Modifications

An alternative to dextran is the microinjection of 20–25 ng of fluorescein-labeled inulin, which unlike dextran is freely filtered but not taken up or secreted by the tubule (Hentschel et al. 2005). In zebrafish, laterality and body curvature defects serve as surrogate markers for kidney cyst formation. Taking advantage of these easily detectable phenotypes, Cao and colleagues (2009) performed a chemical modifier screen in two polycystic kidney disease models and identified compounds capable of inhibiting cyst formation. This screen demonstrates the potential for high-throughput screening in zebrafish models of renal disease.

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Models of Infection and Immunity

Purpose and Rationale

Zebrafish have both innate and adaptive immune systems that closely resemble our own (van der Sar et al. 2004). However, they exhibit no adaptive immunity markers until 4 days post fertilization, when T and B cells are developed in preparation for adaptive immunity, which is not fully functional until 4–6 weeks post fertilization (Sullivan and Kim 2008). As such, zebrafish larvae can be used to study the vertebrate innate immune response in vivo, in isolation from the adaptive immune response (Novoa and Figueras 2012). Furthermore, zebrafish offer unique opportunities to visualize pathogen-host dynamics, innate immune responses, and the activation of immune signaling pathways in vivo through the use of fluorescent transgenic lines. It is also feasible to perform high-throughput drug screening (Meijer and Spaink 2011).

Procedure

Infection by Immersion – Embryonic, larval, or adult zebrafish are incubated at 28 °C in a container suitable for their size, such as a 2 mL microplate well for embryos (O'Toole et al. 2004) or a 600 mL tank for adults (Pressley et al. 2005). The fish are immersed in fish water and a prepared bacterial suspension at a concentration of roughly 10^6–10^8 CFU/mL for larvae

(O'Toole et al. 2004) or 10^5–10^7 CFU/mL for adults (Harriff et al. 2007; Pressley et al. 2005), depending on the bacterial strain. The effects of static immersion can be examined acutely, for example, at 2 or 6 h post infection (O'Toole et al. 2004), or chronically by returning fish to clean water following a period of acute immersion (Harriff et al. 2007; Pressley et al. 2005). Control fish are handled in the same manner but immersed in a sham bath with the bacterial suspension medium (Harriff et al. 2007; O'Toole et al. 2004).

Infection by Microinjection – For viral inoculation, embryos are injected with viral DNA at a concentration of 1 ng/μl into the blastomere at the one to eight cell stage (Ding et al. 2011). For bacterial inoculation, 28 hpf embryos are dechorionated, anesthetized with 0.02 % tricaine, and injected with 1–2 nL of bacterial cells into the axial vein (van der Sar et al. 2004) or yolk circulation valley (Clatworthy et al. 2009) under a stereomicroscope. Inoculum size can be determined by duplicate injection into phosphate-buffered saline and counting colony-forming units on LB agar (Clatworthy et al. 2009). Injected embryos are then returned to clean embryo medium and incubated at 28 °C.

For bacterial or viral infection of adult fish, the animal is anesthetized by immersion in 160 μg/mL tricaine and then inoculated via either intraperitoneal or intramuscular injection with 10 μL of pathogen suspension administered via a 29-gauge needle (Burgos et al. 2008; Neely et al. 2002). For intraperitoneal injection, the fish is positioned supine and held in place with a moistened gauze-covered hemostat. The needle is aligned with the spine and inserted up to the end of its bevel, 1.5 mm, cephalad into the midline of the abdomen posterior to the pectoral fins (Neely et al. 2002). For intramuscular injection, a hemostat is used to position the fish prostrate, and the needle is inserted immediately anterolateral to the dorsal fin, cephalad at 45° relative to the spine (Neely et al. 2002). Pharmacological compounds can be tested either by co-injection with the pathogen (Burgos et al. 2008) or by immersion following injection (Clatworthy et al. 2009; Ding et al. 2011).

Evaluation

Mortality, gross pathology, and histopathology can be examined as described in Pressley et al. (2005), Harriff et al. (2007), and Burgos et al. (2008). Pathogenic DNA or RNA can be quantified by PCR, and spatiotemporal expression can be assessed by organ-specific PCR (Burgos et al. 2008; Clatworthy et al. 2009; Ding et al. 2011; Neely et al. 2002; Pressley et al. 2005) or visualized by in situ hybridization (Ding et al. 2011). The expression of pathogenic proteins can be quantified by Western blot (Ding et al. 2011), and localization can be observed via immunohistochemistry (Burgos et al. 2008). Alternatively, the use of florescent transgenic bacteria (Clatworthy et al. 2009; O'Toole et al. 2004; van der Sar et al. 2003) or viruses (Ding et al. 2011; Palha et al. 2013) enables the visualization of infection dynamics in vivo including localization, spread, and pathogen load. Bacterial burden can be determined by the isolation and culture of bacteria from either whole fish or organ homogenates on selective agar (Clatworthy et al. 2009; Harriff et al. 2007; Pressley et al. 2005; van der Sar et al. 2003). Viral burden can be examined by the in vitro infection of cultured cells (Burgos et al. 2008).

Critical Assessment

The zebrafish model has been deployed to study a range of bacterial, fungal, and viral infections. Targeted drug testing in these models has demonstrated conserved responses to both antibiotic and antiviral compounds (Burgos et al. 2008; Carvalho et al. 2011; Clatworthy et al. 2009; Ding et al. 2011; Gabor et al. 2014). Furthermore, high-throughput screens in tissue injury models have identified novel anti-inflammatory compounds (Hall et al. 2014; Robertson et al. 2014; Wang et al. 2014). Because mammalian pathogens are adapted to thrive at 37 °C, inoculation at 28 °C in the fish is likely to affect disease outcome. While the use of natural fish pathogens mitigates this problem, their relationship to human disease remains unknown (Goody et al. 2014). A second problem is that specific viral receptors may not be conserved or expressed in the zebrafish, perhaps limiting the study of

some viruses in zebrafish (Goody et al. 2014). However, sub-replicon constructs for viral gene expression can be utilized when the human viral pathogen is unable to replicate in the fish (Palha et al. 2013). A useful flowchart of questions to consider before attempting to model a virus in zebrafish can be found in Goody et al. (2014).

Modifications

To mitigate concerns regarding diffusion through the chorion, embryonic zebrafish can be dechorionated prior to infection by immersion (Pressley et al. 2005). The throughput of infection by microinjection, and hence drug screening, can be dramatically increased by the use of a robotic yolk injector, which can process 2,000 embryos per hour (Carvalho et al. 2011).

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Neuroscience

Visual and Nonvisual Light Responses

Introduction

Zebrafish express a great many opsins throughout the eye, brain, and other tissues and, perhaps not surprisingly, exhibit a large number of light-mediated behavioral responses (Fernandes et al. 2013). Some of these behaviors require the image-forming system of the retina, but some of these behaviors do not. The photomotor response (PMR) is a vigorous twitching behavior of embryonic zebrafish to an intense light pulse during a narrow developmental (30–42 hours post fertilization, hpf) window (Kokel et al. 2010). The dark photokinesis response (also called the visual motor response or VMR) is a strong locomotor behavior upon light to dark transitions that also does not appear to require, but is modulated by, visual input through the retina (Burgess and Granato 2007a; Emran et al. 2007; Fernandes et al. 2012). The visual system becomes functionally wired at 3 days post fertilization and allows zebrafish to respond to moving objects (Fadool and Dowling 2008). Two assays test larval and adult zebrafish responses to moving stripes, the optokinetic reflex (OKR) and the optomotor reflex (OMR). In the OKR, eye saccades in response to moving stripes are recorded in immobilized zebrafish, while in the OMR, fish locomotor responses to moving stripes are detected (Zou et al. 2010; Huang and Neuhauss 2008). Over longer time intervals, zebrafish larvae and adults increase their spontaneous locomotor activity in light and lower their spontaneous activity in the dark (Rihel and Schier 2012).

The Photomotor Response (PMR)

Purpose and Rationale

The PMR is a vigorous twitching response to high-intensity light followed by an extended refractory period during which embryos are incapable of responding (Kokel et al. 2010). Occurring from roughly 30–42 hpf, the PMR is one of the earliest zebrafish behaviors reliably invoked in a high-throughput fashion, making it an attractive assay for seeking neuromodulatory compounds through screening assays.

Procedure

Eight to ten 30-hpf-old embryos with intact chorions are placed into embryo water in each well of a flat-bottomed 96-well plate and then exposed to small molecules dissolved in DMSO (final DMSO concentration <1 %) for 1–10 h prior to the assay. Embryos are placed in total darkness (or illuminated with infrared light (>650 nm) to obtain continuous video) for at least 10 min, and the PMR is elicited by exposing the embryos to bright white light (100–300 W) for 1 s (Kokel et al. 2010). The PMR, which lasts for about 2–5 s following an approximately 2 s latency, is video recorded (33 fps) for analysis. A second light pulse during the refractory period (10–30 s) following the PMR fails to elicit a motor response in untreated larvae (Kokel et al. 2010).

Evaluation

Movement is detected by frame-by-frame changes in the average pixel intensity (API) within multiple regions of interest within each well. The average API per well (the Motion Index) is plotted across time to observe the changes in activity. The Motion Index is divided into phases – baseline prior to stimulus, latency (0–2 s poststimulus), excitation (2–5 s post stimulus), and refractory phases (10–30 s post stimulus) – and each phase is statistically compared to vehicle-treated controls. For large datasets, the phases can be represented as behavioral fingerprints that can be clustered to identify small molecules that elicit similar responses (Kokel et al. 2010; Rihel and Schier 2012). Small-molecule hits are then

retested in five to ten replicate wells on multiple experimental days.

Critical Assessment

Since the retina is not yet functional, the behavior is mediated by nonvisual photodetection, and imaging studies have mapped the response to neurons in the larval hindbrain (Kokel et al. 2013a). The biological purpose of this behavior is unknown. Even still, the PMR screen has been used to identify a novel small-molecule photoactivatable regulator of TRP channels, optovin (Kokel et al. 2013b). In addition, of all the behavior-based pharmacological assays in the zebrafish, the PMR is capable of one of the highest throughputs – the initial screening was conducted on more than 14,000 compounds (Kokel et al. 2010).

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Visual Motor Response (Light and Dark Photokinesis)

Purpose and Rationale

Zebrafish larvae respond to transitions from lights on to lights off with vigorous swimming that lasts for 10–15 min. In the literature, this response is known variously as the visual motor response (VMR) or dark photokinesis (Burgess and Granato 2007; Emran et al. 2007; Fernandez et al. 2012). Larvae also acutely respond to dark to light transitions. For neurotoxicity and neuromodulatory testing, the VMR is a reliable method to elicit a behavioral response that may or may not be modulated by the visual system (see section "Critical Assessment").

Procedure

Single 4–7 dpf larvae are placed into fish water in each well of a 96-well plate and then exposed to small molecules dissolved in DMSO (<0.1 % DMSO final concentration; six to ten animals per treatment). The larvae are illuminated with infrared light (>650 nm) for continuous video monitoring and acclimated in white light (69–130 W/cm^2 measured at 495 nm) for 2 min–3 h (Burgess and Granato 2007; Emran et al. 2007, 2008; Fernandez et al. 2012). Larvae are then exposed to alternating 30-min periods of lights on and lights off, typically for three to five cycles, while being video recorded.

Evaluation

For each treatment, the average movement per second is calculated across the assay using either pixel subtraction (Emran et al. 2007, 2008, 2010) or centroid tracking (Burgess and Granato 2007; Fernandez et al. 2012). Each light transition phase and the light and dark baseline phases are separately averaged and statistically compared to vehicle control-treated larvae on the same plate. Untreated larvae will show very strong locomotor activity in response to the lights off transition, lasting for 10–15 min, followed by a suppressed baseline in continued dark. Untreated larvae will transiently respond to the lights on transition for a few seconds and then increase

their spontaneous locomotor activity during continued light exposure.

Critical Assessment

Within the literature, there is some confusion about the extent to which the light-dark transitions depend on the visual system (i.e., the retina). Emran et al. (2007) showed that mutants lacking eyes and pineal photoreceptors failed to show light/dark locomotor responses. Fernandez et al. (2012) show that genetic or surgical ablation of the eyes had only a modest effect on the magnitude of locomotor responses to lights out, and instead they implicated deep brain photoreceptors in this process. In either case, this assay has been used to investigate neurotoxic and neuroactive compounds (Ali et al. 2011, 2012; Akhtar et al. 2013; Deeti et al. 2014; Gao et al. 2014; Long et al. 2014; Spulber et al. 2014). Many studies conflate the use of the VMR to study spontaneous swims; in particular, very short light/dark intervals (e.g., 5 min) do not allow the larvae to reach baseline activity states (see section "Arousal States, Spontaneous Swimming, and Sleep").

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Optomotor Reflex (OMR)

Purpose and Rationale

When presented with moving stripes, zebrafish larvae and adults will show locomotor activity in the perceived direction of motion. The OMR requires visual acuity and the coordination of motor patterns in response to visual input, making

it a sensitive assay for probing visually mediated behaviors.

Procedure

Groups of ten zebrafish larvae are placed in embryo water at 3 dpf and exposed to small molecules for 5 days (compounds can be replenished at 6 dpf). At 8 dpf, the larvae are placed in shallow embryo water at one end of narrow, 23–26 cm long acrylic tanks. OMR is initiated by playing a video of a moving black and white grating (black bars, 2 cm wide; drift speed, 1–2 Hz) for 30–45 s toward the other end of the tank (Orger et al. 2000, 2004). At the end of the assay, the distance that larvae traveled to the other end of the tank is recorded. Each treatment is tested in sets of six runs.

Evaluation

Typical untreated larvae at 8 dpf will travel to the farthest 25 % of the channel. Statistical comparison (by ANOVA) of the average distance traveled compared to matched vehicle controls is used to determine an effect on visual acuity.

Critical Assessment

Blind zebrafish mutants fail to exhibit an OMR in response to stripes (Orger et al. 2004), and the assay performed well when challenged with a set of known visually toxic compounds (Richards et al. 2008) or with ethanol (Bilotta et al. 2002). As coordinated motor function is essential for completing the OMR, false positives, i.e., compounds with no effect on visual function, may arise, necessitating cross-validation with other visual tests such as the optokinetic reflex (OKR) or with electrophysiological recording of the retina.

Modifications

OMR can be adapted for adult zebrafish by using a circular arena with rotating stripes along the outside (Zou et al. 2010). A high concordance of the adult swim with the rotating stripe is a measure of a successful adult OMR. Adult OMR is a promising assay for investigating regeneration of the visual system after optic nerve damage (Zou et al. 2013), although no pharmacological screens based on this method have been reported to date.

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Optokinetic Reflex (OKR)

Purpose and Rationale

All vertebrates, including zebrafish, display smooth pursuit eye movements in response to moving stimuli. Zebrafish larvae have large eyes and will display this optokinetic reflex (OKR) soon after the visual system develops at 3 dpf, making this assay easy to score manually or with automation (Brockerhoff et al. 1995). As complex motor programs are unnecessary for this response, the OKR is a sensitive and direct measure of visual responsiveness in zebrafish larvae and adults.

Procedure

Zebrafish larvae (4–6 dpf) are embedded in 6 % methylcellulose and exposed to a moderately lit rotating drum (6–18 r.p.m.) with alternating 1 cm

wide black and white stripes (Brockerhoff 2006; Zou et al. 2010; Mueller et al. 2011). Eye movements are recorded with a video camera for 1 min. Zebrafish larvae can be exposed to small molecules for a few hours to several days prior to the OKR analysis.

Evaluation

Defects in smooth pursuit eye movements can be detected by manual observation of video (Brockerhoff 2006) or through automated video analysis of total eye distance moved or eye velocity (Mueller and Neuhauss 2010; Mueller et al. 2011; Huber-Reggi et al. 2013). The identification of small-molecule hits can be made by statistical comparison to age and time-of-day-matched vehicle controls.

Critical Assessment

The OKR is a highly sensitive and reliable measure of zebrafish visual acuity. Numerous visual mutants have been identified using the OKR assay (Brockerhoff et al. 1995; Neuhauss et al. 1999). The effects of oculotoxic drugs are easily identified in the larval OKR (Bilotta et al. 2002; Emran et al. 2007; Richards et al. 2008; Deeti et al. 2014), although to date no high-throughput screening of small molecules has been performed with this assay. The OKR can be followed up with secondary testing by in vitro recording of zebrafish retinal responses by electroretinogram (ERG).

Modifications

The OKR can also be performed in adult zebrafish (Zou et al. 2010; Mueller and Neuhauss 2010; Tappeiner et al. 2012; Cameron et al. 2013). Immobilization of adult zebrafish is achieved by pinning or clamping the fish between sets of sponge or foam within a water-filled tank.

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Electroretinogram (ERG)

Purpose and Rationale

In zebrafish, the electroretinogram (ERG) can be used to assess the activity of the outer retina in response to visual stimuli. Analysis of the ERG can provide details about the underlying neuronal subtypes and electrical signaling events that are altered in zebrafish with visual defects due to genetic, pharmacological, or experimental manipulation.

Procedure

For adult or larval zebrafish, the animal is anesthetized under dim red light and placed on its side on a damp sponge with streaming fish water (Brockerhoff et al. 1995; Li and Dowling 1997; Seeliger et al. 2002; Fleisch et al. 2008). A suction pipette filled with Ringer's solution is placed on the cornea, and the reference electrode is placed into the bath solution or directly under the sponge. Light flashes (100–1,000 ms) are directed to the eye, and standard electrical recordings are taken (amplification, 1,000X; band pass 1–100 Hz; scan rate 1,000 Hz).

Evaluation

The zebrafish ERG consists of a negative a-wave, which is dominated by signal from the photoreceptor cells; a large, positive b-wave, which is dominated by the depolarization of the ON bipolar cells; and (detectable only for longer light flashes) a smaller d-wave, which is dominated by depolarization of the OFF bipolars (Wong et al. 2004; Brockerhoff et al. 1995). The amplitudes of each wave can be statistically compared to controls. In larvae, the ERG is cone-dominated, while in adults, the response is rod-dominated.

Critical Assessment

The ERG is a straightforward way to examine the function of the outer retina of various visual mutant or drug-treated zebrafish. For rapid screening, behavioral tests like the optokinetic or visual motor reflex can be first used, with the ERG used as a direct follow-up assay. One problem for small molecules is that, in the intact preparation, many compounds will not easily diffuse into the eye (Wong et al. 2004). To overcome this, an isolated preparation has been shown to be more accessible (see section "Modifications").

Modifications

To enhance drug delivery to the retina, an ex vivo preparation of the retina can be used (Wong et al. 2004; Emran et al. 2007, 2010). The eye is completely removed (including all muscle tissue) and placed on a damp filter paper. A Ringer's solution-filled 10–20 uM glass electrode is pressed through the cornea to just above the lens, and the whole eye is superfused with Ringer's. The eye is submerged with a flow rate of 0.2 mL/min. The reference electrode is placed under the filter paper.

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Other Sensory Systems

Acoustic Startle

Purpose and Rationale

The acoustic startle response is highly conserved among vertebrates. Zebrafish have both short-latency (7.0 ms) and long-latency (28.0 ms) "C-bend" startle responses to auditory stimuli (Burgess and Granato 2007). Modifications to the acoustic startle such as the prepulse inhibition and habituation assay can be used to study schizophrenia and learning, respectively (Burgess and Granato 2007; Wolman et al. 2011).

Procedure

Larval zebrafish (>6 dpf) are arranged either individually into the wells of a plate or in groups of 25–30 larvae per 6 cm Petri dish (Bhandiwad et al. 2013; Burgess and Granato 2007). Adult zebrafish are placed individually into a 10 cm diameter container with 150 mL of fish water (Burgess and Granato 2007). The fish is positioned under a high-speed camera while maintaining constant temperature to avoid changes in startle response latency (Burgess and Granato 2007; Preuss and Faber 2003). The experimental apparatus can be housed in a sound attenuation chamber on a vibration-isolation air table to reduce external vibratory noise (Bhandiwad et al. 2013). Following an acclimation period, a startle inducing acoustic/vibrational stimulus is applied at regular intervals. Stimuli can be applied using a small vibration exciter, impact taps delivered by a tubular solenoid (Burgess and Granato 2007), or by conventional speakers (Bhandiwad et al. 2013). Fish are exposed to repeats of each condition, but the interval should be spaced to avoid habituation (Best et al. 2008; Wolman et al. 2011). Compounds can be tested for their impact on acoustic startle by addition to the water prior to the trial onset (Burgess and Granato 2007).

Evaluation

Tracking software can determine the position of each fish over sequential video frames. To characterize C-start responses, it is necessary for the software to compute head orientation, either by a local density search (Burgess and Granato 2007) or by tracking multiple points on the fish as in Hedrick (2008). Knowledge of head orientation also enables the determination of other changes in body orientation and head-tail distance, including quantification of C-start responses (Bhandiwad et al. 2013; Burgess and Granato 2007), and startle latency and duration. Short-latency responses are separated from long-latency responses computationally (Burgess and Granato 2007).

Critical Assessment of the Method

The speed, ease, and reliability of the acoustic startle response have allowed for a number of pharmacological investigations, including single compound studies (Roberts et al. 2011) and screens based on two modifications of this assay, prepulse inhibition (Burgess and Granato 2007) and acoustic habituation (Best et al. 2008; Wolman et al. 2011).

Modifications

Prepulse inhibition is a neurological phenomenon associated with conditions including schizophrenia and Alzheimer's disease, in which a weak prestimulus (prepulse) dampens the response to a subsequent startling stimulus. The proportion of larvae who respond to a startling stimulus can be reduced by prepulse inhibition (Burgess and Granato 2007). To investigate prepulse inhibition, weak auditory stimuli (prepulses) are pseudorandomly presented prior to the startle stimuli (Bhandiwad et al. 2013; Burgess and Granato 2007). Acoustic habituation is a form of nonassociative learning in which the response to a stimulus is diminished by repetitive presentation. Acoustic habituation can be examined by presenting a train of acoustic stimuli in succession at 1, 5, or 20 s intervals and comparing the decrease in responsiveness across progressive trials (see section "Learning and Memory"; "Nonassociative Learning") (Best et al. 2008; Wolman et al. 2011).

Acoustic startle and prepulse inhibition can be studied in individual agarose-embedded larvae by removing the agarose caudal to the fins to allow the imaging of free tail movements. This enables

optogenetic manipulation and imaging of neural activity during behavior (Bergeron et al. 2014).

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Olfactory Explants

Purpose and Rationale

How the brain encodes signals from the outside world is a fundamental question of neuroscience. The zebrafish olfactory system is an excellent model to study neuronal coding because its experimental simplicity allows for a high degree of control over stimulus and drug delivery (i.e., odorants and small-molecule modulators dissolved in water) combined with accessible neuronal imaging and electrophysiological recording of signals in the olfactory bulb (Friedrich and Laurent 2001).

Procedure

A cold-anesthetized adult zebrafish is decapitated, and the ventral forebrain is exposed by removing the jaws, eyes, and palate (Friedrich and Laurent 2001; Friedrich et al. 2004). This preparation is then superfused with artificial cerebrospinal fluid (Mathieson and Maler 1988) at approximately 3 mL/min (total volume: 0.5 mL) and brought to room temperature (Bundschuh et al. 2012). Odorants are delivered for 2.5 s to the inflow naris via an electronically controlled injection valve (Friedrich and Laurent 2001), with an inter-odorant interval of at least 40 s to minimize habituation (Blumhagen et al. 2011). For pharmacological manipulation, the dura mater is carefully removed to enhance drug exposure (Zhou et al. 2012; Tabor et al. 2008). Compounds can be bath applied, allowing 5 min of exposure for electrophysiological recording and 8 min for imaging studies (Zhou et al. 2012).

Evaluation

The activity of mitral cells in the olfactory bulb during odorant and drug exposure can be monitored using standard electrophysiological (Friedrich and Laurent 2001) or calcium imaging techniques (Friedrich 2014). For the latter, mitral cell responses in the olfactory bulb can be observed with bolus injection of the calcium indicator dye, Rhod-2 AM ester (Yaksi and Friedrich 2006; Yaksi et al. 2009). Alternatively, the olfactory sensory neuron terminals can be loaded with Calcium Green-1 dextran by application of 6–8 % solution into each naris of an anesthetized adult zebrafish for 5 min, followed by 3–6 days before imaging (Tabor et al. 2008; Friedrich and Korsching 1997).

Critical Assessment

The zebrafish olfactory explant is an excellent system for examining neuronal population dynamics in response to stimuli. Several studies

have coupled this preparation with pharmacological investigation of the role of glutamatergic (Tabor et al. 2008), GABAergic (Tabor and Friedrich 2008), and dopaminergic (Bundschuh et al. 2012; Schärer et al. 2012) signaling in olfactory bulb odorant processing.

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Arousal States, Spontaneous Swimming, and Sleep

Larval Short-Term Tracking

Purpose and Rationale

Zebrafish exhibit spontaneous swims in a burst and glide pattern. Disruption to the amount of these swims is a sensitive readout for impaired motor neuron and muscle function and coordination as well as neurological effects (Grillner and Manira 2015).

Procedure

Zebrafish larvae (4–7 dpf) are singly or in groups placed into multi-well plates. The larvae can either be chronically exposed to toxicants or small molecules added to their water from 6 hpf, or compounds can be added just prior or during the test (Renier et al. 2007; MacPhail et al. 2009). The plate is then illuminated with infrared light and either white light or placed in constant darkness. Larvae are allowed to acclimate for 5–20 min, and larval movements are recorded with video cameras for 20+ min. Alternatively, the use of infrared microbeams allows activity to be inferred through the number of beam breaks per unit time (Bichara et al. 2014).

Evaluation

Tracking software is used to determine the total distance moved during the assay. This can be

represented for each treatment condition as a mean cumulative distance traveled or plotted as average distances traveled per epoch (e.g., seconds or minutes). Distance moved or cumulative activity is then statistically compared to vehicle-treated controls (ANOVA).

Critical Assessment

Short-term swimming assays can be strongly affected by prior handling and lighting conditions. Many papers that describe spontaneous swimming in zebrafish use 5 or 10 min alternations in light/dark to elicit robust swimming behavior (MacPhail et al. 2009; Irons et al. 2010, 2013; Duan et al. 2013). However, these short-duration light phases do not allow the larvae to return to their natural, baseline locomotor states and are most likely measuring visual motor/dark photokinesis events (see section "Visual and Nonvisual Light Responses"). Finally, head-embedded and fictive preparations coupled with higher-speed videography (60–120 Hz) give the experimenter better stimulus control and finer quantification of swim speed, coordination, and duration.

Modifications

Screening can be improved with automated image analysis of spontaneous swims of groups of wild-type, mutant, and drug-treated larvae (Mirat et al. 2013).

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Head-Embedded, Tail-Free Swimming

Purpose and Rationale

Zebrafish larvae (4–7 dpf) can perform normal swim bouts in a head-embedded, tail-free preparation. This assay gives the experimenter greater control over pharmacological manipulations, as drugs can be directly injected while monitoring the tail for swim behavior.

Procedure

The zebrafish larva is embedded in low melt agarose (1 %) with the tail free to move in embryo water (Budick and O'Malley 2000; Brustein and Drapeau 2003). After an acclimation period, drugs dissolved in Evan's solution and 1 % Fast Green (to assess injection quality) are then pressure injected with a fine glass needle into the pericardial sac (Brustein et al. 2003; Brustein and Drapeau 2005). Swimming behavior is recorded with a video camera (60–120 Hz) before, immediately after, and 30–60 min after injection.

Evaluation

For each spontaneous bout, zebrafish swimming frequency can be estimated from the number of

left-right alternations of the tail (tail beat frequency). For each treatment, the number of swim episodes, duration, frequency, and the inter-bout intervals can be statistically compared.

Critical Assessment

Experimental work has suggested that tail-free preparations behave similarly to freely swimming larvae (Budick and O'Malley 2000; O'Malley et al. 2004). As drugs can be directly injected into the larva, this method offers more experimental control (e.g., better timing and bioavailability of the compounds) but a much lower throughput than methods that observe freely swimming fish bathed in drug, especially those that track groups of larvae (Mirat et al. 2013).

Modifications

By projecting images on the zebrafish retina, visually evoked swims, including the optokinetic (OKR) and optomotor reflex (OMR), can be studied in larval tail-free preparations (O'Malley et al. 2004; Portugues and Engert 2011). More complex behaviors, such as hunting, can be studied using a higher-speed camera (80 Hz) to capture behaviorally characteristic tail bends (Bianco et al. 2011). The tail-free preparation can be combined with functional calcium imaging to observe neuronal activity during spontaneous or visually evoked swims (Wyart et al. 2009; Portugues et al. 2014; Severi et al. 2014). For example, simultaneous videography of tail movements plus the imaging of neuronal activity using the transgenic expression of the genetically encoded calcium indicator, GCaMP, allows for the correlation of tail movements with neuronal activity (Wyart et al. 2009; Portugues and Engert 2011; Portugues et al. 2014).

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Larval Fictive Swimming

Purpose and Rationale

To combine electrophysiological recordings with observations about swimming pattern and frequency, a larval fictive swimming preparation can be used. Paralyzed zebrafish larvae will still initiate swims by generating motor commands, which can be observed by recording motor nerve activity in the trunk.

Procedure

Larvae are anesthetized in tricaine and then paralyzed by immersion in 1 mg/mL

alpha-bungarotoxin in Hank's solution (Masino and Fetcho 2005), 15 μM D-tubocurarine (Drapeau et al. 1999; Knogler et al. 2010), or by injection of 125 μM alpha-bungarotoxin into the heart (Trapani and Nicolson 2010). Once immobile, the larva is pinned through the notochord and the peripheral nerves are exposed by removing the skin. For recording, the larva is continuously perfused with extracellular recording solution without anesthetic. Peripheral nerve activity is extracellularly recorded (current clamp mode) with a 20–50 μM diameter glass micropipette filled with extracellular recording solution and positioned with light suction at the peripheral nerve in the dorsoventral midline of the myotomal cleft (Drapeau et al. 1999; Masino and Fetcho 2005). The preparation can be stable for several hours. Spontaneous fictive swim bouts can be recorded or swims can be evoked with a briefly shined light stimulus or a touch (e.g., a gentle squirt of bath solution) applied to the base of the tail (Knogler et al. 2010; Knogler and Drapeau 2014).

Evaluation

Patch clamp cellular recordings of hindbrain and spinal cord neurons during fictive swims can be used to observe a number of parameters during swim bout generation including the duration of swim episodes and rest intervals, number of swim episodes, and fictive swim frequency (Brustein et al. 2003; Brustein and Drapeau 2005; Masino and Fetcho 2005; Ahrens et al. 2012), under wild-type and pharmacological blockade of various neurotransmission events.

Critical Assessment

Although unsuitable for high-throughput screening, the zebrafish fictive swimming prep has been used to pharmacologically dissect numerous features of swim pattern generation and speed selection, including the roles of serotonin (Brustein and Drapeau 2005), glycine, glutamate (Knogler et al. 2014), potassium and calcium channels (Buss and Drapeau 2001), and tumor necrosis factor alpha (TNFalpha; Knogler et al. 2010).

Modification

In one recent advance, real-time evaluation of fictive swim events can be used to update the visual scene presented to the larva to create a virtual world (Ahrens et al. 2012). Whole-brain calcium-imaging techniques can then be used to assess neuronal dynamics as zebrafish larvae navigate virtual worlds, and pharmacological methods could be used to dissect the contributions of neurotransmitter systems to these processes (Ahrens et al. 2012; Vladimirov et al. 2014).

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Long-Term Tracking: Sleep

Purpose and Rationale

Zebrafish will exhibit 24 h rhythmicity and sleep-like states as soon as they begin spontaneous swimming at 4 days post fertilization (Zhdanova et al. 2001; Prober et al. 2006; Rihel et al. 2010a). Tracking of individual larval zebrafish over several days allows for the simultaneous investigation of multiple sleep/wake parameters including sleep latency, the structure of sleep bouts, and waking activity levels (Rihel et al. 2010b).

Procedure

Zebrafish larvae (4 dpf) are individually placed into the wells of a 96-well plate with embryo water. Eight to 12 animals per treatment are dosed with small molecules dissolved in water or DMSO (final DMSO concentration <0.1 %). Larvae are then continuously illuminated with infrared light and a 14 h/10 h white light/dark cycle while a circulating water bath maintains the temperature at 28 °C. The plate is then continuously monitored with a video camera for several days (Rihel et al. 2010a, b).

Evaluation

Total movement is calculated for each fish with either commercial (e.g., Viewpoint Lifesciences, Noldus) or custom-made software. For each day and night epoch, multiple parameters can be extrapolated and statistically compared among treatments. Periods of quiescence lasting for 1 min (in larvae) are associated with increased arousal thresholds (Prober et al. 2006) and are considered to be sleep-like states. Other extractable features include the number and length of sleep bouts, sleep latency, and average waking activity (Prober et al. 2006; Rihel et al. 2010a, b). Together, these features can be collected into a single vector or behavioral fingerprint, which allows for the clustering of compounds with similar behavioral effects (Rihel et al. 2010a; Rihel and Schier 2012).

Critical Assessment

The large number of features that can be extracted from longer time course experiments allows for the discovery of small molecules with specific effects that would be missed in shorter trials. For example, in a screen, Rihel and co-workers (2010a) identified small molecules that had effects only at specific times of the 24-h day, including compounds with opposite effects during the day and night (e.g., sedating in one phase, arousing in the other). One caveat is that, over the course of the long tracking period, the continuous exposure of small molecules may induce complex secondary effects that are difficult to interpret. Similarly, metabolism of the compounds may enhance or eliminate their biological activity over the course of the experiment.

Modifications

Adult zebrafish sleep can also be monitored for several days with tracking software, using larger arenas (Yokogawa et al. 2007; Sigurgeirsson et al. 2013). In adults, 6 s of continuous inactivity are considered sleep-like states (Yokogawa et al. 2007). In the larval sleep assay, paramecia, undetectable by the camera, can be added daily into the water for feeding, which allows the continuous tracking of larvae in a 96-well plate from 7 up to >14 dpf. The 96-well plate can also be covered with a transparent, oxygen permeable film to better maintain water levels throughout the tracking experiment (Gandhi et al. 2015). Additionally, larvae and adults can be switched from a light/dark cycle to continuous light or darkness to measure the period and amplitude of circadian rhythms (Cahill et al. 1998; Hurd and Cahill 2002; Prober et al. 2006; Yokogawa et al. 2007; Sigurgeirsson et al. 2013).

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Novel Tank and Anxiety

Purpose and Rationale

When introduced to a novel tank, adult zebrafish exhibit a robust anxiety-like response that includes diving, freezing, and reduced exploration. This test can be employed to assess stress and anxiety in either genetically or pharmacologically manipulated zebrafish.

Procedure

Adult zebrafish are introduced to a 1.5-L plastic tank filled with fish water, with a line delineating the upper and lower portions of the arena. A camera video records the fish's behavior for 6 min or observers score behavior manually based on predefined ethograms (Kyzar et al. 2012; Cachat et al. 2013). Compounds can be tested for their anxiogenic or anxiolytic effect by immersing the fish in a beaker with the dissolved drug solution prior to introduction to the novel tank (Levin et al. 2007; Bencan et al. 2009), with vehicle exposed fish serving as handling controls.

Evaluation

The critical parameters to evaluate stress are the number of entries and latency to enter each area, the time and distances traveled in the upper and lower areas, as well as the number and duration of freeze bouts (Cachat et al. 2010). Computerized video tracking can objectively assess these parameters, but many are also quantifiable manually with ethograms (Kyzar et al. 2012; Cachat et al. 2013). The impact of any given compound on parameters such as swim distance or velocity should be taken into consideration when assessing its anxiolytic or anxiogenic effect.

Critical Assessment

The ease and speed of this methodology have been employed to examine the anxiolytic and anxiogenic effects of a number of compounds including alarm pheromone (Cachat et al. 2010), nicotine (Levin et al. 2007), and hallucinogenic agents (Kyzar et al. 2012; Cachat et al. 2013) and to demonstrate the beneficial effect of fluoxetine in glucocorticoid receptor mutants (Ziv et al. 2013).

Modifications

Thigmotaxis (edge preference) and scototaxis (dark/light preference) are alternative anxiety-like behavioral measures that can be assayed in

both adult (Blaser et al. 2010; Grossman et al. 2010; Maximino et al. 2010a, b) and larval zebrafish (Amir-Zilberstein et al. 2012; Schnörr et al. 2012; Ahmad and Richardson 2013) through methodologies similar to the novel tank. For thigmotaxis, the time spent near or away from the wall is calculated, while for scototaxis, the time spent in dark or light areas is determined.

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Seizures and Epilepsy

Introduction

The tendency to have recurrent seizures, known as epilepsy, can be modeled in zebrafish either pharmacologically by the application of convulsant drugs or genetically by morpholino knockdown or mutagenesis (Grone and Baraban 2015). Zebrafish models exist for a range of inherited epilepsies including Lowe syndrome (Ramirez et al. 2012), Dravet syndrome (Baraban et al. 2013), and epilepsy, ataxia, sensorineural deafness, and tubulopathy (EAST) syndrome (Mahmood et al. 2013). Zebrafish experiencing seizures display the characteristic molecular, electrographic, and behavioral changes observed in rodent models and human patients. Furthermore, anticonvulsant drugs ameliorate these symptoms, underscoring the direct relevance of screening for antiepileptic compounds in the zebrafish.

Electrophysiology

Purpose and Rationale

Zebrafish epileptic events can be unambiguously identified in electrical field potential recordings. Electrographic activity can be examined in genetic or pharmacologically induced seizure models before, or in response to, bath applied antiepileptic drugs.

Procedure

Zebrafish larvae (3–7 dpf) are immobilized in 1.2 % low-melt agarose with their dorsal side exposed to the surface (Baraban et al. 2005, 2007). Anesthetic compounds, which may influence synaptic function and convulsant activity, are not necessary (Baraban et al. 2005). The embedded fish is transferred to a microscope stage and perfused with Ringer's medium with or without a dissolved convulsant drug, such as 15 mM pentylenetetrazole (PTZ)(Baraban et al. 2005). A 1 μm tip glass microelectrode filled with 2 M NaCl is inserted into the optic tectum (Baraban et al. 2005, 2013) or telencephalic forebrain structures (Baraban et al. 2007), and the electrical activity is recorded, amplified, and filtered by standard electrophysiological techniques (Baraban et al. 2005, 2007, 2013). Once a stable pattern of epileptiform activity has developed, test compounds can be added to the bathing medium and evaluated for anticonvulsant effect (Baraban et al. 2013). Alternatively, larvae can be preincubated in the drug, as in Afrikanova et al. (2013).

Evaluation

Epileptiform events, defined as membrane deflections greater than twice baseline noise, can be analyzed by software and classified as ictal (lasting 1,000–5,000 ms) or interictal (lasting 100–300 ms) (Baraban et al. 2013). Measures including burst amplitude, duration, and frequency (Baraban et al. 2005), as well as time spent seizing (Baraban et al. 2013), can be calculated across recording periods and compared between groups (Baraban et al. 2007).

Critical Assessment

Zdebik et al. (2013) noted that fish movement and/or electrode placement into the optic tectum can induce seizure-like activity, even in untreated, wild-type fish. Artifacts can be avoided by surface recordings (Afrikanova et al. 2013; Zdebik et al. 2013). While important for the validation of anticonvulsants discovered by higher-throughput means, electrophysiological methods are likely too cumbersome as a primary assay for small-molecule screening.

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Electroencephalogram

Purpose and Rationale

The electroencephalogram (EEG) can be used to noninvasively examine electrographic activity in genetic or pharmacologically induced seizure models before or in response to antiepileptic drugs.

Procedure

Larval zebrafish are paralyzed by 10-min immersion in a 2 mM solution of D-tubocurarine, rinsed, and mounted in 1.5 % low-melting temperature agarose (Zdebik et al. 2013). A pulled glass micropipette filled with 1 M NaCl with a tip diameter of 10–15 μm is placed on the skin above the optic tectum and used to measure the field potential between this electrode and a reference electrode placed into the agarose (Zdebik et al. 2013). The signal is amplified, filtered, and digitized by standard techniques. The impact of test compounds can be evaluated by addition to the water surrounding the fish (Zdebik et al. 2013).

Evaluation

For statistical comparisons between groups, amplitude can be averaged across recordings. Additionally, Fourier analysis can be used to demonstrate increased power in the 2–4 Hz frequency band, a frequency band typical for human seizure activity (Zdebik et al. 2013).

Critical Assessment of the Method

While overly laborious for high-throughput pharmacological screening, this assay can be employed to validate potential anticonvulsants identified through other means, such as the seizure behavioral assay.

Modifications

A modified method for recording electroencephalograms in adult fish is described by Afrikanova et al. (2013).

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Seizure Behavior

Purpose and Rationale

Genetic and pharmacologically induced zebrafish seizure models display a characteristic sequence of seizure-like behaviors that result from abnormal neuronal activity. These behaviors are observable manually or by video recording and, unlike electrophysiological or electrographic methods, are amenable to high-throughput pharmacological screening for the discovery of novel antiepileptic or convulsant drugs (Grone and Baraban 2015).

Procedure

Individual zebrafish larvae are aliquoted into the wells of a microplate filled with fish water. Adult fish should be placed into a container of suitable size, for example, a 1.5 L trapezoidal tank (Wong et al. 2010). Seizure behavior is monitored either by eye (Baraban et al. 2005; Wong et al. 2010) or through the use of automated video tracking software (Afrikanova et al. 2013; Baraban et al. 2005, 2013; Baxendale et al. 2012; Berghmans et al. 2007; Wong et al. 2010). In a genetic model of epilepsy, the exhibition of spontaneous seizures is examined, and then antiepileptic drugs are applied after a period of recording (Baraban et al. 2013). If a pharmacologically induced model is used, fish can be preincubated in test antiepileptics prior to the addition of a convulsant (e.g., 10 mM PTZ) following baseline recording (Afrikanova et al. 2013; Baxendale et al. 2012; Berghmans et al. 2007).

Evaluation

If manual observations are made, then behavior can be characterized according to both

non-seizure (Wong et al. 2010) and seizure-like ethograms (Baraban et al. 2005; Wong et al. 2010). For example, if seizure behavior is staged as in Baraban et al. (2005), then statistical comparisons can be made between groups for several parameters, including the timing and number of fish reaching each seizure stage. Alternatively, the use of automated tracking software enables the calculation and comparison between groups of parameters including distance traveled, average velocity, and path tracing (Afrikanova et al. 2013; Baraban et al. 2005, 2013; Baxendale et al. 2012; Berghmans et al. 2007; Wong et al. 2010).

Critical Assessment

This assay has been employed by both targeted (Afrikanova et al. 2013; Berghmans et al. 2007) and high-throughput drug screens (Baraban et al. 2013), which have both identified a novel anticonvulsant. While studies show a good correlation between behaviorally and electrographically relevant antiepileptic drugs (Afrikanova et al. 2013), it remains possible that reductions in seizure-like locomotion reflect general toxicity or sedation. To minimize this problem, compound doses can be chosen that minimally affect normal behavior, and potentially novel antiepileptics should be validated by electrographic methods.

Modifications

In an attempt to improve the predictive potential of the zebrafish assay to mammalian convulsants, Koseki et al. (2014) conducted flashlight stimulation, a known seizure induction stimulus.

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Learning and Memory

Introduction

Zebrafish share neuroanatomical and neurochemical homology with the mammalian brain and are able to perform both nonassociative and associative learning (Blaser and Vira 2014a; Roberts et al. 2013). This makes the zebrafish a potentially powerful model for the pharmacological study of neurological, neurodegenerative, and psychiatric diseases that have deficits in learning and memory.

Nonassociative Learning

Purpose and Rationale

Nonassociative learning can be studied in zebrafish in response to pharmacological manipulation.

Procedure

Larval zebrafish (7 dpf+) are placed either individually into the wells of a plate (Best et al. 2008; Roberts et al. 2011; Wolman et al. 2011) or in groups into a Petri dish (Burgess and Granato 2007a, b; Wolman et al. 2011). The fish are allowed to acclimatize for an hour (Roberts et al. 2011) and then presented with either acoustically (Best et al. 2008; Burgess and Granato 2007a; Roberts et al. 2011; Wolman et al. 2011) or visually startling stimuli (Burgess and Granato 2007b; Wolman et al. 2011). Stimuli are repetitively presented with a short interstimulus interval, usually less than 20 s (Best et al. 2008; Wolman et al. 2011), in either a single-massed session or spaced training blocks (Best et al. 2008; Roberts et al. 2011; Wolman et al. 2011). Pharmacological compounds are applied by bath immersion prior to testing (Best et al. 2008; Roberts et al. 2011; Wolman et al. 2011).

Evaluation

Automated behavioral tracking allows the high-throughput identification of both short- and long-latency startle responses (Burgess and Granato 2007a), enabling changes in responsiveness to be examined across stimulus presentations (Best et al. 2008; Roberts et al. 2011; Wolman et al. 2011). As compound exposure can alter initial startle amplitude, changes should be normalized to this value (Wolman et al. 2011).

Critical Assessment

Targeted pharmacological studies have demonstrated that conserved pathways mediate nonassociative learning in zebrafish (Best et al. 2008; Roberts et al. 2011). Taking advantage of the high-throughput potential of this assay, Wolman and colleagues (2011) screened nearly 2,000 compounds for their impact on short-term habituation. This study demonstrated conservation of zebrafish short-term learning with mammalian models and implicated novel compounds in learning (Wolman et al. 2011). Together, these studies illustrate the relevance and ease of large-scale screening for compounds that alter nonassociative learning tasks.

Modifications

Habituation to startle can be studied in adults as in Eddins et al. (2010). Habituation to novelty can also be studied in adults (Wong et al. 2010).

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Associative Learning-Classical Conditioning

Purpose and Rationale

One form of associative learning is classical conditioning, the ability to associate a neutral, conditioned stimulus with a reinforcing stimulus. Classical conditioning can be examined in both larval and adult zebrafish through a variety of related procedures, including conditioned place preference, appetitive conditioning, and fear conditioning (Blaser and Vira 2014).

Procedure

Conditioned Place Preference – an animal learns to associate a specific environment with the delivery of a behaviorally altering drug. A single adult fish is placed into the center of a 2 L tank divided into two visually distinct areas. Fish are allowed to habituate to their new environment for 5–10 min, and then their baseline place preference is determined by observing the percentage of time the fish spends in each area across a 2-min (Darland and Dowling 2001; Kily et al. 2008) or 15-min period (Ninkovic and Bally-Cuif 2006; Webb et al. 2009). Fish are subsequently conditioned by restriction to their least preferred side either in conjunction with (Darland and Dowling 2001; Kily et al. 2008; Lau et al. 2006; Mathur et al. 2011) or following drug exposure (Ninkovic and Bally-Cuif 2006; Webb et al. 2009). Finally, the fish are reintroduced to the tank, allowed to habituate, and recorded for place preference.

Appetitive Conditioning – an animal learns to associate a previously neutral, conditioned, stimulus with an unconditioned reward. Adult zebrafish are placed into a tank of appropriate size, such as a 4 L tank, and presented with a neutral stimulus, for example, a behaviorally inert odor (Braubach et al. 2009) or a light cue (Cerutti et al. 2013), followed by a food reward 4–35 s later. Fish are trained by repetitive trials conducted in short sessions and then tested in probe trials in which the unconditioned stimulus is absent.

Fear Conditioning – an animal learns to associate a neutral stimulus with an aversive, unconditioned stimulus. Individual larval (Aizenberg and Schuman 2011; Valente et al. 2012) or adult fish (Hall and Suboski 1995a, b; Agetsuma et al. 2010) are presented with a neutral visual or olfactory stimulus. Following a brief interval, for example, 10 min (Agetsuma et al. 2010), fish are reexposed to the stimulus but this time in conjunction with an aversive, unconditioned stimulus such as an electrical shock (Agetsuma et al. 2010; Valente et al. 2012) or exposure to alarm pheromone (Hall and Suboski 1995a, b). Paired presentations are repeated over a training period, for example, ten pairings separated by a 6-min interval for larval fish (Valente et al. 2012) or five trials separated by 180 s delivered in two sessions for adult fish (Agetsuma et al. 2010). Finally, retrieval is tested by presentation of the conditioned stimulus alone.

Evaluation

Learning is evaluated by comparing the individual behavioral responses of larval or adult fish during the retrieval trials to those responses during the training trials (Agetsuma et al. 2010; Braubach et al. 2009; Darland and Dowling 2001).

Critical Assessment

Blaser and Vira (2014) make numerous critical recommendations for current and future approaches to zebrafish learning and memory studies, including the need to better understand both the intrinsic preferences and drug pharmacokinetics in zebrafish. Another problem for chemical screening is the difficulty thus far of performing classical conditioning in larval zebrafish, with only one report in 6–8 dpf larvae (Aizenberg and Schuman 2011) and one report in 1-month-old juveniles (Valente et al. 2012).

Modifications

Neuronal activity in response to presentation of the conditioned stimulus can be examined in vivo through the use of calcium imaging in restrained larvae (Aizenberg and Schuman 2011).

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Associative Learning: Operant Conditioning

Purpose and Rationale

Operant conditioning is a form of associative learning in which an association is made between a given behavior and its consequence and can be tested in both juvenile (3–8 weeks of age) and adult zebrafish.

Procedure

Positive Reinforcement – juvenile (Williams et al. 2002) or adult zebrafish (Al-Imari and Gerlai 2008; Bilotta et al. 2002; Colwill et al. 2005; Mueller and Neuhauss 2012; Parker et al. 2012; Pather and Gerlai 2009) are allowed to habituate to a tank or maze. Fish are trained to discriminate between areas of the apparatus by pairing visual cues such as a red card (Williams et al. 2002) with a food (Bilotta et al. 2002; Colwill et al. 2005; Mueller and Neuhauss 2012; Parker et al. 2012) or social reward (Al-Imari and Gerlai 2008; Karnik and Gerlai 2012; Pather and Gerlai 2009). After a set number of training sessions, for example, 20 trials across five consecutive days (Karnik and Gerlai 2012), or upon reaching a predetermined learning criterion (Parker et al. 2012; Williams et al. 2002), fish are examined for their response to the discriminative stimulus in a probe test.

Negative Reinforcement – juvenile (Lee et al. 2010; Valente et al. 2012) or adult zebrafish (Blank et al. 2009; Gleason et al. 1977; Pradel et al. 1999; Xu et al. 2007; Yang et al. 2003) are placed into a tank divided into two areas either physically by a partial barrier or hurdle or virtually by the presentation of visual stimuli (Valente et al. 2012). A neutral stimulus, such as a light, is presented to the fish on one side of the tank followed, after a period of 12 s, by an electrical

shock that terminates either after a brief interval (e.g., 12 s) or when the fish escapes to the other area (Pradel et al. 1999; Xu et al. 2007; Yang et al. 2003). This procedure should be repeated in a training period with trials separated by a variable inter-trial interval to prevent habituation (Lee et al. 2010; Xu et al. 2007).

Evaluation

Learning can be evaluated between groups by comparing either the percentage response in each training session (Xu et al. 2007) or the number of fish who respond correctly during probe trials (Lee et al. 2010). Memory can be tested by calculation of a retention score, which relates the number of trials to reach the learning criterion in the test session to the number of trials required in the training session per fish (Pradel et al. 1999).

Critical Assessment of the Method

A limitation of these methods is that long training periods, in some cases across multiple days, are required to elicit a reliable response. As such, these methods will be difficult to perform as a chemical screen. Furthermore, operant conditioning has been difficult to assess in larval zebrafish. The youngest age with robust learning under operant conditioning has been older than 21 days post fertilization (Valente et al. 2012).

Modifications

Adult fish show an increased latency to cross to the other side of a shuttle box following a single trial exposure to an inescapable electrical shock (Blank et al. 2009).

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Social Interaction

Introduction

Zebrafish are highly sociable animals that form shoals in both natural and laboratory environments (Oliveira 2013). Structured social relationships in the form of dominance hierarchies exist within shoals (Paull et al. 2010). Zebrafish exhibit a number of quantifiable social interactions including aggressive behavior, courtship displays, social preference, and shoaling.

Aggression: Mirror

Purpose and Rationale

When confronted with their own mirror image, adult zebrafish display aggressive behavior resembling that which they show to rival fish. Either manual or video observation of this behavior enables both qualitative and quantitative descriptions of aggression.

Procedure

A water-filled tank (e.g., 20 × 14.5 × 12.5 cm) is set up with a mirror attached to one side (Pham et al. 2012; Teles et al. 2013). The mirror can be fixed in place with suction cups (Toms and Echevarria 2014). If mirror aggression is scored manually, desired zones are delineated on the outside of the tank. Typical zones include the mirror approach zone at 2.5 cm from the mirror and mirror contact zone at 0.5 cm from the mirror (Pham et al. 2012; Cachat et al. 2013). If video tracking is used, these areas can be defined virtually. The tank is equally illuminated across the entire tank, and the apparatus is surrounded with blackout curtains to avoid background objects appearing in the mirror. The fish is placed into the tank and recorded for 5 (Toms and Echevarria 2014; Weber and Ghorai 2013) to 30 min (Teles et al. 2013). For pharmacological manipulations, fish are dosed by bath immersion during embryonic stages or just prior to testing (Cachat et al. 2013).

Evaluation

Manual observations can be made based on a predefined ethogram that includes mirror biting (Pham et al. 2012; Cachat et al. 2013; Weber and Ghorai 2013), mirror contacts (Pham et al. 2012), and aggressive tail beats or thrashing against the mirror (Pham et al. 2012; Toms and Echevarria 2014). Automated tracking software can be used to quantify temporally based endpoints, including the latency to enter, the amount of time spent in zones, the number transitions between zones (Pham et al. 2012; Cachat et al. 2013), and the total distance traveled (Weber and Ghorai 2013).

Critical Assessment

To date, only single compounds have been evaluated for their impact on mirror aggression (Cachat et al. 2013; Weber and Ghorai 2013). Furthermore, as Oliveria et al. (2011) note, mirror fighting elicits neither the full agonistic repertoire of fish nor the brain activation pattern or hormonal response associated with fighting a live opponent (Oliveira et al. 2005; Desjardins and Fernald 2010; Oliveira and Canário 2011). Similarly, in zebrafish no significant changes in monoamine levels are observable in mirror fights (Teles et al. 2013), suggesting that zebrafish can differentiate between real and mirror fighting.

Modifications

In this assay, fish are subject to potential stress from both their novel environment and the presence of the mirror (Pham et al. 2012). One solution to this is to allow the fish to habituate to the tank, which can be seen as quickly as 5–6 min (Pham et al. 2012). An alternative is to introduce a mirror into the habituated tank either manually (Pham et al. 2012) or using a draw string cover, as in Toms and Echevarria (2014). The effect of environmental stimuli on aggression can be studied by designing specific test chambers. For example, Weber and Ghorai (2013) examine the effect of introducing a refuge into the tank.

Rather than testing individual fish, multiple fish can be tested simultaneously if a large enough tank is used; for example, in Cachat et al. (2013), 15 fish were tested in a 1.5 L trapezoidal tank. Way et al. (2015) compared several methodologies and concluded that a flat mirror elicits more bite attempts than other methods, while use of an inclined mirror provokes more darts. These differences should be taken into consideration when interpreting data from these assays.

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Conspecific Aggression

Purpose and Rationale

When placed together, zebrafish will display symmetrical aggressive behavior until one establishes dominance, after which asymmetric behaviors, including chasing and fleeing, are observed (Oliveira et al. 2011).

Procedure

To reliably elicit agonistic behavior, fish are size matched to differ in length by no more than 1 mm (Larson et al. 2006; Oliveira et al. 2011). To distinguish between fish, cuts can be made under anesthesia into alternating fins of each fish (Colman et al. 2009). The fish are then isolated visually (Oliveira et al. 2011; Teles et al. 2013) or both visually and chemically (Larson et al. 2006) from their counterpart overnight, for example, by placing the fish on either side of a 700 mL polycarbonate tank with a removable partition. Following the isolation period, the partition is removed, and the fish are allowed to interact for a period of 30 min (Oliveira et al. 2011) while under either manual or video observation. Compounds for testing are administered by bath immersion (Colman et al. 2009).

Evaluation

Aggression is scored by use of a predefined ethogram, including both agonistic behaviors (e.g., biting, chasing, circling, displays, nipping, and striking) and submissive behaviors (e.g., fleeing, freezing, and retreating) (Larson et al. 2006; Colman et al. 2009; Oliveira et al. 2011).

Conflict resolution can be defined as the time at which these behaviors switch from being symmetrically displayed by both fish to asymmetrically displayed (Oliveira et al. 2011; Teles et al. 2013).

Critical Assessment

Unlike mirror fighting, this assay observes a natural aggressive behavior. However, few pharmacological studies have employed this methodology, probably because of the challenges of pairing fish fairly and unambiguously tracking both fish during the encounter.

Modifications

The effect of conflict outcome on the results of subsequent fights can be studied by re-isolating fish for an hour after conflict resolution followed by repairing each fish to a second naïve, size-matched opponent (Oliveira et al. 2011).

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Competitive Spawning

Purpose and Rationale

Competitive spawning can be used to examine how the aggression and territoriality of male fish impacts their social status and subsequent reproductive success.

Procedure

Two size-matched males and females are introduced into a 15 L tank (Paull et al. 2010) including an appropriate spawning site such as a marble-filled plastic box with a basal mesh separator (Spence and Smith 2005; Spence 2006; Colman et al. 2009; Paull et al. 2010; Filby et al. 2012). Setting up the assay on the evening prior to spawning allows the fish time to habituate to the environment and to establish a dominance hierarchy and territoriality (Spence and Smith 2005; Colman et al. 2009; Paull et al. 2010; Filby et al. 2012). To facilitate observation, fish can be marked using either distinctive fin clips performed under anesthesia (Filby et al. 2012) or Floy fish tags (Delaney et al. 2002). The fish are allowed to spawn in the morning, and then the eggs are collected, washed, and counted. Unfertilized or infected eggs are discarded. The fertilized embryos are incubated for 24 h and then stored in 100 % ethanol for parentage analysis by transgenic expression or by DNA microsatellite analysis.

Evaluation

Dominance can be ascertained by the use of either an agonistic ethogram (Paull et al. 2010), a courtship ethogram (Spence and Smith 2005; Colman et al. 2009b), or by spawn site monitoring to establish territoriality (Spence and Smith 2005; Paull et al. 2010). The reproductive success of each male can be determined through parentage analysis of the collected eggs. If one male in each colony expresses a fluorescent transgene, then parentage can be assigned using florescence microscopy (Colman et al. 2009). Alternatively, parentage can be assigned on the basis of microsatellite markers using software such as Probmax (Danzmann 1997). Using this method, approximately 95 % of embryos can be assigned to a single parental pair (Coe et al. 2008, 2009; Paull et al. 2010; Filby et al. 2012).

Critical Assessment

This assay has been employed to study the impact of environmentally relevant concentrations of synthetic estrogens on reproductive success (Coe et al. 2008, 2009; Colman et al. 2009; Filby et al. 2012). Care should be taken to consider and control for the inherent variability in measures of reproductive success, as discussed by Paull et al. (2008).

Modifications

To control for the effects of handling and anesthesia, Filby et al. (2012) also anesthetize the experimental fish's counterparts. An alternative is to expose an individual male to a compound following a baseline trial to establish reproductive success (Colman et al. 2009; Filby et al. 2012).

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Social Preference

Purpose and Rationale

Zebrafish are highly social animals and prefer to be part of a shoal. Social preference assays examine the impact of genetic, pharmacological, or behavioral manipulation on the tendency of adult zebrafish to seek out and remain close to conspecifics.

Procedure

The apparatus consists of a rectangular tank divided into five separate areas, in which the two outermost segments are separated from the rest of the tank by double panes of transparent plexiglass to minimize chemical or auditory cues (Engeszer et al. 2004). The two central dividers are opaque and removable, and their location is marked on the outside of the tank to give three demarcated areas (Pitcher 1993). Either a single shoal of four or more fish is placed into a random outer segment (Grossman et al. 2010; Riehl et al. 2011; Savio et al. 2012; Seibt et al. 2011; Sison and Gerlai 2011; Wright et al. 2003) or shoals are randomly placed into each outer segment (Braida et al. 2012; Engeszer et al. 2004; Spence and Smith 2007). A single test subject is introduced to the central area, and, following a period of habituation, the central dividers are removed to allow the fish to explore the apparatus. The fish's behavior is manually or automatically observed with tracking software. In paradigms with two shoals, the test period begins once the test fish has recognized both shoals (Braida et al. 2012; Engeszer et al. 2004; Spence and Smith 2007). Pharmacological compounds are given prior to testing by either immersion (Riehl et al. 2011;

Sison and Gerlai 2011) or injection (Braida et al. 2012).

Evaluation

Social preference is calculated by comparing the amount of time a test fish spends in proximity to the social stimulus versus away from the social stimulus. If automated tracking is used, the distance traveled and velocity are calculated to ensure pharmacological effects are not due to toxicity or sedation. Excursions (i.e., the number of times that the subject fish ventures away from the stimulus shoal) can also be evaluated (Moretz et al. 2006; Xia et al. 2010).

Critical Assessment

To date this assay has been employed to study the impact of a range of compounds including lysergic acid diethylamide (Grossman et al. 2010), MK-801 (Riehl et al. 2011; Seibt et al. 2011) and embryonic ethanol exposure (Fernandes and Gerlai 2009). As noted by Sison and Gerlai (2011), care must be taken in these studies to ensure that doses do not impair motor coordination or other potentially confounding variables.

Modifications

An alternative to the use of live stimulus fish is the presentation of animated fish images via screens placed on either side of the test tank (Fernandes and Gerlai 2009; Saverino and Gerlai 2008). Similarly, Abaid et al. (2012) describe the use of robotic fish. The advantage of these methods is that they enable consistent stimulus delivery across sessions and test subjects. However, social interaction is a two-way process, so the presentation of live, reactive fish is more likely to elicit natural social behavior than artificial shoals.

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Shoaling

Purpose and Rationale

A shoaling assay can be used to evaluate the impact of pharmacological manipulation on group dynamics in adult zebrafish.

Procedure

A group of four adult zebrafish are introduced into a novel tank such as a 1.5 L trapezoidal container (Green et al. 2012) and video recorded for 6 min. Either individual fish or the whole shoal (Maaswinkel et al. 2013a) are exposed to test compounds by bath application prior to testing (Grossman et al. 2010) or by addition to the novel tank after a short habituation period (Green et al. 2012).

Evaluation

Data from this paradigm can be evaluated either by manual inspection or by automated video tracking analysis. For example, Green et al. (2012) analyzed 24 screenshots made every 15 s and had blinded observers measure the average inter-fish distances per screenshot (Cachat et al. 2013; Green et al. 2012; Grossman et al. 2010; Riehl et al. 2011). Alternatively, the coordinates for each fish at each time point can be recorded and used to calculate various group parameters (Buske and Gerlai 2011). Automated video tracking methods enable the determination of individual fish trajectories and evaluation of parameters such as distance traveled, velocity, relative turning angle, polarization, and freezing duration (Maaswinkel et al. 2013a, b; Miller et al. 2013; Pérez-Escudero et al. 2014). Key measures of social cohesion include nearest neighbor distance, interindividual distance, distance from the center, and the shoaling index, a discrete variable which describes the number of fish within one body length of each other (Maaswinkel et al. 2013b). The calculation of excursions, as defined by a fish exceeding the mode of all nearest neighbor distances, is also possible (Miller et al. 2013).

Critical Assessment

While automatic tracking allows more precise and difficult measurements to be made, most automated group tracking systems frequently confuse individual fish, severely impairing statistics such as distance traveled (Maaswinkel et al. 2013a). However, recent advances in tracking are rapidly rectifying this problem (Dolado et al. 2014; Pérez-Escudero et al. 2014) and extending methods to larvae (Martineau and Mourrain 2013). To date, studies have examined the impact individual compounds including ethanol (Buske and Gerlai 2011; Maaswinkel et al. 2013b; Miller et al. 2013), ketamine (Riehl et al. 2011), and lysergic acid diethylamide (Grossman et al. 2010). In the future, advances in group tracking methods will permit high-throughput pharmacological testing of group behavior.

Modifications

An alternative measure of shoaling behavior is cluster analysis, in which fish are released into a tank (divided into distinct areas for analysis), recorded, and scored at time points for the number of fish per area (Parker et al. 2013).

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Associative Learning-Classical Conditioning

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Oncology Activity

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Overview of Assays for Anticancer Drug Development

The development of therapeutics to treat cancer is conceptually more difficult than for nonlife-threatening diseases for several reasons, including its complex pathophysiological nature, the molecular individuality of each tumor, and the robustness and predictability of preclinical models toward determining efficacy and safety. A major limitation to development of a "blockbuster" therapeutic strategy is the infinite combination of cellular and molecular perturbations and associated heterogeneity of causative genetic factors driving disease progression. Although challenging, the diversity of drug targets, coupled with the lethality of the disease, has encouraged studies of a vast array of approaches and opportunities for disease treatment over the years.

As a consequence of the panoply of putative cancer drug targets and expansion of strategies from traditional antiproliferative cytotoxic agents toward focused "molecular-targeted," cytostatic, or biotherapeutic approaches, a number of preclinical screening assays are necessitated within this therapeutic area. In contrast to many other diseases, which are restricted to only a few tissues and organs, preclinical efficacy and safety studies in cancer need to have versatility across organ systems and be able to accommodate evaluations of systemic disease dissemination and subsequent drug effects.

Preclinical *in vitro* and *in vivo* tumor models and methodologies are employed across the drug development cycle, from target validation to identification of hit and lead compounds, drug disposition and metabolism, drug safety, and eventually dose optimization and response biomarker analyses for clinical trial progression. Subsequently, there are several "stages" of preclinical pharmacological screening and treatment avenues associated with the malignant phenotype which require exploration to achieve these objectives.

This chapter details the wide range of current preclinical assay procedures, including their strengths and weaknesses, used within the preclinical setting for assessment of potential cancer therapeutic approaches.

Nonclinical *In Vitro* Assessment of Cellular Toxicity

General Considerations

One of the initial stages of pharmacological screening within cancer therapeutic drug programs is to determine the anticancer potential of a test therapeutic against cancer cells *in vitro*.

Many screening tests have been developed for *in vitro* evaluation of cytotoxicity over the years, ranging from dye exclusion techniques of vital stains such as trypan blue or neutral red (Cavanaugh et al. 1990), to bioluminescent detection of released intracellular proteases following loss of membrane integrity (Cho et al. 2008) or quantitation of cellular ATP levels (Crouch et al. 1993; Shukla et al. 2010), to the most widespread technique which involves metabolism of tetrazolium salts by intracellular organelles of viable cells (Mosmann 1983; Shoemaker 2006). At early stages in the drug discovery process, the putative therapeutics are screened against a panel of human cancer cell lines to evaluate both patterns of tumor selectivity and potency (Shoemaker 2006). To improve screening efficiency and increase study throughput, the US National Cancer Institute (NCI) developed a 60 human tumor cell line screen (NCI60) to fulfill this objective (Shoemaker 2006). In these screens, therapeutics with common mechanisms of action demonstrate similar profiles of growth inhibition (Holbeck et al. 2010). In addition to ascertaining the therapeutic potential and confirming proof of drug–target interaction, the same assays as used for efficacy can also be utilized to determine the effect of the therapeutic on non-cancer cell types, as either a secondary or off-target pharmacological effect.

In recent years, technological advances and improved understanding of cellular behavior have also allowed new screening approaches to be developed, which expand upon the established cellular and biochemical assays, and use biophysical properties such as cellular impedance to be exploited for evaluation of cell viability and

posttreatment survival (Khoshmanesh et al. 2011; Limame et al. 2012; Park et al. 2009; Roshan Moniri et al. 2015). Such methodologies, such as the xCELLigence analytical system, permit real-time and label-free evaluation of these effects and extend the level and depth of information that can be gained from these in vitro studies.

Assessment of Cellular Toxicity: MTT Assay

Purpose and Rationale

As previously detailed, there are several biochemical cellular viability assays which can be used to ascertain drug-induced cytotoxicity. The most popular of these assays, the MTT assay, measures the reduction of the yellow MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to dark purple formazan by succinate dehydrogenase present in mitochondria of metabolically active cells, with the amount of formazan created being directly proportional to the viable cell number. Importantly, no interference of the product with the substrate is observed with this assay, allowing it to be conducted in a 96-well plate format with spectrophotometric detection (Mosmann 1983; Shoemaker 2006).

Procedure

The method for the MTT assay developed by Mossmann et al. is described below (Mosmann 1983; Shoemaker 2006). Cells are seeded into 96-well cell culture plates at a density dependent upon the cell type and intended duration of therapeutic exposure, with the target of approximately 70 % cell confluence at time of analysis. In the case of primary cells, the plate may be pre-coated with a matrix protein to aid attachment, if necessary. Following a period of monolayer establishment, normally 24 h, cells are then exposed to a dose range of the therapeutic for the experimental duration, which can range from 1 to 72 h dependent upon the objective of the study. After this exposure period, the MTT solution is added to each well and the plate incubated at 37 °C for 4 h. The insoluble formazan (purple) is then solubilized in dimethyl sulfoxide (DMSO) and the well absorbance read at 540 nm. The color generated is stable for a few hours at room temperature but can be read directly following the crystal solubilization. Since the reduction of MTT varies from one cell type to another, standard curves showing the different metabolic activities must be determined for each cell type.

Evaluation

The MTT assay is selective for metabolically active cells, showing a direct correlation between formazan absorbance at 540 nm and viable cell number (Mosmann 1983; Shoemaker 2006). From this data, dose–response relationships can be determined, as well as the IC_{50} (dose causing 50 % reduction in cell number). In this context, an *in vitro* therapeutic index of a putative cancer therapeutic can be determined, through comparison of the IC_{50} of cancer cells versus that of normal cells. Ideally, a clear differential in cytotoxicity between the cancer and non-cancer cells is required, since agents anticipated to reach therapeutic plasma concentrations that are greater than or equal to the toxicity IC_{50} of the non-cancer cells have a high probability of dose-limiting *in vivo* toxicity (McKim 2010).

Modifications of the Method

One limitation of the MTT assay is the production of an insoluble formazan product, requiring solubilization in DMSO prior to spectrophotometric measurement. Consequently, water-soluble analogues of MTT have been developed, such as XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Scudiero et al. 1988). These water-soluble variants do not require solubilization in organic solvents prior to colorimetric analysis, allowing immediate reading of cell number in the culture well without the requirement for further processing. Relative to MTT, the use of XTT also offers the advantages of being suitable for automation and being more reproducible. On a negative note, a high background is obtained with XTT, and this substrate is not reduced as easily as MTT by mitochondrial enzymes, often requiring electron couplers such as phenazinemethosulfate to promote rapid electron transport to XTT. Consequently, convenience must be balanced with lack

of sensitivity when considering XTT versus MTT for this option.

The relative lack of stability of MTT and XTT and consequent requirement for preparation immediately prior to use, coupled with the fact that they have inherent toxicity, resulted in the development of several further improvements to this methodology, such as Alamar Blue (Hamid et al. 2004). Whereas MTT was colorimetric, Alamar Blue is converted by reduction in viable cells from a nonfluorescent substrate to a fluorescent pink nontoxic product (λ_{ex} 530 nm, λ_{em} 590 nm). Consequently, the Alamar Blue assay is both sensitive and highly reproducible (Hamid et al. 2004). The disadvantage to this method is the requirement for a fluorescence plate reader, rather than a spectrophotometer.

Critical Assessment of the Method

The use of cytotoxicity assays, such as the MTT assay, is widespread across the drug discovery and development sector and is a valuable tool for initial evaluation of a therapeutic index. In order to interpret this data and relate it to the *in vivo* situation, it is important that reference factors are also taken into consideration, including compound solubility and metabolic stability, and not just the value obtained from the MTT assay. Additionally, it is important to remember that the MTT assay and the majority of *in vitro* cytotoxicity assays do not fully recapitulate the *in vivo* situation, in that they often do not address protein binding, metabolite activation and stability, and systemic pharmacology. These limitations must always be borne in mind or resolved when attempting to directly extrapolate *in vitro* observations to the *in vivo* setting (McKim 2010).

In order to improve assessment of drug efficacy, it is strongly suggested that the MTT assay be integrated into a multiple parameter package of *in vitro* cytotoxicity assays (for instance, addition of a membrane integrity screen) (McKim 2010). This is especially important when appreciating that therapeutics can affect several subcellular targets, with varying exposure concentrations and times. The reliance specifically on the MTT assay (or any other single *in vitro* cytotoxicity assay) for predicting *in vivo* toxicity is beholding to the toxicity mechanism itself. For example, a therapeutic which inhibited mitochondrial reductases would provide erroneous results in the MTT assay (Hamid et al. 2004).

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In Vitro Assessment of Inhibitors of Cancer Cell Invasion

General Considerations

With an increasing focus in cancer drug discovery on the development of agents that can interfere specifically with the tumor dissemination process, there is a strong requirement for assays which can screen if novel compounds are effective in this space. The dissemination process can be broken down into the following steps: initial molecular signaling to a cancer cell to detach from its neighbor, degradation of the basement membrane, migration and invasion of the surrounding tissues, intravasation into blood and lymph vessels, travel through the circulation to distant sites, impact in a capillary, extravasation through the vessel wall, and migration through the surrounding tissue to form metastases.

Central and essential for malignancy is the initial gain of functional ability of the cancer cell to migrate away from the primary tumor mass toward a blood or lymphatic vessel, leading to dissemination to another site in the body where it can establish a secondary metastatic deposit. There are several *in vitro* assays used for drug screening which set out to capture and monitor the cell migration process (as reviewed in Kramer et al. (2013)). The majority of these methodologies evaluate two-dimensional migration within a cellular monolayer, including the cell exclusion assay (Fougerat et al. 2012), fence assay (Elbjeirami and West 2006), capillary chamber assay (Zicha et al. 1991), capillary tube assay (George and Vaughan 1962), colloidal particle assay (Takaishi et al. 1995), and more commonly the scratch wound healing assay (Liang et al. 2007) or transwell assay (Poujade et al. 2007).

Cell Scratch "Wound Healing" Assay

Purpose and Rationale

One of the most straightforward cell migration assays to use is the scratch or scratch wound healing assay (Liang et al. 2007; Lingen 2003; Menon et al. 2009), developed based on cell migration *in vivo* during the wound healing process (Menon et al. 2009; Roshan Moniri et al. 2015). In this situation, due to contact inhibition, proliferation is inhibited upon the cellular monolayer attaining confluence (Liang et al. 2007; Lingen 2003). However, scratching the monolayer creates a "wound" and offers the opportunity for these cells to migrate and "heal" the exposed area (Liang et al. 2007; Lingen 2003). The rate of migration of cells across the wound over time in the presence or absence of compound can then be monitored and any anti-migratory effect of the compound assessed.

Procedure

The standard wound scratch assay as performed in our laboratories (Al-Saraireh et al. 2013) is described here.

The assay is carried out in 6-well cell culture plates (9.5 cm^2 well surface area), with cell seeding densities optimized for each individual

cancer cell line in preliminary studies with determination of the number of cells required to form a confluent monolayer after 24 h incubation under standard conditions (37 °C, 5 % CO_2 humidified atmosphere).

Before starting the experiment, a straight line is drawn below each well so as to bisect the well area, which acts as a guide for image capture in the experiment. Cells are seeded in the 6-well plate at the desired concentration (usually between 2×10^5cells/ml and 1×10^6cells/ml) and allowed to adhere for 24 h under standard incubation conditions. After 24 h, a uniform single scratch of 1 cm in length, with a width of 800 μm, is made across the center of each well using a sterile pipette tip guided against the scale of a sterile ruler placed across the top of the well. Following washes in PBS (pH7.4) to remove any cell debris from the scratching process, 2 ml of fresh complete culture medium with or without the compound under test is added to each well. Images of the scratch area either side of the guideline are then taken using a microscope with ×10 objective lens (t = 0 images). The plates are then incubated under standard conditions for 16–48 h (dependent on the time it takes for each individual cell line to "heal" the wound when untreated), and images of the scratch area are again captured at the same position as the initial images (t = *z* images). For each compound under test, the assay is repeated in triplicate.

Evaluation

For each captured image, the scratch width (i.e., area remaining cell free) is measured at five equally spaced locations across the image, and the mean width of the two images is calculated. Migration as a percentage is then calculated as follows:

$$\text{Migration}(\%) = \left[\frac{S_0 - S_1}{S_1}\right] \times 100\,\%$$

where S_0 is the mean width of initial wound at t = 0 and S_1 is the mean width of wound at t = *z*. The migration of the treated scratches is then expressed as a percentage relative to the untreated scratches. We have demonstrated that statistically significant compound effects on migration can be seen where there is inhibition of wound healing of as little as 20 % compared to the untreated control (Al-Saraireh et al. 2013).

Modifications of the Method

In order to improve consistency in the size of the wound area, electrical wounding can be used instead of the standard mechanical wounding. When a pulse of high current is applied, cells in contact with an electric cell–substrate impedance sensing electrode will die, leaving a well-defined wound area (Lo et al. 1993). In addition to consistent creation of wound areas, this system has the advantage in that it can be used to quantitatively monitor healing across the wound by measuring changes in impedance due to the presence or absence of cells (Gorshkova et al. 2008).

Critical Assessment of the Method

While this assay is highly attractive to use as it is technically nondemanding, relatively quick to set up, and does not require any specialist equipment, there are a few drawbacks with performing and interpretation. Making the wound manually can often result in an uneven scratch, and therefore it is important that when conducting measurements that these are taken at the same distances away from the guideline for both the t = 0 and t = *z* images. Additionally, although this technique is highly capable of evaluating mechanistic processes involved with the migratory machinery, it does not directly translate to the processes related to the invasive activity of cancer cells, and complementary techniques are required (e.g., Boyden chamber assays, described below) for full interrogation of the malignant phenotype.

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Microwell Boyden Chamber for Cancer Cell Invasion

Purpose and Rationale

Cancer cell migration per se can be easily monitored using basic monolayer methodologies, such as the scratch "wound healing" assay described above (Albini et al. 1987; Albini and Noonan 2010). However, the scratch assay is not suitable for studying chemotaxis, with its utility focused primarily upon the processes involved in cellular movement, cell–cell or cell–matrix interactions, and therapeutic strategies to intervene and modify these activities. In this context, the most widely used method for studying cancer cell invasion is the Boyden chamber assay (Albini et al. 1987; Albini and Noonan 2010; Shaw 2005). This assay involves addition of cells to an upper chamber of a microwell insert from where they are encouraged to "move through" a cell permeable membrane and "invade" toward a chemoattractant located in the lower microwell chamber (Shaw 2005). Dependent upon the rationale for the study and the proposed molecular target of any investigated compound, the lower chamber can be adapted to include different chemoattractant stimuli: chemokinesis (random migration), haptotaxis (movement toward substratum-bound attractant), or chemotaxis (movement toward a soluble gradient, e.g., growth factor or chemokine) (Albini and Noonan 2010; Shaw 2005). After a defined period of time, the cells that have "invaded" through the membrane into the lower chamber can be easily quantified either colorimetrically in situ on the underside of the membrane via microscopy (Shaw 2005) or using radiolabeling, fluorescent, or biochemical

methods (Albini et al. 1987; Albini and Noonan 2010; Sasaki and Passaniti 1998).

The basic Boyden chamber assay described above is a stepwise advancement on the scratch assay but remains focused on cell migration. The movement, invasion, and subsequent metastatic dispersion of cancer cells from one tissue compartment to another are dependent upon the ability of the cells to move through the extracellular matrix (ECM) and "invade" through basement membranes into blood vessels and secondary tissues. Evaluation and investigation of the mechanistic basis of cancer metastasis are now feasible using *in vivo* models, as addressed later in this chapter. However, the use of these animal models for procedural interrogation of the process and screening of new therapeutic entities is particularly limited, labor intensive, and low throughput. Therefore, the development of *in vitro* assays to recapitulate and simulate the process of cancer cell invasion and screen for molecules which can retard and prevent such activities is highly important. Several assays have now been developed to address this objective involving introduction of a physical protein barrier into the assay (reviewed extensively in Albini and Noonan (2010)). Transwell assays (such as Boyden chamber) can be used for this purpose, by coating the upper surface of the cell-permeable membrane with an extracellular matrix (ECM) protein gel. The composition of this gel can be variable, with early studies using basement membranes extracted from tissues, including chicken chorioallantoic membrane, chick heart, and amnion (Albini and Noonan 2010; Hart and Fidler 1978; Mignatti et al. 1986). These ECM components have now been superseded by the use of Matrigel, a tumor extract rich in basement membrane proteins including laminin, type IV collagen, and heparin sulfate proteoglycans (Albini et al. 1987; Albini and Noonan 2010; Kleinman et al. 1986).

For comprehensive analyses, we suggest that cell migration and cell invasion should be conducted in parallel using the same chemoattractant to evaluate and discriminate between chemotaxis and chemoinvasion. The ratio between invaded and migrated cells in the absence of the matrix barrier defines the "invasive index," the relative number of "invasive" cells that crossed the barrier.

Procedure

Quantification of cell invasion and the effect of drugs therein are performed using the modified Boyden chamber assay, as described here.

There are a vast array of chemicals with utility as chemoattractants within the lower chamber of these assays, with single growth factors offering weaker targeted activity, and more complex attractants (e.g., conditioned media from diverse cell sources) representative of physiological "cocktails" active on several cell types (Albini and Noonan 2010). The choice of attractant is highly dependent upon the objective and focus of the study.

For the invasion assays, the concentration of Matrigel is important as it provides a differential barrier between invasive and noninvasive cells but should not be too high as to prevent all cellular invasion, with a dilution of one part Matrigel to two parts base medium recommended (Albini and Noonan 2010; Marsh et al. 2008; Shaw 2005). An important characteristic of Matrigel is that it is liquid at 4 °C and polymerizes at 37 °C to form a barrier similar in composition to basement membrane (Albini and Noonan 2010; Shaw 2005). The thickness of the Matrigel layer is also a determinant of cellular invasive transit time, thus a uniform coating is required for comparative analysis. There are now several of these Matrigel systems described, with the 96-well plate format the commonest. Furthermore, these plates are now widely available commercially as pre-coated products, ensuring uniformity of Matrigel coating and subsequent standardization of the assay (detailed in Albini and Noonan (2010)).

The "standard" invasion assay using transwell inserts and the modified Boyden chamber as described herein is as reported by Shaw (2005) and reviewed by Albini and Noonan (2010).

If Matrigel coating is required (unrequired for commercial systems), it is thawed at 4 °C and then kept on ice. The 96-well plate containing the transwell inserts is then also placed on ice to chill. An aliquot of Matrigel, diluted 1:2 in cold media, is added into the upper chamber and

allowed to equally cover the porous membrane. The plate is incubated at 37 °C for at least 30 min to allow the Matrigel to form a solid barrier.

The 96-well plate containing the transwell chamber is transferred to the cell culture cabinet, either at room temperature if without Matrigel (cell migration assay) or at 37 °C if containing Matrigel (cell invasion assay). A chemoattractant is added to the lower chamber (500 μL), containing either the attractant of choice or more commonly complete serum containing tissue culture medium. For negative control wells, serum-free media containing 0.1 % bovine serum albumin (BSA) are used.

Cells are plated in the upper chamber of quadruplicate wells in serum-free medium (total volume 200 μL) at the desired concentration (normally 5×10^4 cells). If evaluation of a drug or investigational compound is required, this is added to the chamber, with those acting upon the chemoattractant added to the lower compartment while those targeted at the cell or cell products applied to the upper chamber. The plate is then incubated at 37 °C for the desired time, normally 4–24 h for migration and 24–72 h for invasion, dependent upon cell characteristics and mechanistic basis of therapeutics under evaluation. The number cells migrated/invaded into the lower chamber (including those attached to the undersurface of the membrane) are then determined.

Evaluation

Cell migration and invasion (Matrigel) into the lower chamber is assessed either via trypsinization and counting with a hemocytometer or using colorimetric or fluorometric methods (Albini and Noonan 2010; Sasaki and Passaniti 1998; Shaw 2005).

Commonly crystal violet staining is used to quantify cell transit in migratory and invasion studies, for shorter-term assays (wherein cells remain attached to the membrane) as follows. The media (and Matrigel) is removed from the upper chamber, and using a cotton swab, non-invaded cells are removed from the upper portion of the transwell membrane. The transwell insert is then transferred to a new 96-well plate containing methanol for 30 min to fix the cells that have invaded onto the lower surface of the membrane. Methanol is removed from the wells, and crystal violet stain is added (200 μL) and incubated at room temperature for 15 min. The insert is then removed, washed in water to remove excess stain, and allowed to air dry. The number of migrated cells can then be counted using an inverted microscope and eyepiece reticule, making sure to discriminate between cells (pink/purple) and filter pores (colorless) (Shaw 2005).

A discrimination of dead and necrotic cells, indicative of compound cytotoxicity, and viable cells can also be analyzed by standard methodologies while still on the membrane, such as MTT (Sasaki and Passaniti 1998), or post-removal by methodologies such as flow cytometry (Albini and Noonan 2010).

An invasion index can be calculated and indicates the specific contribution of matrix degradation as follows:

$$\text{Invasion index} = \frac{\text{Invaded cells}}{\text{Migrated cells}} \times 100$$

Modifications of the Method

Matrigel used in the invasion assays is known to contain numerous growth factors entrapped within the matrix, particularly on the heparin sulfate side chains of proteoglycans. For analyses of the effects or interference of specific growth factors or ECM components, there are now several "factor-depleted" Matrigel variants available (Albini and Noonan 2010).

Critical Assessment of the Method

The metastatic process involves an intimate relationship between the tumor cells and other cell types within the microenvironment, including fibroblasts, macrophages, neutrophils, and endothelial cells (Fridlender et al. 2009; Hanahan and Weinberg 2011; Joyce and Pollard 2009; Kalluri and Zeisberg 2006), factors which are not recapitulated in the invasion assay limiting its full translational validity (Albini and Noonan 2010).

The Boyden chamber assays for both migration and invasion are limited by the fact that they do not give a fully accurate depiction of cells'

behavior throughout the process, being largely endpoint assays. The optimal timepoint for these assays is being determined empirically and applied generically across experiments (Albini and Noonan 2010; Roshan Moniri et al. 2015; Shaw 2005). The development of real-time label-free assays with improved potential for accuracy and applicability, particularly impedance-based technologies (e.g., xCELLigence; described later in this chapter), is offering opportunities to address these deficiencies (Limame et al. 2012; Roshan Moniri et al. 2015; Scrace et al. 2013).

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Real-Time *In Vitro* Analysis of Cell Survival and Behavior: Label-Free Technologies

General Considerations

Modulation of cancer cell survival, motility, and dissemination by anticancer therapeutics is known to involve a multitude of mechanistic pathways both chronologically and pathophysiologically. Consequently, in order to improve cancer treatment and therapeutic efficacy, it is now becoming important to understand these mechanisms and the transient nature of their interplay. Additionally, it is now appreciated that similar drugs may exert their maximal effects at

different posttreatment timepoints or induce a range of therapeutic responses in different tumor types. Using current conventional *in vitro* biochemical or proliferative endpoint assays (described in section "Nonclinical In Vitro Assessment of Cellular Toxicity"), such investigations are particularly difficult. Therefore, the development of assays and methodologies to monitor cellular behavior continuously and in real-time is vitally important to interrogate the pharmacological mechanism and cellular responses of putative cancer therapeutics.

Over the past decade, opportunities and advancements in biophysical methodologies, micromachining technologies, and cell culture techniques have led to the development of a range of label-free cell-based assays and devices (Ligler 2009; Ona and Shibata 2010; Roshan Moniri et al. 2015). In contrast to conventional endpoint determination of cellular viability or invasion, these assays involve dynamic monitoring of cellular status without addition of exogenous enzymes, addressed in terms of changes in extracellular proton (H^+) fluxes, oxygen levels, or mitochondrial membrane potentials ($\Delta\Psi_m$) (Ona and Shibata 2010) and changes in cell–environment interactions or cell morphologies (Ona and Shibata 2010; Roshan Moniri et al. 2015).

Label-free cell-based sensors to monitor extracellular pH have been used in several screening studies of anticancer drugs, utilizing a H^+-ion-sensitive microelectrode (ISM), H^+-ion-sensitive field effect transistor (ISFET), and light-addressable potentiometric sensor (LAPS) (Ona and Shibata 2010). The biological parameter underpinning these particular technologies relates to the increased acidification of the extracellular environment, as a consequence of the efflux of protons arising from increased respiration and glycolysis (Ona and Shibata 2010). Although inexpensive, ISMs have a slow response time and low reproducibility (Bobacka et al. 2008; Ona and Shibata 2010) and have the potential to cause cell damage (Franks et al. 2005; Ona and Shibata 2010). In contrast, ISFET and LAPS measure a field effect which is less stressful to the cells and more practical (Ona and Shibata 2010). However, in preclinical studies of cancer agents, ISFET has failed to determine the extracellular pH changes in many studies, and LAPS was comparable to the MTT assay, although no quantitative relationship was identified (Ekelund et al. 2000; Koebe et al. 2000; Ona and Shibata 2010).

Similar to the measurement of extracellular pH, measurement of extracellular O_2 consumption arising as a result of increased aerobic glycolysis of cancer cells has also been employed as a noninvasive methodology for determining dynamic screening of anticancer drugs (Bonnet et al. 2007; DeBerardinis et al. 2007; Gohil et al. 2010; Ona and Shibata 2010). Studies using amperometric microelectrodes or scanning electrochemical microscopy have not as yet demonstrated a quantitative relationship or good accordance with results from the endpoint MTT or other such assays (Andreescu et al. 2004; Ona and Shibata 2010; Torisawa et al. 2005).

The approaches for anticancer drug sensitivity screening which have demonstrated most success and positive application within the label-free technologies have been those which monitor changes in cell morphology and cellular interactions with other cells or their environment (Ona and Shibata 2010; Roshan Moniri et al. 2015). Sensors for these methodologies include acoustic wave sensors, such as quartz crystal microbalance (QCM) and magnetoelastic resonance sensors (MRS), and surface plasmon wave sensors, such as surface plasmon resonance (SPR) and resonant waveguide grating (RWG) (Ona and Shibata 2010). The technological approach demonstrating greatest success and versatility within this area is electrochemical detection of cellular growth and behavior, using open circuit potentials (OCP), electrochemical impedance spectroscopy (EIS), and microelectrode arrays (MEA) (Adlam et al. 2008; Arias et al. 2010; Ke et al. 2011; Kustermann et al. 2013; Martinez-Serra et al. 2014; Ona and Shibata 2010; Roshan Moniri et al. 2015; Xing et al. 2005). Whereas OCP occurs as an electrochemical potential at an electrode against a reference electrode immersed in the cell culture solution (Ona and Shibata 2010; Woolley et al. 2002), EIS and MEA measure electrochemical changes occurring across

electrodes in the flow of an alternating current which is detected as either resistance or capacitance, referred to as impedance (Ke et al. 2011; Lee et al. 2009; Ona and Shibata 2010; Roshan Moniri et al. 2015). The impedance is sensed as the flow block of an electric current by cells which act as excellent electrical insulators at low signal frequencies. Consequently, cellular viability and pathophysiological functions can be monitored using this technology, allowing parameters such as cell density, morphology, adhesion, and growth to be determined (Ke et al. 2011; Lee et al. 2009; Ona and Shibata 2010; Roshan Moniri et al. 2015). The close relationship of results obtained from MEAs with those obtained from endpoint viability assays strongly supports the utility and predictability of these assays, with the added benefit of being able to dynamically monitor cell responses noninvasively in real time (Kho et al. 2015; Kustermann et al. 2013; Martinez-Serra et al. 2014; Roshan Moniri et al. 2015).

Purpose and Rationale

The impedance-based technology with widest published use for anticancer drug evaluation is the xCELLigence real-time analyzer system (ACEA Biosciences), which allows continuous label-free and dynamic monitoring of cellular phenotypic changes (Kho et al. 2015; Kustermann et al. 2013; Martinez-Serra et al. 2014; Roshan Moniri et al. 2015). This system uses custom-designed cell culture plates which have a high-density interdigitated gold electrode array upon which the cells adhere and grow (Ke et al. 2011; Roshan Moniri et al. 2015). When cells are seeded onto the plate, the signal increases as cells adhere to the bottom of the well because of impedance of the current between the electrodes. However, the impedance value is actually an indication of net cellular (focal) adhesion rather than cell number specifically, which additionally provides information relating to cellular morphology and adhesion. The attachment and detachment of cells from the surface, and consequently the interdigitated electrodes, change the impedance value within the well. This impedance value is converted within the integrated software of the system, using complex-defined algorithms (Atienza et al. 2006; Ona and Shibata 2010; Solly et al. 2004) and calculated as a cell index (CI) value. There is a direct correlation between the number of cells attaching and the CI value and vice versa (Ke et al. 2011; Roshan Moniri et al. 2015). Therefore, an increase in CI over time indicates an increase in cell number, indicative of cell proliferation, until the well is fully confluent whereby the CI value plateaus (Ke et al. 2011; Roshan Moniri et al. 2015). Conversely, a decrease in the CI value indicates cytotoxicity of an added agent or overgrowth and over-confluence of the well (Ke et al. 2011; Roshan Moniri et al. 2015). This monitoring of cell growth and viability is shown to correlate very well with cell number calculated by endpoint biochemical assays (Ke et al. 2011; Kho et al. 2015).

A major advantage of the continuous noninvasive monitoring afforded by the xCELLigence system is the ability to schedule drug addition or cellular analyses based upon behavioral and empirical data of the cells rather than an arbitrary or fixed timepoint based on generalized cell response or convenience. Similarly, real-time kinetic cell viability measurements following exposure to putative anticancer agents provide temporal information as to when maximal effect is induced, which can be predictive of a therapeutic mechanism of action (Abassi et al. 2009; Ke et al. 2011, 2015; Kustermann et al. 2013).

Procedure

The assay is performed using specialized xCELLigence "E-plates" designed for the particular version of the system you are using, either a 96- or 16-well format (Kho et al. 2015; Roshan Moniri et al. 2015). For growth characterization and proliferation rate studies, an initial titration of different cell concentrations is required to identify the optimal seeding density, with a range between 40×10^3 and 2.5×10^3 cells/well suggested. For cytotoxicity assays, cells are seeded at a density (determined by prior titration), whereby a dosing window and duration are available prior to full

confluence and resulting cellular compromise and decline are observed (Kho et al. 2015). For instance, an optimal density for a 72 h drug exposure study would be one in which the CI value increased to maximal value at a timepoint after the final assay point (i.e., 72–96 h).

In terms of the experimental procedure, the xCELLigence analyzer is housed within the 37 °C incubator to permit continuous studies of cell behavior. Before beginning the study, the experimental details, recording schedule, and the layout of the cell densities/treatments are added to the appropriate page of the xCELLigence software. In general, the schedule includes a minimum of three steps: background and baseline measurement, cell adhesion and monitoring step, and compound activity monitoring step (for drug evaluation). An additional cell recovery step may also be added to monitor cell behavior after removal of the drug, a step proving important for monitoring cytostatic versus cytotoxic therapeutic mechanisms. However, variations in the interval measurements required and the duration of the experiment are dependent upon the actual study, cell identity, and treatment regimen under evaluation.

Prior to cell addition, it is important that a background reading and baseline impedance of each well of the plate are determined to ensure that all electrode connections are functioning within acceptable limits and to provide a reference point for calculation of the CI during the experiment. Practically, to each well of the E-plate, cell culture media (50 μl) is added and the plate returned to the incubator for 20 min to equilibrate at 37 °C. The plate is then inserted into the xCELLigence analyzer cradle and the background impedance signal measured.

When the background measurement is completed, the machine is paused and the E-plate returned to the tissue culture hood. The final volume of medium in each well is then adjusted to 150 μl by adding 100 μl medium containing the appropriate density of cells (i.e., 2.5×10^4 cells/ml for a final well density of 2500 cells), the plate returned to the analyzer cradle within the incubator, and the machine restarted. As a general method for the cell monitoring step, readings every 5 min over the initial 2 h of the study (to evaluate cell adhesion) and then every 15 min over the period prior to drug addition (normally 24 h) are ideal in our experience.

After completion of the cell monitoring step and confirmation of appropriate cell growth, the test anticancer compounds can be added to the cells on the plate (normally 24 h after cell seeding). Compounds are added to the plate at a concentration tenfold greater than the final required concentration, with a final solvent concentration of 0.1 % DMSO. In general, duplicate or triplicate wells are used for each treatment for statistical analysis. In practical terms, the xCELLigence system is paused and the E-plate returned to the tissue culture hood, and then 16.7 μl of each compound (at 10x concentration) is added to the respective well (final well volume 167 μl). The E-plate is returned to the cradle of the instrument and the activity monitoring step initiated. The length and frequency of this monitoring phase are dependent upon the proposed therapeutic mechanism and cell type being evaluated. In general, monitoring every 15 min for 48–96 h is enough for most cytotoxic compounds, but for compounds expected to mediate cellular morphological changes or immediate cytotoxic effects, more frequent monitoring (e.g., every minute) for the first 1–2 h is recommended (Ke et al. 2011; Roshan Moniri et al. 2015).

If a cell recovery step is necessitated, the plate is removed from the instrument cradle, the media removed (containing drug) and replaced with fresh media, and the plate returned to the instrument. The duration of this step can be variable but is generally 24–48 h with a frequency of 30 min intervals.

An added advantage of this technique is that cells can subsequently be evaluated by an endpoint assay or subject to molecular analyses (e.g., MTT assay or protein analysis), as a consequence of the xCELLigence impedance-based assay being both label-free and noninvasive.

Evaluation

Data collection is integral and automated through the xCELLigence software, which runs the

monitoring procedure. In principle, the impedance value determined by the xCELLigence system is converted using complex algorithms to an arbitrary value termed cell index (CI) (Atienza et al. 2006; Ona and Shibata 2010; Solly et al. 2004). A direct correlation exists between the CI value and the number of attached cells (Ke et al. 2011; Roshan Moniri et al. 2015).

Integral to the software is functionality to plot the data, both in real time and in terms of treatment groupings. Data is displayed as either cell index (CI), normalized-CI, or delta-CI. The CI value displays the actual readings from each well (inclusive of background subtraction via the algorithm), whereas normalized-CI relates to comparison of the value to a particular timepoint (normally reading previous to drug treatment) which is then set as 1.0 by the software. The delta-CI value represents the change in values from a designated timepoint. The software also includes functionality to allow more advanced data analysis to be conducted, such as calculation of IC_{50} values and cellular doubling times, etc.

The inbuilt monitoring aspects of the xCELLigence software also give an indication of errors or faults detected with the equipment and study, such as connection issues, disruptions to power supply, and errors with specific wells within the plate, offering quality control of the technology throughout the study.

Modifications of the Method

The xCELLigence impedance-based technology provides a real-time measurement of cellular adhesion and morphology, which allows a diverse and ever expanding range of *in vitro* cellular studies and behaviors to be monitored in oncology research, beyond that of straightforward cytotoxicity evaluations. Importantly for analysis of oncology therapeutics, this technology has the capability of discriminating between a cytotoxic and a cytostatic therapeutic effect through its real-time functionality of cellular kinetics, permitting interrogative assessment of molecular mechanisms and drug safety liabilities (Kustermann et al. 2013). Similarly, it is now clearly demonstrated that therapeutics triggering different cellular reactions and mechanisms, e.g., kinase inhibition, interruption of cellular cytoskeletal infrastructure, and microenvironmental interactions, produce distinctive impedance profiles and graphical "shapes" and thus the technology has significant value for interpretation of therapeutic mechanisms (Abassi et al. 2009; Ke et al. 2010, 2015).

The ability to apply extracellular matrix proteins to the E-plates has extended the scope of studies that can be conducted in the oncology therapy field using this technology. It is well established that *in vitro* growth of particular cell types, including primary cancer cells and malignant cell lines, is positively affected by inclusion of a growth matrix (e.g., collagen or fibronectin), resulting in enhanced cellular adhesion and development of the correct cellular phenotype and polarity. This approach has been used to optimize and create an *in vitro* endothelial cell model of the blood–brain barrier, with clear utility for evaluation of both pharmacological and oncological studies (Kho et al. 2015). Similarly, pre-coating of the wells with fibronectin has been shown to induce the adhesion of many leukemic/lymphoma cell types, which otherwise are non-adherent, permitting cell growth, viability, and drug responsiveness of these cell types to be robustly monitored (Martinez-Serra et al. 2014).

A defining characteristic of malignancy is cellular invasion and metastasis, and there are now several methodologies and approaches developed to study these factors *in vitro*, as described in earlier sections of this chapter (Hulkower and Herber 2011; Limame et al. 2012). The commonest methodology used for determining cellular invasive capability still remains the filter-based Boyden chamber, involving chemotactic-driven cell migration through a porous membrane and colorimetric-based endpoint detection (Boyden 1962; Hulkower and Herber 2011; Limame et al. 2012). However, real-time noninvasive detection of migration is now possible using the xCELLigence technology (Limame et al. 2012; Roshan Moniri et al. 2015). Determination of invasive capacity is achieved through use of a modified E-plate in which the

sensor electrodes are integrated into the lower face of a membrane suspended within the well (termed a CIM plate) rather than the conventional arrangement at the bottom surface of the wells within the E-plate (Limame et al. 2012; Roshan Moniri et al. 2015). Furthermore, the ability to add extracellular matrix proteins (such as Matrigel) to the upper surface of the CIM plate, with the cells seeded on top, allows the invasive phenotype and drug effects thereon to be assessed additionally to their migratory and proliferative capacity (Limame et al. 2012). This approach was qualified by the demonstration that primary human breast cancer cells but not normal non-tumorigenic breast epithelial cells possess a strong basal migratory and TGF-β_1-inducible invasive potential (Mandel et al. 2013) and the identification of biomarkers and putative therapeutic targets in early-stage colorectal cancer (Dunne et al. 2014).

An upcoming important and critical area in development of oncology therapeutics is their effect upon other body systems, primarily the cardiac system, a major cause of attrition in oncology drug development and a significant concern for longer-term health of patients (Todaro et al. 2013). A major step forward building upon the fundamental impedance technology is the xCELLigence Cardio system, a higher specification system with capability of simultaneously measuring viability and rhythmic beating of cardiomyocytes and therefore prediction of cardiotoxicity and drug-induced arrhythmias (Lamore et al. 2013; Wang et al. 2013). It is anticipated that this approach and area will become a major factor for oncology therapeutics over the coming decade.

Critical Assessment of the Method

As a relatively new technology, there are many applications which have been demonstrated and many more which have yet to be developed for this system. The potential and the disadvantages therein have yet to be fully identified and realized. The lack of familiarity and widespread use of these technologies is currently the issue, which can only be resolved by time.

The main reported criticism of this technology is the inappropriate or inexperienced interpretation of the data obtained. This primarily occurs as a consequence of analyses of "normalized" data rather than the raw CI data, which fails to account for variations in cellular adhesion and can artificially "hide" variations that may exist intra- and inter-experimentally (Kho et al. 2015). The solution proposed being to assure data interpretation is valid and methodologies justified.

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Nonclinical *In Vivo* Pharmacological Assessment of Oncology Therapeutics

General Considerations

While *in vitro* evaluations can give significantly useful information about drug–target interactivity and toxicity against specific cell types, what they cannot account for is the complexity of the cancer tissue, comprising not only the cancer cells but the supporting environment of stromal fibroblasts, endothelial cells, and extracellular matrix which all interact to support tumor growth. In addition, the majority of agents to treat cancer are administered systemically, and drug metabolism and pharmacokinetics and off-target toxic effects need also to be evaluated.

This complexity can be best captured through evaluation of the test compound in living animal models. Mice are the least sentient species of mammal that are most frequently used for cancer therapy studies which are acceptable to regulatory bodies worldwide. This is due to their genetic similarity to humans and relatively small size, lifespan, large and frequent litter sizes, and cost. As compounds progress toward clinical evaluation, then testing is carried out in another animal model, usually rat, dog, pig, or monkey.

In performing animal testing, one must be aware of one's ethical obligations to using animals and also the 3Rs guidelines (Russell et al. 1959). The first "R," "Replacement," challenges the experimenter to assess if there are nonanimal alternatives that can be used to provide the outcome of a particular test. This could be through the use of human tissue, computer modeling, or cell-free or cell-based assays, e.g., the use of human keratinocyte–melanoma co-cultures (Dekker et al. 2000). If there are no alternatives to using animals, then the next challenge is "Reduction." Through statistical power analysis the number of animals to be used can be reduced to the minimum that will permit robust statistical significance, e.g., the use of repeat micro-sampling for DMPK analysis (Chapman et al. 2014). The final "R," "Refinement," concerns the welfare of the animals undergoing procedure in the experiment, with refinement strategies including provision of environmental enrichment in the living area, the appropriate use of analgesia and anesthesia and minimizing the length of exposure to procedure, e.g., surgical methodology and analgesia for vasectomy (Miller et al. 2012). Based on the 3Rs' philosophy, several local and national organizations have published guidelines relating to good

practice for *in vivo* experimentation including the Federation of European Laboratory Animal Science Associations (Guillen 2012) and the UK National Institute for Cancer Research (Workman et al. 2010).

Taking the considerations covered above into account, the following section sets out to cover the assays one would use for the evaluation of experimental agents against the majority of cancer types where a clear primary focus of tumor growth is observed, followed by formation of secondary metastatic deposits. Models will be described in order of consideration in the screening process, with an increase in sophistication and mimicry of the clinical situation. Noninvasive imaging methods will also be described which assist in reducing the numbers of animals used in a particular experiment, allowing tracking of tumor growth in the same animals throughout the study.

The majority of these models are unsuited to hematological malignancies and these malignancies will be considered separately at the end of the chapter.

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Determination of Nonclinical Maximum Tolerable Dose

Procedure

Initial evaluation of maximum tolerated dose for the investigative agent should be conducted in mice. The maximum tolerated dose (MTD), in respect of cancer pharmaceuticals, is being defined as the highest dose of a therapeutic in which the clinical condition of the animal is maintained and which is independent of the mechanism of drug action (Workman et al. 2010). These tolerability studies must be conducted using the same strain as will be employed for efficacy studies, which in the majority of cases will be an immunocompromised model such as nude or SCID mice. With a few exceptions, these mice must also be non-tumor bearing.

Although these studies are not tightly prescriptive in terms of their design, the consensus across several groups and organizations is the use of two mice per dose level with a doubling dose-escalation or dose-halving de-escalation design (Workman et al. 2010). Such a study design agrees with the suggested guidelines for nonclinical rodent studies and fulfills a major aim of the S9 guidelines to reduce and refine experimental animal numbers (ICH 2010; Russell et al. 1959; Workman et al. 2010).

An important criterion when conducting these tolerability studies is to characterize the efficacy and more importantly the safety profile within the therapeutic dose range of the therapeutic, using a schedule which reflects that intended for the clinic, particularly the duration of treatment. The suggested dose schedules, as outlined in the ICH S9 guidelines, are identified in Table 1.

In the majority of cases for a new therapeutic, whereby the ideal dosing regimen is not yet identified, the starting point for MTD assessment should be set as a single dosing event. In these cases, escalation to a second dose level requires a minimal interval of 24 h to allow acute toxic or safety concerns to be identified. For therapeutics intended to be delivered chronically or over a longer time period (e.g., daily for a month, orally or intravenously), the interval is suggested to increase to at least 5 days (Workman et al. 2010).

Table 1 Example schedules for oncology therapeutics to support clinical trials (Adapted from ICH S9 guidelines (ICH 2010)). These schedules are intended for evaluation of conventional cancer therapeutics and should be modified accordingly for biopharmaceuticals with extended pharmacodynamics or half-lives and potential for immunogenicity. In addition, the schedules outlined in Table 1 do not identify recovery periods which should be incorporated into study design

Clinical schedule	Nonclinical study schedule
Single dose every 3 weeks	Single dose
Daily for 3 days, repeated every 3 weeks	Daily for 3 days
Daily for 5 days, repeated every 3 weeks	Daily for 5 days
Daily for 7 days, repeat alternating weeks	Daily for 7 days, repeat week 3
Single dose every 2 weeks	Repeat dose, 14 days apart
Single weekly dose for 3 weeks, 1 week rest	Once a week for 3 weeks
Two doses per week	Two doses per week for 4 weeks
Continuous daily	Daily for 28 days
Continuous weekly	Once a week for 4–5 doses

Additional to the dosing route and treatment schedule, toleration is also dependent upon the composition of the therapeutic solution being administered. Delivery of the therapeutic as an acidic or basic solution commonly is associated with complications due to the irritant nature of the vehicle, limiting the maximum deliverable dose. Therefore, the vehicle in which the therapeutic is delivered should be as close to physiological pH as possible, for example, 0.9 % saline or 5 % dextrose/saline. In cases where the therapeutic is poorly soluble in aqueous solution, a vehicle comprising low concentration organic solvent, not exceeding 10 % (or 5 ml kg^{-1}) of the total administered volume, could be used. A commonly used example is dimethyl sulfoxide (DMSO). Chemical composition of the therapeutic may also necessitate inclusion of a detergent (such as Tween), an emulsifier, or a solubilizer, although these should not exceed 20 % of the total delivered volume. Additionally, cyclodextrin to a maximum of 45 % total volume is also an accepted inclusion for drug administration of poorly soluble therapeutics, although the use of this compound necessitates that the animals be rehydrated 2–4 h posttreatment (Workman intraperitoneal dosing in mice of 10 ml kg^{-1} and intravenous dosing in mice of 5 ml kg^{-1} (equating to 200 μl and 100 μl for a 20 g mouse, respectively) (Workman et al. 2010).

The volume of therapeutic solution to be administered is also of consideration for assessment of tolerability, with the smallest possible volume being used in order to keep solvent-related issues and subsequent toxicity. Although no definitive guidelines are available, the following suggestions are widely accepted as standard; oral/

Evaluation

Clinical signs of toxicity observed during these nonclinical rodent tolerability and efficacy studies may manifest as either a behavioral, physiological, or pathological change. In agreement with the guidelines for the welfare and use of animals in cancer research, all mice should be visually examined twice daily as a minimum (Workman et al. 2010). In addition to the legislative requirements for evaluation of animal physical and psychological distress or suffering, mice should be weighed frequently (i.e., daily) and their behavior observed. A median body weight loss of 15–20 % of the weight prior to treatment requires cessation of dosing and monitoring of the mouse for weight recovery. Mice with weight loss exceeding 20 % should be sacrificed.

The MTD for the therapeutic is the highest dose of a drug in which the clinical condition of the experimental animal is maintained, and no significant drug-induced adverse effects are observed. Specifically, a dose of the drug causing weight loss less than 15 % of the weight before treatment, not causing death due to toxicity events, or causing significant detrimental changes in animal well-being within 1 week after administration. The dosing schedule should reflect that used for the proposed efficacy studies.

Modifications of the Method

The conventional methodology for evaluation of the MTD is to use healthy non-tumor-bearing immunocompromised mice, which in the vast majority of cases is applicable. Despite this, the

advent of "targeted molecular therapeutics" will in some cases require the additional use of tumor-bearing or immunocompetent mice for tolerability studies. Studies requiring immunocompetent mice include those involving biotechnologically derived peptides and antibodies. The use of tumor-bearing mice is potentially necessary for determination of the tolerability of tumor-activated prodrugs, although an alternative strategy may be to evaluate the active moiety in the "standard" tolerability model.

As opposed to evaluation of therapeutic tolerability in non-tumor-bearing mice (or for non-oncological conditions), care should be adopted when identifying apparent drug-induced adverse effects in tumor-bearing models as these may be a result of tumor presence or response rather than a true drug safety concern. Although such events are rare and avoidable by appropriately designed experimental studies, they should not be overlooked and should be appraised within all studies as standard. For example, dermal distension and ulceration and cachexia can both be the result of tumor presence.

Critical Assessment of the Method

The MTD in mice provides the foundation for calculating the first in human starting dose for the therapeutic, indicating the significant importance of this approach. The downside to this methodology is that the majority of clinical signs indicative of toxicity do not provide mechanistic information, but simply identify the presence of a toxicity either warranting further investigation or in severe cases animal termination.

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Hollow Fiber Assay

Purpose and Rationale

The hollow fiber assay (HFA) was developed by the NCI in the mid-1990s as a way of expediting the screening process as its *in vitro* screens were generating more compounds than it could cope with in its standard xenograft screens (Hollingshead et al. 1995).

In the assay, cells are enclosed in biocompatible polyvinylidene (PVDF) hollow fibers which have a 500 kDa pore size which allows free movement of compounds and macromolecules in and out of the fiber, but restricts cell movement. The fibers are transplanted intraperitoneally (i.p.) and subcutaneously (s.c.) and then treatment administered i.p., thus any activity seen in cells from the subcutaneous sites will give an indication that the compound is not metabolized to an inactive form in the circulation. At the end of the experiment, the fibers are removed and the cells evaluated for survival compared to untreated controls using a modified MTT assay. The assay length is usually 7 days, and due to the fibers being available in different colors, three different cell lines can be analyzed in one mouse at the same time. Therefore, compared to standard xenograft testing, several more compounds can be assessed using fewer animals in a shorter time, thus fulfilling 3Rs criteria of Reduction and Refinement.

The assay has been broadly adopted for screening compounds whose mechanism of action results in cytotoxicity (Bueno Perez et al. 2013; Mi et al. 2009; Park et al. 2014). Given the ability to readily retrieve a pure cancer cell population, which can be analyzed by many standard molecular biological or immunodetection techniques, the assay has also been adapted to evaluate for pharmacodynamic endpoints for compounds where the mechanism of action is less straightforward (Suggitt et al. 2006; Veiga et al. 2011).

Procedure

The standard hollow fiber assay protocol as developed and used by the NCI (Hollingshead et al. 1995) and as performed in our laboratories (Shnyder et al. 2006) is described below.

Freshly harvested tumor cells are resuspended in complete cell culture medium and loaded into 15 cm lengths of sterilized color-coded PVDF Spectra/Por hollow fibers (Spectrum Medical Inc, Houston, TX, USA) at the required cell density (cell line-dependent and usually ranges from 10^6 to 10^7 cells/ml). The ends of the fiber are clamped and heat-sealed using heated forceps. The fiber length is then cut up into 1.5 cm lengths which are again heat-sealed at both ends and then transferred to 6-well plates containing complete culture medium for an overnight incubation under standard cell culture conditions before implantation the next day.

Immunodeficient mice of either sex aged 8–12 weeks, maintained under standard husbandry conditions, are used in these experiments. All procedures were carried out under a UK Home Office Project License, following UK National Cancer Research Institute Guidelines for the Welfare of Animals (Workman et al. 2010). Under general inhalation anesthesia (2 % isoflurane), the mouse is positioned lying on its back, and a small incision is made through the skin and abdominal wall in the left ventral flank, below the spleen. One loaded hollow fiber for each of up to three cell lines is transplanted intraperitoneally, then the incision in the abdominal wall closed and sealed with surgical glue, and the skin incision closed with surgical glue and stapled. The mouse is then repositioned lying on its front, and a small incision is made in the skin of the lower right dorsal flank, and each of up to three loaded hollow fibers is placed subcutaneously so that they lie parallel with the mouse's spine. The skin incision is closed with surgical glue and analgesia administered. The mouse is then monitored during recovery from the anesthesia. This is designated day 0.

Mice are housed in groups of 5 or 6, and treated groups receive treatment on days 3, 4, 5, and 6 post-implantation. Treatment is usually as i.p. administration, but this can alter if an alternative administration route is already in place for a compound. On day 7 post-implantation, mice are sacrificed by cervical dislocation and the hollow fibers excised, wiped to remove excess host tissue, and placed in 6-well plates in 2 ml of complete cell culture medium containing 20 % FCS. The plates are then placed on an orbital shaking platform for 30 min. 1 ml of a prewarmed 1 mg/ml MTT solution in complete cell culture medium containing 20 % FCS is then added to each well and the plate incubated under standard culture conditions at 37 °C for 4 h. The solution is then aspirated from each well and 2 ml of a 2.5 % protamine sulfate solution in saline added. The plate is then stored for a minimum of 24 h in the dark at 4 °C. When ready to complete processing, the solution is aspirated and 2 ml of fresh protamine solution added and the plate incubated for a further 2–4 h in the dark at 4 °C. The fibers are then singularly transferred to a well in a 24-well plate, and each fiber is cut in half with scissors so that it can lie flat along the bottom of the well. The plate is left overnight in a safety cabinet in a darkened room to give the fibers time to dry out. Once the fibers have dried out, 300 μl of DMSO is added to each well and the plate is placed on an orbital shaking platform at 100 rpm for 4 h to dissolve and extract the formazan formed in the MTT exposure.

Evaluation

One hundred and ninety microliters of solution is then transferred to a well of a 96-well microplate, and the plate is read at 540 nm on a microplate spectrophotometer. Percentage cell survival is calculated by comparing mean absorbances obtained for the treated group hollow fibers implanted at either the subcutaneous or i.p. site to the mean obtained for the untreated or vehicle-treated control group at the same site. The NCI have determined a scoring method for evaluating efficacy in the hollow fiber assay as follows (Hollingshead et al. 1995). A $\geq$50 % reduction in survival for the treated group compared to the control group is considered as a positive result which is given a score of 2. The scores are totaled for a given compound for all assay runs, and if a compound has either a sum score $\geq$21 %, or a subcutaneous score $\geq$8 % of the total possible score, or 100 % cell kill is seen

at either site, then the compound is considered for further *in vivo* evaluation.

Modifications of the Method

As a further refinement of the method, we have reduced the fiber length to 1.5 cm from 2 cm in order to reduce inflammation due to the serrated seals of the fibers at the subcutaneous site rubbing against the skin when the animal moves around.

We have also demonstrated that the assay can be performed in normal as well as immunodeficient mice without any loss of effect for a 7-day assay (Shnyder et al. 2006) which has significant cost benefits due to the reduction in husbandry requirements for immunocompetent as opposed to immunodeficient animals.

As stated above, there have been reports where the endpoint assessment has been changed from analysis of cytotoxicity to more sophisticated readouts such as cell cycle inhibition (Hall et al. 2000), regulation of gene expression (Krauthauser et al. 2001), DNA cross-linking (Suggitt et al. 2006), and DNA strand breaks (Veiga et al. 2011); however in many cases, this presents a technical challenge due to the relatively small number of cells that can be obtained from a single fiber (typically $<10^5$ cells).

Critical Assessment of the Method

While the assay is a robust early-stage *in vivo* method for demonstrating proof-of-principle activity, due to its nature, it cannot model the complexities of the disease or reproduce the interactions between tumor and host cells. Therefore, it is clear that agents demonstrating promise in this assay will need to be progressed to more complex screening models before entering clinical trials.

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Subcutaneous Transplantation Models

Purpose and Rationale

As has been discussed in the previous section, while the hollow fiber assay is a good intermediate between *in vitro* and *in vivo* assessment, the tumor cells are grown in isolation from the host cells, and so there is no opportunity to assess the

influence of tumor structure on compound effects. This is observed in models where tumor cells are transplanted at assorted sites in the host body and the tumor grows with the support of the host cell infrastructure. The most common site for transplantation of tumor fragments or inoculation of cancer cells is subcutaneously in the dorsal or ventral flanks of mice. This is a relatively (compared to the techniques described in the following sections) minimally invasive transplantation technique, and as the tumor is grown just under the skin in the flanks of the mouse, it is easily accessible for measuring. Thus any delay in tumor growth compared to untreated control animals caused by administration of the agent under investigation can be directly assessed. In addition, as compounds are administered systemically any drug metabolism and off-target toxicity can be readily evaluated. At some stage in the preclinical development of all clinically used anticancer agents, subcutaneous transplantation models will have been used, from established cytotoxic agents such as doxorubicin (Balkwill and Moodie 1984) and 5-fluorouracil (Nowak et al. 1978) to more modern targeted agents such as bevacizumab (Damiano et al. 2007) and afatinib (Li et al. 2008).

The most readily used of the subcutaneous transplantation models is the human tumor xenograft model, where immortalized human tumor cell lines are transplanted into immunodeficient mice, and which is described in detail in this chapter. Other alternatives are covered below in the Modifications subsection.

Procedure

The standard subcutaneous human tumor xenograft transplantation methodology as performed in our laboratories (Gill et al. 2014; Harvey et al. 2011; Pors et al. 2011; Shnyder et al. 2007) is described below. All procedures were carried out under a UK Home Office Project License, following UK National Cancer Research Institute Guidelines for the Welfare of Animals (Workman et al. 2010). The tumor fragments used are sourced from subcutaneously implanted tumors (either sourced from previous liquid nitrogen stocks or from cultured cells) grown in either flank of two donor animals. Donor tumors are usually harvested when they have a volume of ~500 mm^3 to ensure that there is a good source of viable tumor tissue to transplant.

Immunodeficient mice of either sex aged 8–12 weeks, maintained under standard husbandry conditions, are used in these experiments. Under brief general inhalation anesthesia (2 % isoflurane), 2–3 mm^3 tumor fragments are implanted subcutaneously in the lower right and/or left ventral flank using a 3 mm diameter trocar. Following implantation, mice are monitored frequently, and once palpable and measurable tumors are evident (approximate tumor volume of 32 mm^3), the mice are allocated into groups of 8–10 (depending on tumor take rate) by restricted randomization. Treatment, which can be single or multiple intravenous (i.v.), intraperitoneal (i.p.), oral (p.o.), intramuscular (i.m.), or subcutaneous (s.c.) administrations, then commences on what is designated day 0 of the experiment. Treatment is usually administered at the previously determined MTD and fractions of that. In addition, a reference untreated control group which usually receives drug solvent, plus a positive control agent clinically used for treating the tumor xenograft type are included.

Mice are examined daily for any deleterious effects, and up to five times per week, bodyweight and tumor volume are recorded. Tumor volume is measured using calipers, with the longest diameter of the tumor, b, and a reading taken perpendicular to this, a, recorded. The experiment is terminated when either the longest tumor diameter reaches 17 mm in length or earlier if deleterious effects are seen.

Evaluation

Tumor volumes for day x are calculated using the formula: $(a^2 \times b)/2$, and relative tumor volumes (RTV) for each animal are normalized to the respective volume on day 0. From plots of experiment time versus RTV for each animal, the time for the tumor to double from its size on day 0 (RTV2) is calculated, and using the RTV2 value, Mann–Whitney U tests are performed to determine the statistical significance of any differences in growth rate between control and treated groups and between the different compounds.

Novel compounds are considered worthy of further evaluation if they have a $p < 0.01$ significance and demonstrate efficacy at least equal to the positive control treatment.

Modifications of the Method

Established human tumor cell lines grow as xenografts with considerable variability in "standard" immunodeficient mice, and there are modifications such as co-inoculation of cells with Matrigel to provide a ready matrix for the cells to grow in (Tomlinson et al. 2009), or the use of severe combined immunodeficient (SCID) mice strains, where the increased lack of immune response aids in facilitating tumor growth (Rader et al. 2013).

While the example given here uses established human cancer cell lines, there are two other sources of cancer tissue that can be utilized in the subcutaneous transplantation model: allograft transplantation of mouse cancer cell lines and early passage tumor material obtained from human patients (patient-derived xenografts; PDX).

In the allograft approach, immortalized cancer cell lines derived from spontaneous or induced tumor tissues in a specific immunocompetent mouse strain are transplanted subcutaneously back into mice of the same strain. This model has the advantage of the host environment in having the same immune profile as the transplanted tumor, and therefore, as well as there being less chance of graft rejection, there is no compromise in the interactions between the tumor cells and supporting cells in the tumor environment. The main disadvantage of this model is that the therapeutic target will be a murine mimic of the human one; therefore it is important to take this into account when evaluating efficacy (Shnyder et al. 2003).

PDX tumors are becoming more commonly used (Burgenske et al. 2014; Rosfjord et al. 2014). The rationale for this is that there is a tendency for immortalized tumor cell lines to mutate over time such that the phenotype is no longer the same as the source clinical material. This can pose a major problem in evaluating molecular-targeted therapies where expression of the target has been mutated out or expression levels decreased over time. Therefore, if early passage PDX are utilized, target expression should be the same or similar to the clinical situation and therefore give a truer reflection of clinical efficacy (Rosfjord et al. 2014). A major disadvantage with these models is the poor take rate, with it sometimes taking several patient samples to be tried before a successful PDX is established (Rosfjord et al. 2014; Siolas and Hannon 2013).

Critical Assessment of the Method

While it has been demonstrated that the subcutaneous model has merit as a predictor of clinical efficacy, certainly for cytotoxic agents (Suggitt and Bibby 2005), there are several disadvantages to using this model when evaluating more "sophisticated" targeted therapeutic strategies.

As the tumor cells or fragments are transplanted subcutaneously, the tumor cells are not maintained in their natural environment and thus not exposed to the same interactions with normal epithelial, stromal, and immune cells which are important for the process of tumor dissemination and metastasis (Hanahan and Weinberg 2011). Consequently, it is very rare for tumors transplanted subcutaneously to disseminate and metastasize, and hence these models are of little use in evaluating molecules which work by controlling tumor dissemination.

Another concern is alteration of genotype in tumor models repeatedly passaged *in vitro* or transplanted *in vivo*, such that target molecule expression seen in the clinical situation is no longer observed in established allografts or xenografts (Talmadge et al. 2007).

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Creation of Orthotopic Cancer Models

Purpose and Rationale

As stated above, while subcutaneous models can give a good picture of the effects of systemically administered therapy on the control of a primary tumor, the tumor is not being grown at its clinically relevant site, and thus the interactions with the host cell infrastructure do not mimic the clinical situation. In addition, these tumors rarely metastasize, and thus subcutaneous models are not ideal for assessing the effects of antimetastatic agents.

More relevant to the clinical situation is to orthotopically transplant the tumor pieces, or

inoculate the cells, directly at the site where the tumor was derived from. This often involves invasive surgical procedures and a more intense regimen of post-operative husbandry than required for subcutaneous models but has the advantage of use for evaluation of antimetastatic agents, as tumors implanted orthotopically more readily disseminate. What is important though is to confirm that the patterns of dissemination mimic the clinical situation. There are several established models for orthotopic transplantation covering a broad range of solid tumor types. The tumor sample used can be either allogenic or xenogenic, and PDX tissue has also been utilized in orthotopic models (Rosfjord et al. 2014; Sicklick et al. 2014).

Procedure

The example of an orthotopic model for prostate cancer described here was developed by Pavese et al. (2013), using the human prostate cancer cell line PC-3M, which was originally derived from liver metastases following intrasplenic injection of the PC-3 parent line (Kozlowski et al. 1984). This model demonstrates all the criteria one would expect to see for an orthotopic model at any site: local growth in the clinical primary site, plus dissemination to clinically relevant secondary sites including the lungs, lymph nodes, and bone.

As with all cell transplantation techniques, handling of cells from harvesting to injection is crucial to ensure that cells remain healthy, and cells should be prepared as near to transplantation as is practically possible. PC-3M cells are harvested and resuspended in sterile saline and concentration adjusted to 2.5×10^5 cells/20 μl and transferred to a 0.5 ml syringe fitted with a permanent 28½ G needle for each mouse, making sure that no air enters the needle along with the cell suspension. The syringe is then stored on ice until injection.

Immunodeficient male mice aged 6–8 weeks and a bodyweight of 19–21 g are used and maintained under standard husbandry conditions. Following administration of pre-surgical analgesia, mice are fully anesthetized by inhalation of isoflurane, with anesthesia maintained using a nose cone throughout the surgical procedure. Following disinfection of the lower abdomen, a low midline abdominal incision is made of around 3–4 mm in length. Using forceps, the bladder is lifted to reveal the ventral lobe of the prostate (any fat covering the prostate can be gently moved away from the prostate with a sterile cotton swab). 20 μl of the cell suspension is then injected into the ventral lobe, ensuring that there is no leakage of solution and that there is a blistering of the tissue caused by the introduction of the cell suspension. The bladder is then replaced, the muscle layer closed using surgical glue, and the skin incision closed with surgical glue and stapled.

Postsurgical analgesia is administered and animal weight and food consumption monitored frequently, along with palpation to check for the presence of tumors until the experiment is terminated.

As soon as primary tumors can be detected by palpation (~4–5 mm length when measured with calipers) at usually 4 weeks following transplantation, therapy, which can be single or multiple intravenous, intraperitoneal, oral, intramuscular, or subcutaneous administrations, can commence. Mice are then monitored until either bodyweight loss is above 15 % of starting weight or the primary tumor has grown to $\geq$1.5 cm in length, when the animal is terminated and tissues taken for analysis as follows. Through cardiac puncture under terminal anesthesia, whole blood is taken and placed into tubes containing the anticoagulant sodium citrate. The lungs, femurs, and regional lymph nodes are removed and placed into 10 % neutral buffered formalin and then processed and embedded into paraffin wax for histopathological analysis according to local protocols. The primary prostate tumor is removed, ensuring that any surrounding normal tissues are not also taken. The tumor is weighed and the tumor volume calculated from measurements taken with calipers. The tumor is then either formalin fixed as for other tissues or snap frozen in liquid nitrogen if required for other analyses.

Evaluation

Growth of the primary tumor is evaluated as per subcutaneous models, described previously. In order to assess for the presence of metastatic deposits in secondary organs and tissue, serial

sections are taken every 50–200 μm through the formalin-fixed and paraffin-embedded (FFPE) blocks of tissue, and they are either stained with hematoxylin and eosin or immunolabeled with a prostate-specific marker antibody and the number of metastatic deposits present counted. The number of deposits seen in the drug-treated groups is then compared with the untreated control group.

In order to assess for the presence of circulating tumor cells (CTCs), the blood sample is centrifuged at 800 × g for 5 min, the plasma removed, and the red blood cells lysed using a lysis buffer such as ACK Lysis Buffer (Life Technologies, Paisley, UK). After a further centrifugation at 800 × g for 5 min, the cells are resuspended in cell culture medium plus antibiotics and incubated under standard conditions for up to 10 days, frequently checking the flask for the presence of adherent cells. These can then be harvested and immunolabeled as above to confirm that they are CTCs.

Modifications of the Method

Rates of tumor establishment could be improved by further decreasing the innate immunity of the host animal, for instance, using SCID mice or chimeric humanized mice where the mouse's blood is reconstituted with human hematopoietic stem cells or lymphocytes (Legrand et al. 2006; Talmadge et al. 2007).

As discussed later in this chapter, noninvasive imaging techniques can also greatly enhance analysis of drug efficacy. In addition, some of these techniques can be adopted to improve cell transplantation and localized therapeutic delivery, for example, ultrasound-guided injection of cells into the adrenal gland or para-adrenal space to establish a neuroblastoma orthotopic model (Teitz et al. 2011) or between the muscle wall and mucosa to establish a bladder orthotopic model (Jager et al. 2013).

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Site-Specific Cancer Models

Purpose and Rationale

These models are useful if there is a need to monitor therapeutic effectiveness at a specific metastatic site, but models are not readily available, or where there is a very low take rate of the primary tumor disseminating to the specific site, which may be useful for evaluating aspects of tumor biology, but will not have the statistical power for evaluating novel therapeutics.

As with orthotopic models, these models range in complexity and invasiveness from a simple

intravenous tail vein inoculation of cells to establish tumor deposits in the lungs (Liang et al. 2012), through intrasplenic inoculation to establish liver metastases (Fraedrich et al. 2012), intratibial injection of cells to establish bone deposits (Eswaraka et al. 2014), through to intracranial inoculation (Nakayama et al. 2013).

Procedure

The example of a site-specific model for evaluating the effects of therapy on liver metastatic deposits described here is based on the methodology of Fraedrich et al. (2012). In their study, the gastroenteropancreatic neuroendocrine cell line BON1 (Grabowski et al. 2008) is seeded into the liver through intrasplenic injection as follows.

Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice aged 6–10 weeks of either sex are used and maintained under standard husbandry conditions for SCID mice. BON1 cells are harvested as close as possible to inoculation, resuspended in fresh cell culture medium with low (2 %) serum at 5×10^5 cells/200 μl, transferred to a 1 ml syringe attached to a 27 G needle, and kept on ice until use.

Following administration of pre-surgical analgesia, mice are fully anesthetized by inhalation of isoflurane, with anesthesia maintained using a nose cone throughout the surgical procedure. Following disinfection of the left flank, a 5 mm incision is made in the abdominal wall, and the spleen is exposed. 200 μl of the cell suspension is then injected into the spleen, ensuring that there is no leakage of solution and that there is a blistering of the tissue caused by the introduction of the cell suspension. The splenic pole is then ligated to secure hemostasis, and 10 min following cell injection, a splenectomy is performed to prevent tumor growth in the spleen. The abdominal wall is closed using surgical glue, and the skin incision is closed with surgical glue and stapled. Postsurgical analgesia is administered, and animals are then monitored daily for the presence of any deleterious effects. From preliminary studies, liver metastases are clearly detectable after 2 weeks, and at this point, mice are restrictedly randomized into groups of 12 and treatment commences (designated day 0).

Evaluation

On days 0, 4, 8, and 12, 3 mice from treatment and untreated control groups are sacrificed and their livers processed for formalin-fixed paraffin wax-embedded (FFPE) blocks. Serial sections are taken every 200 μm and stained with hematoxylin and eosin. The relative area covered by metastatic deposits in a set of the same number of sections for each group is evaluated using image analysis software, and the amount of deposit in the treated groups is compared to the control. Statistical significance is assessed using a two-tailed Student t test, with a compound considered to be active if there is a significant reduction in metastatic burden of $p < 0.05$.

Critical Assessment of the Method

The same issues are applicable as detailed for orthotopic models, described previously.

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Models of Spontaneous Tumors

Purpose and Rationale

With orthotopic models, there is still a degree of removal from the clinical situation in that cells foreign to the host, whether they be allogenic or xenogenic, are still being used. More realistic in terms of the biology of tumor growth and dissemination would be for the tumor to evolve spontaneously from the hosts' own cells and tissues. In this way, it should be possible to learn about the biology of tumor development and gives the opportunity to treat precancerous as well as cancerous cells.

As with humans, animals also develop tumors spontaneously over time. Spontaneous tumors were among the earliest models used (Levin 1912) and also served as a source of tumor cells for establishing immortalized cell lines. However this is not a very reliable methodology to use when performing drug efficacy studies due to the unpredictability of tumor formation in a cohort of animals, and these models fell out of use.

The advances in transgenic technology over the past few decades have rekindled interest in using spontaneous models. With transgenic models, there is a confidence that most animals will consistently develop tumors at the same site.

Procedure

The example of a spontaneous tumor model for therapeutic assessment which this procedure is based on is a study by Gatti et al. (2014), looking at histone deacetylase inhibitor coadministered with temozolomide in a spontaneous ret transgenic mouse melanoma model (Dragani et al. 2000).

C57BL/6 mice which carry the human ret transgene with control under the mouse metallothionein-I promoter are used and maintained under standard conditions for transgenic mice. The transgenic strain is established by cross-breeding N3/RET male mice with C57BL/6 female mice. The resulting F1 mice are then genotyped in order to select experimental animals which express the ret transgene, with melanoma known to be detectable microscopically in these animals when 6 weeks old. At 7 weeks, these animals are placed by restricted randomization into experimental groups, with 12 animals per group, and treatment commences. Animals are monitored frequently for the presence of macroscopic tumor deposits, and once animals demonstrate macroscopic evidence of disease, e.g., presence of subcutaneous tumors or exophthalmos, then the animals are sacrificed, and tumor, bone marrow, spleen, and lymph nodes are removed and snap frozen in liquid nitrogen for further genomic and proteomic analysis dependent upon the type of therapy. In untreated control animals, disease is evident within 44 days of the commencement of treatment.

Evaluation

Kaplan Meier plots of percentage disease-free mice over time are constructed, and statistical significance is assessed using a log-rank test, with a $p < 0.05$ considered as a significant effect.

Modifications of the Method

In order to improve heterogeneity, a hybrid model approach can be taken where non-germline genetically engineered modela (GEM) are utilized (Das Thakur et al. 2014; Heyer et al. 2010). In these models, chimeric mice are established which can develop spontaneous tumors in a specific tissue. These are formed by implantation of genetically engineered embryonal stem cells (ESC) into preimplantation embryos leading to chimeras which carry a mixture of the ESC which can be activated due to a tissue-specific switch and wild-type host cells. Due to this mixture, tumorigenesis occurs in a more natural manner mimicking the clinical situation (Heyer et al. 2010).

Critical Assessment of the Method

While GEM often develop tumors at the clinically relevant site, which gives an advantage

over some of the models described above, there are still a number of limitations to using these models for drug screening. Considerable time and expense are required to initially establish a GEM, and due to vagaries of breeding, there is unpredictability to the numbers of animals from a brood which can develop tumors and which can be included in a screening study. In addition, there is concern regarding the genotype of the cells forming the tumor, as the artificial promoters which are used in the development of the models can alter cell function and reduce the heterogeneity of the formed tumors, and this can consequently affect tumor progression and metastasis, such that dissemination patterns do not mimic the clinical situation (Talmadge et al. 2007).

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Development of Hematopoietic Tumor Models

Purpose and Rationale

Unlike most solid tumors where there is a definitive primary seeding site with tumor growth, which can be readily mimicked and measured as described elsewhere in this chapter, with leukemia's, the pattern of clinical disease with a bone marrow niche and passage through extramedullary organs presents a different challenge when developing *in vivo* therapeutic screening models. In addition, while a large number of solid tumors can readily grow in standard immunodeficient mice, where T cells are lacking, the presence of intact NK and B cells has prevented the establishment in these mice of hematopoietic tumors which mimic the clinical disease (as reviewed in Jacoby et al. (2014)). The development of the SCID mouse (Bosma et al. 1983), where B cells are lacking as well as T cells, advanced the field such that an orthotopic bone marrow transplantation model was now possible, giving a more clinically relevant disease model (Shultz et al. 2007). However, due to the continued presence of NK cells as well as innate immunity, these models often require conditioning with sublethal irradiation to enable engraftment of the hematopoietic cells or repeated *in vivo* passaging of the cells (Jacoby et al. 2014). Subsequent developments lead to the NOD/SCID (Greiner et al. 1995) and NOS/SCID/γC^{-} (NSG) strains, where IL-2γ knockout mice were crossed with NOD/SCID mice, resulting in innate immunity defects and lack of NK cells, and this has improved engraftment rate and relevancy (Ito et al. 2002).

Procedure

The example of a therapeutic leukemia model described here is based on the methodology of Cocco et al. (2010). Here, the 697 pre-B acute lymphoblastic leukemia cell line (Findley et al. 1982) was systemically inoculated into NOD/SCID mice, and therapy with human recombinant interleukin-23 was assessed.

Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Greiner et al. 1995) aged 4–6 weeks of either sex are used and maintained under standard husbandry

conditions for SCID mice. 697 cells are harvested as close as possible to inoculation, resuspended in fresh cell culture medium with low (2 %) serum at 5×10^6 cells/100 μl, transferred to a 1 ml syringe attached to a 27 G needle, and kept on ice until use. With the animal restrained, 100 μl of cell suspension is slowly injected into the tail vein and the mouse placed back in its cage. Eight hours following cell inoculation, therapy can commence. Blood samples can be collected every 2 or 3 days by puncture of the tail vein with a 27 G needle and collection of 10 μl of blood into a heparinized tube to help monitor the disease progress. In addition, animals are monitored daily for the presence of any deleterious effects, and once these are evident, then the animal is sacrificed (typically ~14 days following tumor inoculation for untreated control animals), and following a collection of a terminal blood sample, necropsy is performed to check for the presence of tumor masses, which are measured using calipers, and bone marrow is harvested from the femur.

Evaluation

Peripheral blood and bone marrow cells are co-labeled with antibodies which identify leukemic cells and markers to confirm treatment effects, and then the cells are analyzed using flow cytometry. A value of $p < 0.01$ is considered statistically significant when comparing treated and control samples when analyzed using a Mann–Whitney *U* test.

Any tumor deposits are measured and comparisons of tumor volume between treated and untreated groups evaluated, with a $p < 0.01$ value considered therapeutically effective when analyzed using a Mann–Whitney *U* test. Serial sections are taken and the number of proliferating and apoptotic cells and number of tumor microvessels assessed following immunohistochemical staining. Treatment is considered statistically significant if $p < 0.05$ compared to the control using the Student *t*-test.

Modifications of the Method

Where there are requirements for an intact immune system in which to evaluate the effects of immunomodulation, then syngeneic transgenic mouse models (Kindler et al. 2008; Zhang et al. 2010) or bone marrow transduction/transplantation models (Chiang et al. 2008; Li et al. 1999) which model genetic changes in leukemia can be utilized. Given the increased duration afforded by the development of disease in these models, they are also better suited for evaluation of therapeutic intervention over the course of the disease compared to xenograft models.

Critical Assessment of the Method

While human xenograft transplantation models such as those described above are highly advantageous as a systemic disease model for evaluating drug therapy, the deficiencies in the host immune system inevitably mean that the role of the immune system in the development of leukemia cannot be monitored, which is a major issue in a disease where immunomodulation is a key component of treatment (Mantripragada et al. 2014). In addition, these models have a short lifespan which can be measured in weeks rather than months, which limits their use for monitoring more sophisticated therapeutic regimens (Jacoby et al. 2014).

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Noninvasive Evaluation of Cancer Models

Purpose and Rationale

While effects of therapy can be readily assessed in subcutaneous tumor models using direct caliper measurement, assessment provides a greater challenge if the primary tumor is growing in a deep-lying organ and is not readily palpable and accessible to caliper measurement and also, it is not possible to track tumor dissemination to other, sometimes unpredictable sites. The only way involves sacrifice of the animal and removal and ex vivo analysis of its tissues and organs for the presence of tumor, and this provides only the snapshot of tumor burden at the time of sacrifice. A more complete picture of the effects of a treatment could be obtained if the tumor could be monitored in real time in the same animal, and this is possible using noninvasive imaging techniques, where the tumor burden can be measured at several times during the experiment.

There are several modalities which can be used for noninvasive imaging, including ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission tomography (SPECT), and optical imaging which can be either bioluminescence (BLI) or fluorescence, of which near-infrared fluorescence (NIRF) imaging is the most sensitive form. All have their own advantages to justify inclusion in a drug development program, but they also all have disadvantages which would preclude selecting just one modality alone (Beckmann et al. 2007; O'Farrell et al. 2013). The advantages and disadvantages of these modalities within the preclinical setting are summarized in Table 2.

Procedure

The most commonly described and readily accessible noninvasive imaging modality used in preclinical evaluation is optical imaging, and a bioluminescent neuroblastoma metastasis model (Daudigeos-Dubus et al. 2014) is used as the basis for the procedure described herein.

A luciferase expressing subline of the IGR-N91 neuroblastoma cell line (Ferrandis et al. 1994) is established by transfecting a PcDNA3-Luc2 vector into the cells using lipofectamine and selecting for neomycin resistance. Success of transfection can be monitored by Western blotting using a luciferase antibody and evaluation of bioluminescence by addition of 150 μg/ml luciferin to cultures of the

Table 2 Advantages and disadvantages of imaging technologies

Modality	Spatial resolution	Advantages	Disadvantages	Use in drug evaluation
Ultrasound	50 μm	Good resolution Anatomical and functional information Fast and portable Relatively inexpensive	Imaging through bone or lungs difficult	(Ingram et al. 2013; Ma et al. 2015)
CT	50–100 μm	High-sensitivity anatomical imaging Provides 3-D image	Limited functional information Poor soft tissue contrast Expensive	(Badea et al. 2012; Fushiki et al. 2009)
MRI	80–100 μm	Good resolution Good soft tissue contrast Anatomical and functional information	Low sensitivity Relatively long acquisition time Expensive	(Garcia et al. 2014; Whisenant et al. 2014)
PET	1–2 mm	Can provide biochemical information High sensitivity Provide 3-D image Can monitor changes in tumor metabolism and drug biodistribution	Requires radio-nucleotide facilities close by due to short half-lives of isotopes Limited anatomical information Expensive	(Couture et al. 2014; Qin et al. 2015)
SPECT	1–2 mm	Can detect multiple probes simultaneously in contrast to PET Radioisotopes have longer half-lives	Lower sensitivity compared to PET Expensive	(Jardim-Perassi et al. 2014; Larimer and Deutscher 2014)
BLI	1–10 mm	High sensitivity Broad applicability Can simultaneously monitor several molecular events Relatively inexpensive	Requires genetic manipulation of cells Provides limited anatomical information Reduced sensitivity with imaging depth	(Chai et al. 2013; Daudigeos-Dubus et al. 2014)
NIRF	1–3 mm	Increased penetration compared to normal fluorescent probes Relatively inexpensive	Requires genetic manipulation of cells Reduced sensitivity with imaging depth	(Agollah et al. 2014; Li et al. 2013)

IGR-N91-luc cells in 96-well plates and imaging using an IVIS50 optical imaging system (Perkin Elmer) to detect light emission above background levels, 2 min following addition of the luciferin substrate.

Immunodeficient mice aged 6–8 weeks of either sex are used and maintained under standard husbandry conditions. IGR-N91-luc cells are harvested as close as possible to inoculation, resuspended in fresh cell culture medium at 1×10^6 cells/100 μl, transferred to a 1 ml syringe attached to a 27G needle, and maintained on ice until use. With the animal restrained, 100 μl of cell suspension is slowly injected into the tail vein and the mouse placed back in its cage. Animals are then monitored daily for the presence of any deleterious effects and at regular intervals of 2–7 days are optically imaged to assess for presence of tumor deposits as follows.

Mice are injected i.p. with 150 mg/kg of D-luciferin and immediately anesthetized with 2.5 % isoflurane. The mice are placed in the imaging chamber of the IVIS 50 system with anesthesia maintained and at the previously determined time of peak bioluminescence (usually 5–15 min and varies for cell lines and site of main deposits) are imaged for between 10 s and 2 min depending upon the signal strength, with the mice placed subsequently on its back, front, and left side in order to optimize imaging of different anatomical structures.

Once bioluminescence signals are clearly established above baseline (approximately 3–4 weeks for the IGR-N91-luc cell model), the mice are placed in groups of five. Treatment with the test compounds then commences, and further imaging is carried out.

Mice are terminated when deleterious effects due to the tumor burden, such as excessive panting or weight loss are evident. Organs are removed and placed in a Petri dish and immediately imaged to check for the presence of bioluminescent signal and then processed for histological analyses of metastases as previously described for orthotopic and site-specific tumors respectively.

Evaluation

Bioluminescence intensity is measured as photons per second, and total mean signal measured for the treated groups is compared to the untreated control group for each day of imaging. Statistical significance is assessed using a nonparametric Mann–Whitney test, with a compound considered to be active if there is a significant reduction in tumor burden of $p < 0.01$.

Critical Assessment of the Method

In optical imaging for evaluation of therapeutic response, the target cells have to be genetically modified in order that they can be visualized. Thus with current technologies, this methodology is not translatable for use in the clinic. Unlike other more sophisticated imaging techniques, optical imaging does not give any information about the depth or shape of the structure which is the source of the detected photons (O'Farrell et al. 2013).

Modifications of the Method

Alternative methods which could be utilized instead of bioluminescence imaging are described and detailed in the descriptive table above (Table 2). Increasingly though noninvasive imaging using combined modalities is being adopted (O'Farrell et al. 2013), with BLI being combined with MRI and/or PET and CT (Downey et al. 2014; Mittra et al. 2013; Thorsen et al. 2013), in order to give structural and spatial information as to the location of the light emission.

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Regulatory Guidance for Clinical Development of Oncology Therapeutics

Purpose and Rationale

Despite high levels of activity and innovation in discovery and development of cancer therapeutics, the approval rate of small molecule drugs is still particularly low and reportedly less than 10 % (Ocana et al. 2011; Walker and Newell 2009; Westhouse 2010). The major reasons cited for limited clinical progression of cancer medicines are often poor selection of preclinical model systems, limited appreciation of drug pharmacokinetics or pharmacodynamics, and poor translation of preclinical observations to the clinical situation (Westhouse 2010; Wittenburg and Gustafson 2011).

To address these deficiencies and uncertainties regarding development of oncology therapeutics, the ICH S9 guidelines were developed (ICH 2010; Rosenfeldt et al. 2010; Senderowicz 2010). These guidelines identify the type and timing of nonclinical studies required for progression of new oncology therapeutic entities into the clinic, with the specific goals of facilitating and accelerating drug development, concomitantly refining and reducing animal use, and protecting patients from unnecessary side effects (ICH 2010). Specifically, nonclinical evaluation studies to identify and interpret the toxicological profile and pharmacological properties of cancer therapeutics, with the ultimate aim of establishing a

safe starting dose for Phase I clinical trial (ICH 2010).

Throughout the ICH S9 guidelines, there is a clear statement of intent to develop therapeutics with a greater tolerance for risk in terms of cancer treatment, exemplified by modification, elimination, or deferral of "standard" nonclinical studies (relative to ICH S6 for biotherapeutics and ICH M3 for non-oncological conditions) and use of nonclinical doses associated with adverse effects as the starting point for first in human clinical trial therapeutic starting dose.

Procedure

The following section identifies the specific study requirements pertaining to efficacy and safety monitoring of oncology therapeutics as identified in the ICH S9 guidelines (ICH 2010; Senderowicz 2010). This section identifies the main principles relating to preclinical studies, with an overview of the requirement for particular studies indicated in Table 3.

Drug Efficacy: Preliminary characterization of mechanism of action, resistance, and schedule dependencies in addition to cancer therapeutic efficacy is required as part of the nonclinical package. This should utilize appropriate models based on the target and mechanism of action of the therapeutic, but does not necessarily require evaluation in the same tumor type as intended for the clinic. Pharmacodynamic (primary and secondary) studies should also be evaluated, as appropriate.

***Drug Pharmacokinetics*:** A limited set of kinetic parameters identified in nonclinical studies, with the objective of facilitating clinical dose-escalation, are required for commencement of clinical studies. These parameters include peak plasma levels, exposure levels and AUC, and therapeutic half-life. Studies in support of drug absorption, distribution, metabolism, and excretion, conducted nonclinically, are required for later stage clinical trials and should be progressed in parallel to clinical development.

***Drug Safety Evaluation Studies*:** The screening of cancer pharmaceuticals will usually require evaluation in two species (rodent and non-rodent), as indicated in the ICH S9 guidelines (ICH 2010).

Table 3 Overview of the requirement for particular studies for oncology therapeutics, as described in the ICH S9 guidelines (ICH 2010)

ICH safety guideline	Requirements for evaluation of oncology therapeutics
S1A–S1C Carcinogenicity studies	Not required for marketing
S2 Genotoxicity studies	Not required for clinical trials, but data may be required for marketing
S3A Toxicokinetic study **S3B** Pharmacokinetic study	S3A: Conducted as appropriate S3B: Partial requirements prior to clinical trial, with further data required for marketing
S4 Chronic toxicity testing	Shorter duration studies (3 months) are generally sufficient for marketing
	Assessment of potential to recover from toxicity is required
S5 Reproductive toxicology studies	No requirement prior to clinical trials, but partial requirements required for marketing
S6 Safety evaluation of biotechnology-derived products	Only required for biotechnology-derived therapeutics
S7A Safety pharmacology for human pharmaceuticals **S7B** The nonclinical evaluation of the potential for delayed ventricular repolarization	Requirement for marketing, but not specifically prior to clinical trial
S8 Immunotoxicity studies	General toxicology studies are considered sufficient, unless immunomodulatory pharmaceutical
S10 Photosafety evaluation	Only required if potential risk is identified
M3 Nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals	Requirement dependent upon route of administration, proposed mechanism of action

An exception to this being the development of genotoxic therapeutics, targeting rapidly dividing cells, in which safety studies in one rodent species may suffice (ICH 2010; Newell 2005). If the

anticancer therapeutic is biotechnology-derived, then ICH S6 guidelines are referred with respect to the choice and number of species to be screened (Food and Drug Administration HHS 2012; Lewis and Cavagnaro 2010). If the initial short-term toxicity studies are comparable between rodent and non-rodent, then longer-term studies can be conducted using only the rodent, on the condition that the rodent is a relevant species.

An assessment of vital organ function, including cardiovascular, respiratory, and central nervous systems, is required prior to initiation of clinical trials. In contrast to other therapeutic classes, stand-alone safety pharmacology studies are not required to support clinical studies in patients with late-stage cancer or advanced disease. Safety parameters should be integrated and included in general toxicology studies.

Critical Assessment of the Method

The ICH S9 guidelines are prescriptive in terms of the "core" efficacy and safety studies required but are generic in terms of the "mechanistic class" of drug being investigated. For instance, the same overarching guidance is applied to DNA synthesis inhibitory cytotoxic agents as to the newer cytostatic molecular-targeted agents. The guidance is also limited with regard to the requirements for recovery and reversibility data relating to posttreatment toxicities. Consequently, guidance and type of studies required have to be applied on a case-by-case basis for regulatory submissions, based largely on experienced interpretation of the guidelines and the therapeutic under investigation (Ponce 2011; Senderowicz 2010; Wittenburg and Gustafson 2011).

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Stem Cells

Benedikt Müller and Suzanne Kadereit

Contents

Introduction

In the last 20 years, costs of pharmaceuticals have grown faster than other parts of health care, with estimated costs for drugs reaching market ranging around 800 million USD (DiMasi et al. 2003). Accumulating data and numerous failed clinical trials indicates that drug development and evaluation in animal models, animal-derived in vitro test systems, and standard human cell lines are not reliably predictive for human efficacy and safety (Perel et al. 2007; Leist and Hartung 2013). Failed drugs increase the general costs of development, particularly if failing in very late stages of development, as clinical trial testing is extremely expensive with phase III trials generating around 40 % of overall drug development costs (Roy 2012). Failure in the clinical trial stage can moreover result in unnecessary suffering due to unanticipated severe side effects, even resulting in life-threatening situations such as in the case of testing of TGN1412 (Attarwala 2010). Also, failures such as in the case of Vioxx, where cardiac toxicity emerged only after large-scale use in humans, are difficult to predict with currently available methodologies (Karha and Topol 2004).

Animal models and in vitro systems with animal cells have failed repeatedly over the years, mainly due to differences in species-specific metabolism, neurogenesis, cellular signaling, and immune function (Dohnal et al. 2014; Hengstler et al. 1999; Lazarov and Marr 2013; Mestas and Hughes 2004). Hepatotoxicity is the

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most frequent reason for withdrawal of already approved drug, followed by hematologic and cardiac toxicity (Fung et al. 2001). One of the most prominent examples of failure of animal models to predict human toxicity was thalidomide, which passed testing in animal testing without any indication of teratogenicity, resulting in thousands of humans born with severe developmental defects in extremity growth (Kim and Scialli 2011). The only currently validated in vitro test with stem cells, the embryonic stem cell test (EST), also failed to detect the teratogenicity of thalidomide in its original format (zur Nieden et al. 2004). The validated EST is using murine, not human, embryonic stem cells (Heuer et al. 1993). When using more sophisticated endpoints or human embryonic stem cells (hESCs), the predictivity of the EST is improved (discussed in more detail later).

Development, particularly of the human brain, is likewise very species specific. The human brain is much more complex than other vertebrate brains, with its development continuing past birth. Due to the complexity of its development, the human brain is particularly sensitive to insults during development. Moreover, it was shown that some receptors that are important during brain development are expressed differently in human and rodent brains. For example, Gassmann and colleagues investigated the role of the aryl hydrocarbon receptor (AhR) in persistent organic pollutant (POP)-induced developmental neurotoxicity (DNT). They found that in contrast to mouse neural progenitor cells that were susceptible to POP-induced DNT, human neural progenitor cells were far less susceptible to DNT due to the absence of AhR (Gassmann et al. 2010). Thus, toxicological pathways are difficult to model with sufficient predictability in rodent systems.

One additional problem arising recently is REACH (Registration, Evaluation, Authorization of Chemicals), a recent European Union legislation mandating that chemicals produced for, or imported into, the European market are evaluated for their safety for humans (Schoeters 2010). The number of chemicals that need to be tested under REACH is daunting, with estimates reaching over 100,000 chemicals, by far exceeding the numbers of available animals required for this evaluation. It is estimated that testing under REACH will use 20 times more animals and cost 6 times as much as estimated during development of the legislation. Currently, regulatory testing has neither the high-throughput methods nor alternatives to animal testing to be able to comply under REACH (Hartung and Rovida 2009).

Drug development and testing taking place in human cells is currently performed on cell lines which are mostly derived from tumor tissues. Accordingly, apart from chromosomal anomalies, these cells also harbor defects in normal regulatory pathways of cell cycle, cell death, and senescence and may be more robust to toxicity than cells in vivo, rendering prediction of toxicity for normal cells difficult. On the other hand, drug development and evaluation in normal human primary cells are also not desirable; as such cells are associated with a far-too-high batch-to-batch variability. Moreover, human primary material is only scarcely available and does not grow well (if at all) in culture and can certainly not supply the quantities of cell materials required to support high- to ultra high-throughput screening in drug development, left alone mandatory testing under REACH. This also holds true for tissue stem cells isolated from human fetal or adult tissue.

The derivation of human pluripotent stem cells (PSCs), which not only are relatively normal but also grow indefinitely in culture and are able to generate all cell types of the human body, has raised high hopes that such cells could support the demands for the cell numbers needed for drug development and testing. Moreover, with such cells, human disease models can be developed which will not only inform on mechanistic aspects of disease etiology but also enable a more targeted development of drugs. It is anticipated that PSCs will in the near future replace classical cell lines used in drug development and testing. Although classical cell lines are much easier and cheaper to culture, they do not allow for a more sophisticated cell type-specific testing, as cell type choice is limited. Often, the cell type of standard, tumor-derived cell lines is also ill defined and does not correspond to fully differentiated cells in vivo. Moreover, for the two main target cell types of human drug toxicity, hepatocytes and

cardiomyocytes, no adequate human cell lines exist.

For their better scalability to high throughput, possibility for disease modeling, and thus applicability to drug development and evaluation, this chapter will focus on PSCs. In contrast to fetal and adult stem cells which can be grown only to a limited extent in culture, PSCs can generate large amounts of progeny in culture and can still be differentiated into all cell types of the human body. Not only does this alleviate batch-to-batch variability considerably, but PSCs can also provide sufficient number of cells for high-throughput screening of the thousands of compounds generated through combinatorial chemistry. They can also generate meaningful disease models in which compounds can be screened for efficacy, moreover with genotype specificity, and thus allow for personalized medicine. As PSC-derived cell culture models can be (and are already partly) characterized as to their molecular underpinnings, they enable identification of target-selective compounds, as well as function-based approaches, where therapeutic effects can be observed by normalizing a disease-specific abnormality.

Pluripotent Stem Cell Properties

Due to their promising properties, human pluripotent stem cells (PSCs) have raised high hopes for both scientific and medical applications since the first successful isolation of an embryonic stem cell (hESC) line from human blastocysts in 1998 (Thomson et al. 1998). PSCs exhibit two unique properties in comparison to all other cells: pluripotency and long-term self-renewal in culture. Pluripotency is the ability to generate cells from all three germ layers (mesoderm, endoderm, and ectoderm) as well as cells of the germ line, i.e., all cell types of the body (Gao et al. 2013). Hence, PSCs can be used to generate any human tissue. Moreover, as the cells are self-renewing, meaning that they can divide indefinitely into identical daughter cells, these cells can be maintained in culture for extended periods of time without losing their stem cell properties. This enables culturing of PSCs over extended passages without loss of their potential to generate all cell types of the body.

Importantly, and in contrast to most other cell lines, hESCs are karyotypically normal and maintain this property for extended periods of culture, if maintained properly (Englund et al. 2010; Wenger et al. 2004). Differences in the karyotype of cancer cell lines were demonstrated to lead to high variation in gene expression patterns, thus making it difficult to reproduce results even when using the same cell line (Abdallah et al. 2013). In order to generate in vitro models as similar as possible to the in vivo situation, a normal karyotype, as provided by hESCs, is essential.

In 2007, so-called "induced" pluripotent stem cells (hiPSCs) were generated for the first time from human adult somatic cells, opening even further horizons (Takahashi et al. 2007). To achieve this, the group of Yamanaka used transcription factors which were already known to regulate pluripotency in hESCs and influence cell proliferation and differentiation: Oct4, Sox2, c-Myc, and Klf4 (Niwa et al. 2000; Bullejos et al. 2000; Chen et al. 2003; Kanazawa et al. 2003; Mitsui et al. 2003). The introduction of these transcription factors into adult, terminally differentiated, fibroblasts induced reprogramming of the somatic DNA of the fibroblasts and led to cells exhibiting properties very similar to embryonic stem cells. During reprogramming, epigenetic marks accumulated during development and further maturation to terminally differentiated cells are stripped off the DNA. At the same time, the DNA is reprogrammed to the pluripotent state. The exact processes and mechanisms operating are still largely unknown and incompletely understood. These induced PSCs expressed pluripotency gene patterns, had an epigenetic profile similar to hESCs, and could differentiate into cells of all three germ layers. Thus, by overexpressing only four transcription factors involved in the maintenance of the pluripotent network, Yamanaka and colleagues succeeded in reprogramming somatic DNA to pluripotency. Since 2007, the approaches used for reprogramming of human somatic cells to generate hiPSCs have become more robust and are

steadily being improved (Ma et al. 2013; Drews et al. 2012).

The generation of hiPSCs caused widespread enthusiasm, as the use of iPSCs for cellular therapies offers a way to prevent immune rejections upon transplantation, by generating patient-specific hiPSCs from the patient's own cells. Such cells can then be used to generate therapeutic cells that presumably would not be rejected by the patient they have been derived from. However, it has also emerged that not all hiPSC lines are truly pluripotent, likely due to incomplete reprogramming of the somatic DNA. A deeper look at the molecular level has furthermore revealed that hiPSC lines differ significantly from hESC lines when analyzing global gene expression and methylation patterns (Kim et al. 2010; Chin et al. 2009). Also, hiPSCs harbor a higher amount of mutations than hESCs, some stemming from the original somatic cells, but many induced through the reprogramming process itself (Lister et al. 2011; Ji et al. 2012). Some of these mutations may be caused by the introduction of viral vectors into the genome during reprogramming and disruption of normal genes or regulatory sequences. Additional mutations include point mutations, nonsense mutations, missense mutations, splice variations, or copy number variations (Hussein et al. 2011; Gore et al. 2011). The type and number of mutations do not seem to correlate with donor cell age, type of reprogrammed cell, or reprogramming method (Ben-David and Benvenisty 2011). To address some of these issues, different methods of reprogramming have been developed, using non-integrating vectors, transposons, plasmids, excisable vectors, transduced proteins, or engineered RNA to reprogram the somatic donor cells. Unfortunately, most of these methods are much less efficient than the transduction with lentiviral vectors (Bayart and Cohen-Haguenauer 2013). During reprogramming of mouse embryonic fibroblast, it could be shown that some mutations could also be traced back to rare somatic cells within the population of starting cells, suggesting selection of rare mutated cells during the reprogramming process (Young et al. 2012). Moreover, it emerged that with increasing time in culture, the newly derived hiPSCs lose much of their mutational load and become more similar to hESCs (Polo et al. 2010; Hussein et al. 2011).

For cellular therapies with hiPSCs, the use and potential activation of oncogenes have raised concerns of tumor development after transplantation (Robbins et al. 2010). Furthermore, due to incorrect reprogramming in certain cells and ensuing defective differentiation with persistence of undifferentiated cells in the therapeutic cell preparations, there is a potential of tumor formation upon transplantation (Miura et al. 2009). It could also be shown that some cell lines demonstrate a high frequency of tumor formation upon chimera formation, attributable to reactivation of the c-myc oncogene (Okita et al. 2007).

While such properties are certainly not desirable for human cellular therapies, mutations may be less relevant for drug screening applications. However, as mutations and/or incomplete reprogramming as well as aberrant methylation patterns may influence differentiation potential of hiPSCs and have an impact on the quality of the cells one generates for testing, such problems have to be taken into account when developing models and systems for drug testing (Miura et al. 2009; Deng et al. 2009; Doi et al. 2009). Due to incomplete reprogramming, hiPSCs retain gene expression patterns of the original cells (Ghosh et al. 2010; Polo et al. 2010; Kim et al. 2010). This epigenetic "memory" can have effects on the differentiation potential of an iPSC line, for example, when epigenetic properties favor the generation of a specific cell lineage (Feng et al. 2010). For example, hiPSCs generated from keratinocytes did not differentiate well into hematopoietic lineage, and cord-blood-derived hiPSCs generated far less keratinocytes than hiPSCs derived from keratinocytes (Kim et al. 2011). Whether or not iPSCs exhibit an overall reduced differentiation potential compared to hESCs is still heavily discussed and may be cell line dependent (Hu et al. 2010; Boulting et al. 2011). It may also become less of an issue as hiPSC lines become more hESC-"like" with prolonged culture time.

Nevertheless, induced PSCs have distinct advantages, compared to hESCs. hiPSCs can be

generated from readily accessible tissue of patients with a particular genetic condition and can therefore serve as a platform for personalized medicine, drug testing, and predictive toxicology studies (Han et al. 2011; Gross et al. 2012; Teoh and Cheong 2012). Patient-derived hiPSCs can also be used to generate disease models for clinical testing, an approach that will be discussed in more detail further down. An additional advantage of hiPSCs is the avoidance of ethical issues raised by the destruction of early embryos to generate hESCs. This has caused research with hESCs to be regulated or even completely prohibited in some countries (Teoh and Cheong 2012). The use of hiPSCs on the other hand is far less regulated and can be carried out in any laboratory that is interested in this field, which led to a very large scientific community involved in hiPSC research in a very short time.

The choice of the type of pluripotent stem cell (hESC vs. hiPSC) depends on the application in which they are going to be used. When developing a patient-specific approach or a disease model, iPSC technology enables the generation of cells with a particular disease background that cannot always be recreated easily with ESCs. On the other hand, when generating a developmental model or defined target cell types for transplantation, hESCs are often preferred. Due to the genetic abnormalities discussed above for hiPSCs, therapeutic cells generated from hESCs would presumably be of higher quality.

Murine ESCs can be used to faithfully recreate in vivo development in the dish (Barberi et al. 2003). Similar behavior is inferred for human ESCs, and accordingly, hESCs now serve to model early human development. For example, embryoid bodies (EBs), small aggregations of PSCs differentiating in suspension, are used as a model of early embryogenesis as the differentiating cells generate spontaneously cells of all three germ layers (Dvash et al. 2004). EBs can also be used for toxicological testing (Meganathan et al. 2012). As EBs can be guided toward specific cell lineages by exposure to specific growth factors, they are widely used as the initial step for directed differentiation and can be used for the generation of developmental models of specific cell lineages (Schuldiner et al. 2000). Cells of the neural cell lineage can be generated by adding growth factors such as retinoic acid, nerve growth factor, or fibroblast growth factor 2 (Schuldiner et al. 2001; Reubinoff et al. 2001). For directed differentiation of human ESCs toward cardiomyocytes, a mix of growth factors such as bone morphogenetic protein 4, activin A, and fibroblast growth factor 2 is needed (Murry and Keller 2008). An overview of growth factors needed to generate specific cell types was generated by Williams and colleagues (Williams et al. 2012).

In combination, hESCs and hiPSCs are expected to offer an unlimited supply of human cells of desired type to generate in vitro models for developmental studies, disease modeling, drug discovery, and development of personalized medicine approaches and offer sufficient human cell material for toxicology and REACH.

Human Versus Animal Models

The major advantage of human PSCs is that they allow to generate human models. The value of animal models in correctly predicting the outcome of treatment strategies in humans is controversial (Perel et al. 2007). The large number of potential treatment modalities that were proven beneficial in animal studies but finally failed in randomized clinical trials can only partly be explained by shortcomings in the design of animal studies, such as insufficient statistical power or overoptimistic conclusions about efficacy (van der Worp et al. 2010). More importantly, animal models can often times simply not predict human responses to drugs (Inoue and Yamanaka 2011). On a genetic level, for example, inflammation-related changes in gene expression in mice were often very different from the changes observed in humans (Seok et al. 2013). Furthermore, as many genetic variants of human diseases are located in noncoding regions of the genome, which have no similarity in other organisms, the introduction of these genetic variants into animal genomes is unlikely to yield a disease phenotype (Merkle and Eggan 2013). Another example that is hard

to replicate in animal models is the impact of diseases on the proteome and its complex regulation which often leads to secondary effects. In case of the neurodegenerative Alzheimer's disease, for example, a toxic effect caused by protein misfolding could not be recapitulated in a transgenic mouse model (Winklhofer et al. 2008).

Recently, a large-scale compound drug screen in neural stem cells and rat mixed cortical neurons was performed (Malik et al. 2014). The researchers tested 2,000 compounds for their species and cell type specificity. From these 2,000 substances, 100 were shown to be specifically toxic for human neural stem cells. In a secondary screen with different human neural cell types, they were able to demonstrate a cell type-specific toxicity for more than 80 % of these compounds. These results illustrate clearly that drugs can have various effects on different cells, depending both on the species and the target cell type, and that in order to predictably identify compounds affecting human cells, human in vitro models are needed with, if possible, the cell type of interest.

There have also been numerous cases in which drugs that were already approved and commercially available had to be withdrawn from the market, after successful preapproval trials. In these cases, animal models did not predict the harmful effects on humans, which were only reported in studies after the damage had already been done. Two widely known examples are the drugs rofecoxib (Vioxx) and thalidomide (Contergan). Rofecoxib (Vioxx) is a nonsteroidal anti-inflammatory drug (NSAID) inhibiting Cox-2 that was approved in 1999 for the treatment of osteoarthritis, acute pain, and primary dysmenorrhea (Roth-Cline 2006). Shortly after approval, thrombotic side effects were postulated (Title et al. 2003), and in the following years, several studies investigating other potential applications for rofecoxib reported cardiac toxicity, particularly in long-term studies (Bombardier et al. 2000; Graham et al. 2005; Juni et al. 2004; Mukherjee et al. 2001). In 2004, the drug was finally withdrawn due to increased cardiovascular risk following long-term use. In this case, cardiac toxicity was only noticed after long-term use and this in millions of users. Such effects are difficult to detect in animal models and initial clinical trials due to small-size samples. In case of thalidomide, the outcome was also severe as it caused malformation of the limbs of around 10,000 infants worldwide in the 1950s and 1960s. The drug was approved in Germany in 1957 and used as a sedative or hypnotic. Later on, it was commonly used by pregnant women to alleviate morning sickness. It then emerged that thalidomide had teratogenic effects during a very narrow time window of pregnancy, between 20 and 36 days postfertilization (Kim and Scialli 2011). The strong teratogenic effect of thalidomide could not be detected in in vivo reproductive or developmental studies in rodents (Fratta et al. 1965; Schumacher et al. 1968; zur Nieden et al. 2004). Even the validated and commonly used mouse embryonic stem cell test (EST), a developmental in vitro model to specifically test embryotoxicity by evaluating the effects on cardiac differentiation of murine embryonic stem cells, is not able to detect the teratogenic effect of thalidomide (zur Nieden et al. 2004). Just recently, researchers have developed an EST based on human hiPSCs with molecular endpoints (Aikawa et al. 2014), which was able to demonstrate the teratogenic effect of thalidomide. This finding highlights the superiority of human stem cell-based models compared to traditional models based on animal models and cells.

One last aspect also worth considering is that it is very possible that a certain number of potential drugs is discarded during their development due to lack of efficacy in animal models but might have beneficial effects in humans, as molecules are more and more designed to impact specific pathways in human.

PSCs for Human Disease Modeling and Drug Development

For many human diseases, etiology and molecular mechanisms are still poorly understood since they can only be studied to a limited extent in vivo in human. For the first time, modern techniques now allow for the generation of disease-specific cell models derived from PSCs. This is particularly the

case with hiPSCs. Cells can be taken from a patient affected by a genetic disease and hiPSCs generated by reprogramming. These hiPSCs can then be differentiated into any cell type of the body and the cells analyzed carefully for disease phenotype and molecular underpinnings (Dimos et al. 2008). By now, a large variety of hiPSC lines has been generated, including disease-specific lines from genetically inherited and sporadic diseases (Onder and Daley 2012). For in vitro models based on disease-specific hiPSCs, it is most important that the cells exhibit an easy observable cellular or molecular disease phenotype. Examples for neuronal disease-related phenotypes in hiPSC-derived models are the generation of motor neurons with a selective deficit in a model of spinal muscular atrophy (Ebert et al. 2009). A model with hiPSCs derived from Rett syndrome patients revealed a smaller soma size and altered calcium signaling in neurons derived from these hiPSCs (Marchetto et al. 2010). Additional effects that were detected in this model included fewer synapses, reduced spine density, and electrophysiological defects when compared to hiPSCs from normal cells. In case of metabolic diseases, patient-specific hiPSCs that were differentiated into hepatocytes were found to recapitulate key pathological features of the diseases affecting the patients from which they were derived. The cellular deffects in these models of inherited metabolic disease observed included altered protein aggregation, deficient receptor function, and elevated lipid and glycogen accumulation (Rashid et al. 2010). The cardiovascular disease long-QT syndrome was also shown to be recapitulated in hiPSC-derived cells. Cardiac myocytes of long-QT patients exhibited prolonged action potentials and an increased susceptibility to catecholamine-induced tachyarrhythmia compared to non-disease control cells (Moretti et al. 2010).

Another significant advantage of using hiPSCs for drug development and testing is the availability of already a large variety of cell lines with genetic defects. In addition, more cell lines are generated in international efforts to generate large banks of hiPSC lines from normal cells as well as from genetically affected cells (McKernan and Watt 2013). Since all these cell lines have different genetic backgrounds, development and testing on a multitude of different hiPSC lines can cover the broad genetic variability in humans and can mimic the various reactions due to genetic predispositions of patients to a drug. A substance that might not show an effect in one cell line might have effects in another line with a different genetic background. As of today, large banks with stored cord blood cells exist. Cord blood cells have been shown to be particularly amenable to reprogramming and moreover harbor fewer mutations than skin cells from a 60-year-old adult (Rocha et al. 2004; Giorgetti et al. 2009). In the foreseeable future, these banks could be used to generate banks of hiPSC lines, which could then be used to evaluate the effects of a drug for a whole population. The results from such studies would provide far more significant data than any traditional in vitro method used in pre-clinical trials today.

Another new development in the field of drug discovery is the use of disease-specific hiPSC lines in high-throughput drug screening approaches. hiPSCs can be grown and differentiated into the target cell type in multiwell microtiter plates. Cells in each well can then be exposed to different compounds, allowing for fast testing of a high amount of substances in a single step. Possible readouts could include cell proliferation, gene expression, protein levels, or enzyme activity. When including control lines in the assay, the outcome of these tests can be immediately compared to the non-disease phenotype.

One condition that is probably better modeled with hESCs than with hiPSCs is developmental neurotoxicity (DNT). DNT is the developmental exposure to neurotoxicants, i.e., toxicants to the nervous system. The nervous system as one of the most complex organs is particularly susceptible to insults during development. During development, cells have to proliferate extensively but in an orchestrated manner; express appropriate receptors and signaling and effector molecules, within the right time frame and in the right location; migrate to their destination; and differentiate and, in the case of neurons, establish complex and far-reaching neuronal networks. These processes are highly regulated and thus prone to

impact of only slight changes within the cellular environment potentially leading to neurodevelopmental disorders in the absence of noticeable cell death or changes in gross brain morphology. In recent years, it has emerged that environmental pollution and other types of exposure are contributing to a rise in neurodegenerative diseases and behavioral problems including aggressivity, learning disabilities, attention deficit disorder, and autism spectrum disorders (Grandjean and Landrigan 2006; Boulet et al. 2009). Also of concern is the possibility that DNT may result in an acceleration of age-related decline in cognitive function. Animal models and regular human cell lines can only inform to a very limited extent about DNT-induced subtle changes in neural functioning and behavior in human. Stuttering, for example, a neurodevelopmental disorder, would be difficult to model in mice. Current, more elaborate in vitro models based on primary neurons, neural cell lines, and immortalized neural precursor cells are only partly satisfactory (Radio and Mundy 2008). While such models have allowed elucidation of molecular mechanisms underlying impairment of differentiation and neurite outgrowth, such models contain usually only one cell type and as such can only poorly model the complex intercellular interactions taking place during in vivo development.

As hESCs can be differentiated into neural progenitors, neurons and glial cells, while recapitulating crucial steps in in vivo neural development, they are particularly attractive for modeling neurodevelopment in vitro (Barberi et al. 2003; Conti and Cattaneo 2010). hESCs differentiating in culture to neural cells better reflect the sensitivity of developing neural cells to insult than assays based on fetal or perinatal cells, such as neurons. Within the same cellular test system derived from embryonic stem cells, terminally differentiated neurons are far less susceptible to toxicity than the still differentiating neurons (Zimmer et al. 2011). Accordingly, hESCs are used increasingly to model DNT, with different assay systems covering different stages during neural development, ranging from very early stages of neural induction and specification of the first progenitors of the brain (Balmer et al. 2012) over stages where neural crest cells migrate to generate peripheral neurons (Zimmer et al. 2012) to the stages where neurons develop (Stummann et al. 2009; Hoelting et al. 2013; Colleoni et al. 2012).

Limitations to Human Disease Modeling with hiPSCs

Although hiPSC-derived disease models are promising to generate human disease phenotypes in vitro, there are also limitations. hiPSCs cannot necessarily be generated from all genetic diseases. In the case of Fanconi anemia (FA), a genetic disease caused by deficient DNA repair (FANC pathway) leading to bone marrow failure and cancer, researchers reported a failure of reprogramming of fibroblasts derived from two different FA patients (Raya et al. 2009). After repair of the genetic defect with lentiviral vectors encoding the affected FANC genes, reprogramming efficiency was restored to normal, non-disease levels. Müller et al. were able to generate iPSCs from somatic FA cells albeit with a decreased efficiency due to an increase in double-strand breaks and senescence (Müller et al. 2012). They as well concluded that complementation of the FA gene defect rescues reprogramming efficiency of somatic FA cells to normal levels. These studies demonstrate that iPSC models for diseases related to DNA damage repair and senescence are not always easily developed and might demand prior repair of the genetic defect.

Another challenge is the modeling of epigenetic disorders during development, such as deficient imprinting. For example, in fragile X syndrome (FXS) patients, the disorder is caused by an expanded CGG trinucleotide repeat in the fragile X mental retardation (FMR1) gene locus, resulting in epigenetic silencing and the loss of expression of the FMR1 protein. Neural cells derived from reprogrammed FXS patient fibroblast lines showed variations in CGG-repeat lengths, with some having shorter repeats than the input population of fibroblasts (Sheridan et al. 2011). This indicates that epigenetic predispositions are not necessarily transferred after

reprogramming of somatic cells. Moreover, in various studies, a clone-to-clone variability of hiPSC lines in exhibiting or developing a disease phenotype was demonstrated. Particularly, two studies carried out by Agarwal et al. and Batista et al. showed that their hiPSC models of dyskeratosis congenita (DC), a premature aging syndrome caused by mutations in telomerase components, displayed different disease phenotypes. Whereas the hiPSCs from Agarwal et al. recovered telomerase activity and were able to regrow telomeres, the hiPSCs from Batista et al. retained the disease-related deficiency and showed telomere shortening (Agarwal et al. 2010; Batista et al. 2011). These studies illustrate the need to keep the variations in viral integration sites, starting cell heterogeneity, passage numbers, and culture conditions from one hiPSC cell line to another in mind when using them as disease models. To alleviate discrepancies between different cell lines, multiple hiPSC lines from multiple patients with the same disease should be generated when developing a disease model. While this clone-to-clone variability can be beneficial in large-scale studies, it does complicate the process of initially developing a reliable disease model.

One approach to address this problem is the generation of genetically defined clones by genome editing. Modern genetics tools such as engineered zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) allow for either a directed correction of an affected gene or the introduction of a disease-specific mutation into the genome (Hockemeyer et al. 2009). This technique has already been successfully used to model susceptibility variants for familial Parkinson's disease, by introducing two different point mutations in the α-synuclein gene which led to the generation of two models with differing susceptibility levels (Soldner et al. 2011). TALENS have also been successfully used to correct disease phenotypes. X-linked chronic granulomatous disease is a defect of neutrophil microbicidal reactive oxygen species (ROS) generation caused by a mutation in CYBB gene. By introducing a single-copy CYBB minigene into X-CGD hiPSCs without off-target insertions, Zou et al. could restore ROS production (Zou et al. 2011). Another example is the in situ correction of sickle cell anemia. Sebastiano et al. generated hiPSC lines from sickle cell anemia patients and corrected the disease-related mutation by using three ZFN pairs and thereby generating transgene and disease-free hiPSC lines (Sebastiano et al. 2011).

Additional limitations to hiPSC-derived disease models are a lack of efficient protocols for the generation of certain cell types affected by a disease, the lack of sensitive detection methods for disease phenotypes, the problems with modeling late-onset diseases, or the inability to model interactions between different cell types. This holds also true for model derivation from hESCs. An example for the lack of suitable differentiation protocols are models for hematological disorders. The establishment of models for sickle cell anemia, β-thalassemia, and ADA-SCID has proven difficult as the blood cell lineages most relevant to these diseases cannot be generated with satisfying efficiency and purity (Onder and Daley 2012). Generation of models for late-onset diseases such as sporadic Parkinson's disease, for example, which not only require the genetic predisposition but also environmental queues, might require experimental induction of the disease as well as an acceleration of the course of the disease (Kim et al. 2013; Cherry and Daley 2012). Finally, in pursuance of modeling non-cell autonomous deficits as occurring, for example, in ALS (amyotrophic lateral sclerosis), a disease where motor neurons die, a disease model derived from cocultures of motor neurons derived from hESCs and mutated astrocytes showed that the motor neurons die due to oxidative stress elicited in the mutated astrocytes (Di Giorgio et al. 2008; Marchetto et al. 2008). While in this case protocols for the generation of motor neurons are well developed, many other cell types have yet to be derived with higher efficacy.

Pluripotent Stem Cell Handling

As discussed above, PSCs can be a valuable source of cells for in vitro models, but they require a very meticulous handling, including stringent

quality control. In comparison to other more robust cell lines, pluripotent stem cell lines are highly susceptible to environmental changes and can quickly lose their pluripotent qualities. Heterogeneous cell populations can develop with spontaneous differentiation from PSCs entailing that cells do not necessarily reach developmental stages in a synchronized manner during directed differentiation (Kitaoka et al. 2011). Crucial for high-quality PSC populations are feeder cells of constant quality, defined media and additives, standardized passaging and seeding techniques, substrate, and particularly the cell or tissue microenvironment. The latter is very complex and plays an important role in the generation of appropriate in vitro models (Viswanathan et al. 2014). In contrast to mouse PSCs, hPSCs cannot be passaged as single cells as the absence of cell-cell contacts leads to apoptosis. Thus, successful passaging of hPSCs depends on the generation of small clusters of defined and, importantly, constant size. The addition of ROCK inhibitor to the culture medium allows for single-cell passaging for a limited number of passages (Watanabe et al. 2007). Another important factor of hPSC culture is oxygen tension. Physiological oxygen tension reduces the incidence of chromosomal aberrations without altering hESC pluripotency marker expression while improving maintenance of pluripotency (Forsyth et al. 2006; Forristal et al. 2010; Zachar et al. 2010). Lastly, the choice of substrate for stem cell culture plays an important role in cell quality. Viswanathan et al. have extensively discussed the different choices of substrates, with a focus on synthetic materials and their effect on self-renewal and differentiation (Viswanathan et al. 2012, 2014).

hiPSC lines show high disparities between cell lines, due to incomplete reprogramming, genetic background variability (Soldner and Jaenisch 2012), epigenetic memory (Kim et al. 2010), or erosion of X-chromosome inactivation (Mekhoubad et al. 2012). These disparities in cell quality can have a great impact on the outcome of in vitro tests and should be characterized very carefully. To fully realize the potential of human PSCs and to benefit from all the potential these versatile cells have, robust differentiation and cell purification protocols, optimal control settings, and validation with human samples and/or other disease models are needed (Inoue et al. 2014).

The Embryonic Stem Cell Test (EST)

As outlined above, the availability of PSCs provides new approaches for disease modeling and drug testing. Yet, despite all the advantages of these cells compared to traditional models and the extensive research regarding the development of more defined cell models, as of date, only one stem cell-based assay has been validated on the regulatory level: the mouse embryonic stem cell test (EST). The EST is a validated assay for potential embryotoxicants (Seiler and Spielmann 2011). For regulatory purposes, it can however not be used on its own, but rather incorporated into an integrated test strategy. The official standard operation procedure (SOP) of the EST can be accessed at the EURL ECVAM online database (http://ecvam-dbalm.jrc.ec.europa.eu/, accessed December 8, 2014).

This test was originally developed because of the need for a reliable, cellular in vitro test for embryotoxicity of chemicals. The reasons for the development of this new cellular assay were the high costs and ethical concerns associated with animal testing, a toxicological study for one chemical which was estimated to require around 4,700 animals, and a paucity of tests specific for early embryonic development (Höfer et al. 2004). Already established in vitro methods such as the frog embryo teratogenesis assay (Bantle et al. 1990), the chicken embryotoxicity screening test (Jelinek et al. 1985), or assays based on murine embryos (Sadler et al. 1982; Flint 1993; Schmidt et al. 2001; Cicurel and Schmid 1988) are based on somatic cells rather than on stem cells and developing cells and thus do not reflect early developmental stages.

The EST was developed in the group of Spielmann and his coworkers in 1993 and was validated in 2002 (Heuer et al. 1993; Genschow et al. 2002). The test is based on the assumptions that in vitro tests of basal cytotoxicity are able to

accurately determine rodent in vivo LD_{50} (Ekwall 1999; Spielmann et al. 1999) and that exposure of ESCs to embryotoxic substances leads to alterations in the differentiation patterns during embryoid body (EB) formation (Wobus et al. 1994). In this test, two mouse cell lines are used that represent both the embryonic and the adult tissue: the mouse ESC line D3 and the mouse 3T3 fibroblast cell line. The D3 ESCs are differentiated to EBs in the absence of the pluripotency cytokine leukemia inhibitory factor (LIF), leading to the formation of beating clusters of cardiomyocytes. The first endpoint investigated in the EST is the concentration of the test substance that is needed to inhibit cardiac differentiation by 50 % after 10 days of exposure (ID_{50}). Further, the concentrations of this substance needed to inhibit proliferation of both D3 ESCs and 3T3 fibroblasts by 50 % after 10 days of exposure lead to two additional endpoints. The three endpoints are used to generate a biostatistical prediction model that assigns the tested substance to an embryotoxicity category (strong, weak or non-embryotoxic) (Genschow et al. 2000, 2002; Spielmann and Liebsch 2001).

The EST became widely recognized when the European Centre for the Validation of Alternative Methods (ECVAM) began seeking for new toxicity tests as alternatives to animal experiments in the mid-1990s and initiated validation studies for various in vitro assays. In 2002, the EST was validated as a screening assay for potentially embryotoxic chemicals by the European Scientific Advisory Committee (ESAC 2002). In the first study, a set of 20 substances with known embryotoxicity were tested. For weak and nontoxic substances, the embryotoxicity was predicted with an accuracy of 78 %, whereas strongly embryotoxic substances were predicted with 100 % accuracy (Genschow et al. 2002, 2004). The poorest accuracy was recorded for the detection of non-embryotoxic compounds.

Although the EST was sufficiently accurate at predicting embryotoxic effects and was ready to be accepted by regulatory commissions (Balls and Hellsten 2002), the ESAC ultimately did not recognize the EST as qualified to replace the already established animal experiments (Spielmann 2009). One of the reasons was that the amount of tested substances was not large enough. In a follow-up study with 13 additional substances, the EST failed to correctly predict the embryotoxic effect of the tested compounds, indicating that the test required further optimizations (Hareng et al. 2005). Further limitations of the original mouse EST have been extensively discussed, including the lack of molecular endpoints, such as differentiation markers for all cell lineages, for example (Schmidt et al. 2001; Piersma 2004), the inability of mouse D3 ESC to detect toxic effects of substances that require metabolic activation (Marx-Stoelting et al. 2009; Verwei et al. 2006) and the inability of the EST to detect teratogenic effects past the early embryonic differentiation stages.

In the past few years, many of these concerns have been addressed, and new variations of the original EST have been proposed. Early on, the use of reporter genes under control of cardiac-specific promoters was discussed when a lineage-dependent effect of retinoic acid on ESC differentiation was reported (Wobus et al. 1994; Kolossov et al. 1998). Reporter gene assays for developmental toxicity have since been implemented (Bremer et al. 2001) and have replaced the rather subjective and poorly quantitative determination of the embryotoxic effect of a substance by microscopical observation of beating EBs. Instead, the expression of the cardiac marker genes α-myosin heavy chain (MHC) and α-actinin is quantified by flow cytometry in labeled cells (Seiler et al. 2004, 2006). This flow cytometry-based EST has successfully been adopted for assessing developmental toxicity and has reduced the test duration while providing a more reproducible screening for developmental toxicity (Buesen et al. 2009). Even more biomarker genes of embryotoxicity have been identified that displayed a significant decrease in expression after exposure to a non-cytotoxic dose of the embryotoxic 5-fluorouracil (Estevan et al. 2011). This effect was already measured 5 days after exposure, reducing the time needed to evaluate the embryotoxic potential of a substance by 50 % compared to the usual 10 days needed to observe beating cardiomyocyte clusters.

In addition to cardiac markers, additional lineages were added to the endpoints of the EST (Spielmann et al. 2006). The inclusion of parameters for neuronal differentiation led to the positive detection of the highly embryo and neurotoxic substance methylmercury, which had not been successfully classified in the conventional EST previously, with cardiac endpoint alone (Stummann et al. 2007). Hayess et al. have developed a test for determining DNT of substances by inducing neural differentiation of mouse ESCs. They were able to determine DNT with a 100 % predictivity and accuracy for nine substances (Hayess et al. 2013). Another endpoint that has been established successfully is testing for the expression of three genes involved in osteogenesis. The results of this osteoblast differentiation assay (ESTo), testing 19 substances, showed a high correlation to results of the conventional EST and allowed for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an embryotoxic substance that could not be detected otherwise (de Jong et al. 2014). These examples show the importance of a broad range of genetic markers for successfully identifying embryotoxic compounds.

Endpoints based on gene expression, protein markers and metabolic activity can potentially indicate embryotoxic properties. A proteomics approach carried out by Osman and coworkers showed that EBs exposed to the embryotoxic monobutyl phthalate for 25 h showed a shift in the intracellular levels of 33 proteins, including cardiac markers and chromatin modulator enzymes (Osman et al. 2010). A recent study investigated the impact of embryotoxic substances on genes of the energy metabolism. The transcriptomics approach of van Dartel et al. revealed dynamic changes in energy metabolism during early ESC differentiation in response to embryotoxic compounds. The test showed activation of glycolysis, truncated activation of the tricarboxylic acid (TCA) cycle, activation of lipid synthesis, and activation of glutaminolysis (van Dartel et al. 2014).

As outlined previously, the use of human cells has important advantages compared to murine cells. Species-specific differences in embryonic development between man and mouse have been reported including DNA methylation, DNA repair, and the expression of genes involved in drug metabolism (Krtolica et al. 2009). Accordingly, an EST using hESCs would significantly enhance the validity as such species-specificity problems would be alleviated (Wobus and Löser 2011). A recent study, adapting the EST to human cells, using hiPSCs and human dermal fibroblasts has successfully detected the teratogenic effects of thalidomide, which is not detected in the validated mouse EST (Aikawa et al. 2014). Additional proof-of-concept studies with the goal to determine the suitability of hESCs for toxicology tests have been performed. Adler and coworkers carried out studies evaluating the effects of all-trans retinoic acid and 13-cis retinoic acid on human ESCs, human ES-derived progenitors, and human foreskin fibroblasts, thereby detecting a toxic effect of 13-cis retinoic acid, which was not detected in previous mouse EST analyses (Adler et al. 2008a). Moreover, in a second study, they established a human EST yielding results very similar to the mouse EST (Adler et al. 2008b). They proposed to use additional markers of undifferentiated ESCs as well as markers of neural plate morphogenesis and early cardiogenesis to expand the amount of molecular endpoints for evaluation of embryotoxicity.

As of today, the validation process of the EST is an ongoing project, and the test is continuously being improved by addition of new endpoints and adjustments for high-throughput screening and the future use of hESCs or hiPSCs. Although studies of an EST with human cells are very promising, still many issues such as insufficient sensitivity and specificity need to be addressed. Although, test systems with hESCs or hiPSCs are very promising, they are also plagued by poor reproducibility due to the inherent difficulties in culturing and differentiating human PSCs in a standardized manner. hPSCs are best passaged by cut-and-paste or as small clumps rendering standardization and scale-up difficult. Currently world-wide efforts are focussed on addressing these issues and numerous research groups are developing scaled-up culture conditions for expansion of PSCs and their improved differentiation towards cells of interest.

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Quantitative Metabolomics

Amrita Cheema and Siddheshwar Chauthe

Contents

General Considerations

Metabolomics is the comprehensive study of the metabolome, the repertoire of small molecules or biochemicals present in cells, tissue, and body fluids, which are end product of cellular processes (Harrigan and Goodacre 2003). The array of small molecules includes metabolic intermediates (α-ketoglutarate, oxalate), hormones (progesterone), other signaling molecules (cAMP, AMP), and secondary metabolites (glucose, cholesterol) (Harrigan and Goodacre 2003). Metabolomics has several advantages over the other "omic" approaches for molecular phenotyping studies. The metabolome represents the final endpoint of cellular expression and hence could be interpreted as a phenotypic readout of a biological system in a dynamic state (Fig. 1). The changes in the metabolome are amplified relative to changes in the transcriptome and the proteome such that minor perturbations lead to significant changes in metabolism thereby affecting the overall physiology (Horgan and Kenny 2011). The metabolome is closest to the phenotype of the biological system and is also influenced by environmental factors (Kaddurah-Daouk and Krishnan 2008). Hence, the study of metabolome can certainly provide more information that could possibly bridge the gap between genotype and phenotype. Metabolomics in conjunction with other system biology approaches has great potential to advance our understanding of human diseases and hence rapidly gaining importance as a

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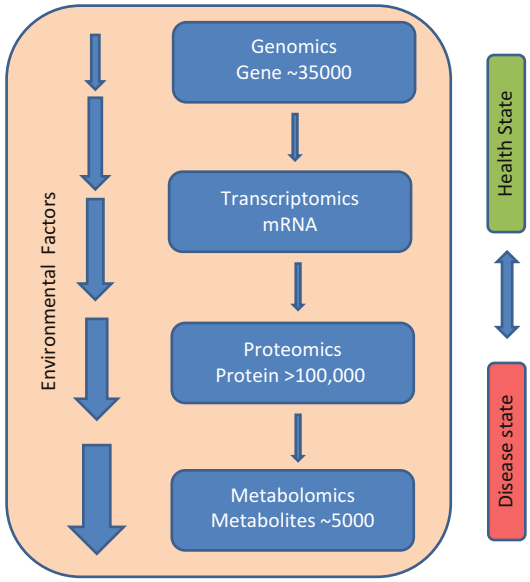

Fig. 1 Schematic representation of relationship between different levels of cellular expression and the influence of environmental factors at metabolic level

powerful tool for biomarker discovery and likely to have a huge impact on understanding of disease onset and progression and can thus augment the development of clinical diagnostics (Kaddurah-Daouk 2006). Moreover, the level of complexity at the metabolite level is much less as compared to proteomic and transcriptomic levels which are more diverse and undergo transient changes making it difficult for use as stable biomarkers that are predictive of a specific pathology (Horgan and Kenny 2011). The most recent release of HMDB (human metabolite database) catalogs 41,992 metabolites of which the total number of endogenous metabolites is approximately 29,328 (Wishart et al. 2007). Metabolomics-based studies require a collaborative and an interdisciplinary effort toward study design, metabolite identification, data analysis, and interpretation. Recent advances in tandem mass spectrometric techniques have enabled the precise measurement of numerous metabolites in various media ranging from cell cultures to biological fluids such as urine, saliva, and blood at sub-femtomolar levels.

Purpose and Rationale

Metabolomics can be used for diverse applications including investigations into metabolic changes that occur in response to biological, toxicological, and environmental stimuli. It can be potentially used for discovering novel pathways or understanding pathway-based perturbations in health and disease. For instance, in current clinical setting, the analysis of different components of blood augments diagnosis of healthy and diseased states for several metabolic diseases like measuring blood glucose to monitor diabetes, measuring cholesterol for predicting risk for cardiovascular disease, and measuring creatinine level for kidney function test. Technological advances in metabolomics technology facilitate the development of metabolic signatures that reveals the global biochemical changes of the disease before and after treatment (Kaddurah-Daouk 2006; Bujak et al.) and can thus be used to diagnose disease, predict patient response toward therapies, and monitor recurrence post therapy. Thus, the metabolic signature is likely to provide valuable information about biomarkers of the disease state; it would also help us to identify drug response phenotype and mechanism of disease onset and progression (Patel and Ahmed 2015). The discovery of prognostic biomarker provides information monitoring disease progression thus facilitate alternate therapeutic regimens, thus giving impetus to drug discovery and development. Metabolomics could be used to characterize phenotype and biological response to genetic modification (Rischer and Oksman-Caldentey 2006) and nutritional imbalance (Zivkovic and German 2009). Metabolomics also has significant application in the area of gastrointestinal disease. Metabolomics sheds light on the regulation of digestion and absorption of food products in the gastrointestinal tract (Martin et al. 2010). For example, it could be used to identify different topographical regions of the intestine through specific metabolic profiles and also to understand spontaneously relapsing and immunologically mediated chronic disorders of the gastrointestinal tract such as irritable bowel syndrome (IBS)

(Madden and Hunter 2002) or inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD). Metabolomics also provides novel insights into the regulation and the etiology and molecular basic of metabolic syndrome and related cardiometabolic, neurological, and psychiatric disorders (Collino et al. 2013). Several groundbreaking studies focused on translational research fueling the hope of moving metabolomics technology to the clinic to guide treatment, monitor disease prognosis, and augment the development of disease modifying therapeutics. Despite great expectations however, only a limited number of biomarkers have been validated via this approach till date. The reasons include lack of population heterogeneity, inconsistencies, and limitations in the study design with limited statistical power and a trend to report metabolomics data without reporting magnitude of perturbation in the relative abundance of the metabolites (Wood 2014).

Procedure

Metabolome is typically defined as the collection of small molecules produced by cells. However, contrary to genome or proteome, human metabolome composition is still not fully defined. Several publicly available databases, such as Human Metabolome Database (HMDB) (http://hmdb.ca), METLIN (http://metlin.scripps.edu), or KEGG (http://www.genome.jp/kegg), provide information on metabolites present in human biofluids. HMBD comprises around 40,000 human metabolites. However, due to the presence of various exogenous metabolites originating from diet, pharmacotherapy, as well as compounds produced by endogenous gut microflora, the size of HMDB is overestimated (Wikoff et al. 2009). There are mainly three research approaches, which have emerged in metabolome analysis: metabolic profiling (Suhre 2014), metabolic fingerprinting (Inoue et al. 2015), and metabolic footprinting (Behrends et al. 2014). Metabolic profiling is also called targeted metabolic approach. Herein, the focus is on identification and quantification of a set of metabolites driven by a specific hypothesis which motivates the interrogation of particular biochemical pathways, e.g., glycolysis, gluconeogenesis, oxidation, or citric acid cycle (Abdel Rahman et al. 2014). In this strategy, the hypothesis is based on change in metabolite profile in response to a specific gene mutation, disease progression, pharmacotherapy, protein dysfunction in a given disease state, or diet intervention.

The identity of the metabolites selected for this approach is initially established by searching online databases and using standard compounds. However, the analysis of these chemically diverse class of compounds using single analytical method is an arduous task, but due to advancement in mass spectrometry and NMR spectroscopy and their hyphenate techniques such as liquid chromatography coupled with mass spectrometry (LC-MS), gas chromatography coupled with mass spectrometry (GC-MS) or capillary electrophoresis coupled with mass spectrometry (CE-MS), supercritical fluid chromatography coupled with mass spectrometry (SCF-MS), and liquid chromatography coupled with nuclear magnetic resonance spectroscopy (LC-NMR) (Zhang et al. 2012), it is now possible to rapidly measure thousands of metabolites simultaneously from minimal amounts of sample. The most critical step in metabolomics is the sample preparation, which is the extraction of a group of compounds with varied physicochemical properties requiring expertise and understanding of the basic organic chemistry. There is a plethora of literature available on optimal protocols for the sample preparation and analysis of specific classes of metabolites (Vuckovic 2012; Want et al. 2006).

Targeted Metabolomics (Metabolic Profiling)

Liquid chromatography coupled with triple-quadrupole mass spectrometry is used extensively for targeted metabolomics. Multiple reaction monitoring (MRM) also known as selected reaction monitoring (SRM) functionality is widely used for quantification of metabolites in biological matrices. These advancements provide

high-throughput, reproducible, sensitive, and robust method for absolute quantification of low-concentration metabolites. The MRM-based methods are routinely used for analyzing metabolites in a variety of biological pathways such as central carbon metabolism, lipid and amino acid metabolism, glycolysis, pentose phosphate pathways, etc. (Abdel Rahman et al. 2014).

A typical workflow (Fig. 2) for targeted metabolic profiling involves the development of MRM assay of metabolite of interest using standard compounds. In a typical MRM method, signal intensity depends critically on the tuning of instrument parameters, such as collision energy and cone voltage, for the generation of maximal product ion signal. Once the standard response curve is generated, then metabolites are extracted from tissues, biofluids, or cell cultures and analyzed. The identified metabolite peaks are then quantified based on internal or external reference standard compounds. The internal standard is normally added in the sample matrix to minimize error in extraction of analyte. Sometimes, sample matrix may interfere with ionization of analyte of interest, in such cases sample preparation protocol is optimized to avoid ion suppression. The resultant quantitative data can be used for pathway analysis or for statistical analysis for feature selection and biomarker development. Targeted metabolomics can provide valuable information about the dynamics and fluxes of metabolites and promises robust statistical models for distinguishing sample classes with better classification accuracy.

Untargeted Metabolic Profiling (Metabolic Fingerprinting)

Untargeted metabolic profiling is also called metabolic fingerprinting. This approach is unbiased and aims to understand the composition of whole metabolome in the sample in response to a specific change or environmental stimuli in a biochemical system. The strength of the approach is that it allows for a comprehensive interrogation of systemic changes in the metabolome without *a priori* hypothesis. The metabolic finger printing is a unique pattern and composition of metabolites in a particular sample in a given condition (Ellis et al. 2007). Due to complexity of the biological matrix, there is no unique method for identification of all possible metabolites in biological fluids. Although analytical techniques such as LC-MS and NMR have been widely used to analyze the untargeted profiling of the biological samples, the former technique is widely used due to more comprehensive coverage of metabolites compared to NMR because of enhanced resolution and sensitivity. Metabolic profiling is the most widely and routinely used technique in metabolomics, and it has wide applications in understanding metabolic difference and selective biomarker identification between disease and healthy subject groups or between different experimental groups (Wikoff et al. 2009; Yanes et al. 2010).

A typical untargeted metabolic workflow involving LC-MS analyses (Fig. 3) involves extraction of metabolites from biological samples and subsequent analysis using high-resolution and high-mass accuracy platform like time-of-flight mass spectrometry (TOFMS). After data acquisition, the data are pre- and post-processed using bioinformatics software such as XCMS (Scripps Center for Metabolomics) and Progenesis QI (Waters), MassHunter (Agilent), etc. A molecular feature extraction (MFE) algorithm is used to find metabolite peaks in raw data sets and perform nonlinear retention time alignment between groups of samples. The m/z values obtained for the peaks of interest are searched in metabolic database such as MMCD, Lipid Maps, METLIN, and HMDB and later confirmed by comparing tandem mass spectrometry (MS/MS) data and retention time data to that of standard compounds. Several multivariate statistical methods have been used for selective and differentiating diagnostic and prognostic biomarkers (Patti et al. 2012).

Metabolic Footprinting (Extracellular Metabolomics)

Metabolic footprinting is also known as exo-metabolome. This methodology is concerned with secretory products or metabolites in

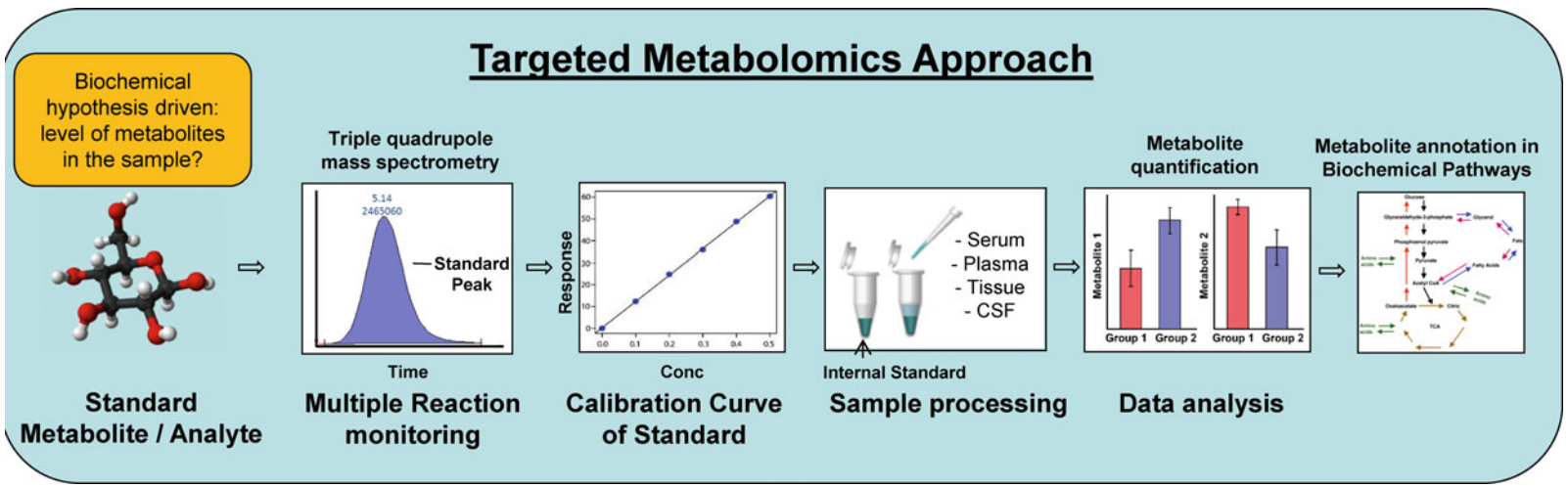

Fig. 2 A typical workflow for a targeted metabolic profiling experiment

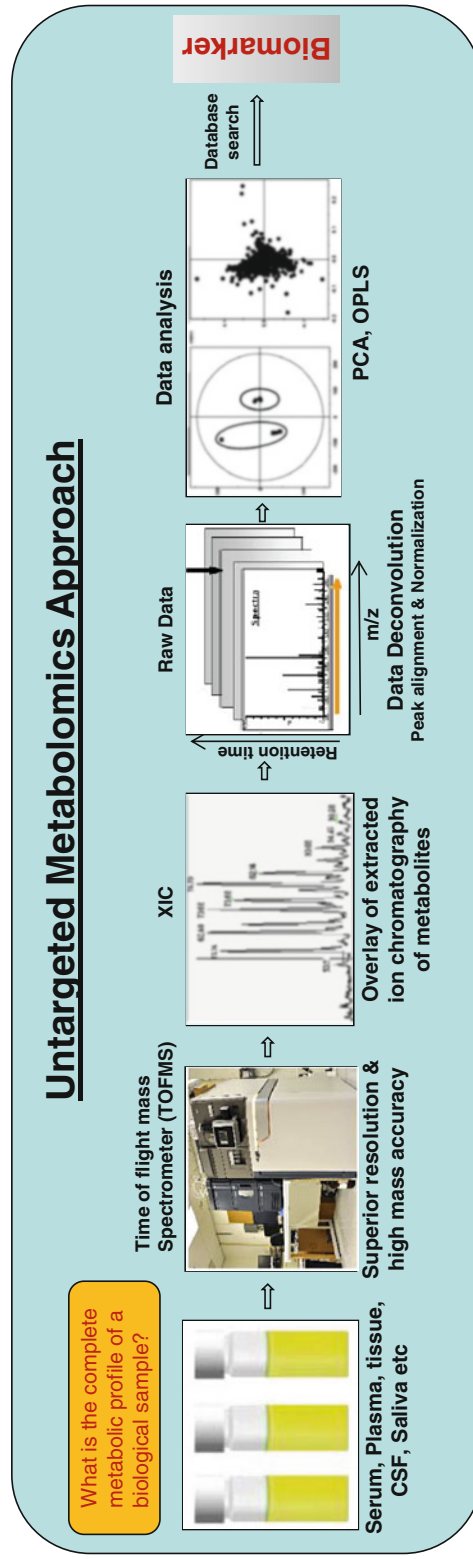

Fig. 3 A typical workflow for an untargeted metabolic profiling study

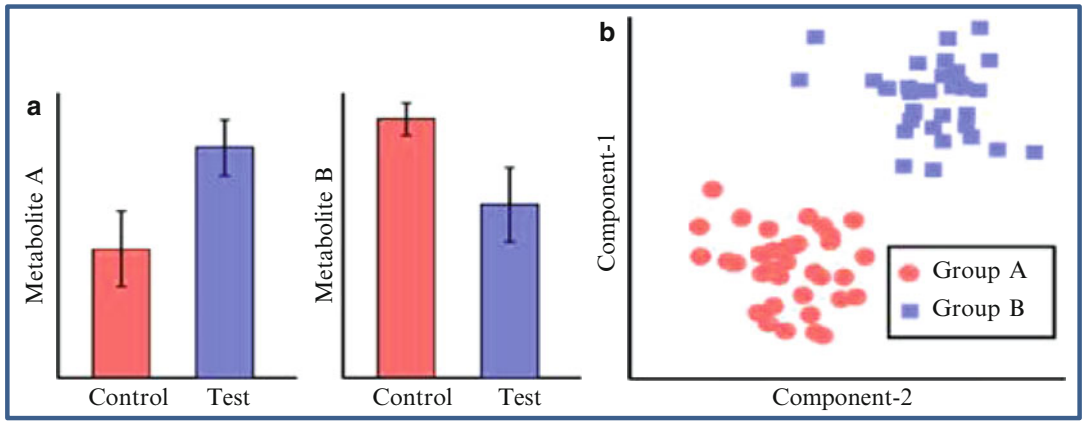

Fig. 4 Common statistical Methodologies used to analyze metabolomics data. (**a**) Depict the variances in the concentration level of single metabolites between two or more groups (e.g., *t*-test, ANOVA). (**b**) Multivariate approaches like PCA and PLS model the relationships between metabolites

extracellular medium of the cells or microorganism (Mapelli et al. 2008). A living cell secretes enzymes and excretes metabolites to the extracellular medium, these metabolites are continuously taken up by the living cells, and those metabolites which fail to uptake by living cells interact with component of the medium resulting in metabolic profiles that are highly specific to a particular species or genetic background of the cells. Thus, we are able to distinguish different metabolic states of the cells, different microbial species, and even different strains or mutants from the same species (Sue et al. 2011). Although metabolic footprinting represents only a fraction of the entire metabolome, it offers valuable information for understanding of intercellular communication mechanism and interpretation of different metabolic networks (Mapelli et al. 2008).

However, measurement of a complete set of extracellular metabolites is a tough task due to dynamics of extracellular metabolism and complex media components. Although, Several diverse techniques have previously been used for this purpose which include nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis coupled with mass spectrometry (CE-MS), gas chromatography coupled with mass spectrometry (GC-MS), and liquid chromatography coupled with mass spectrometry (LC-MS), none of these techniques completely fulfill the criteria of an accurate, simple, rapid method with a broad dynamic range (Kell et al. 2005).

Statistical Analysis of Metabolomics Data

Metabolomics is a relatively new field that uses measurements on metabolite abundance as a tool for biomarker discovery and general metabolic profile. Statistical analysis in metabolomics is simpler compared to proteomics because of significant knowledge of domain of metabolites. Current metabolomics data analysis methodologies can be placed into two general categories, if the aim is to analyze a small group of specific metabolites, usually the more abundant ones (targeted metabolomics) in such case statistical significance in targeted variable can be tested by univariate, i.e., parameter-by-parameter fashion (e.g., *t*-test, analysis of variance (ANOVA)). Univariate methodologies reduce a large set of measured metabolites data to a small number of metabolite with the strongest response under the investigated conditions (Xia et al. 2013; Commisso et al. 2013) (Fig. 4a). In contrast, if the aim of the metabolic study is to understand the complete metabolic profile of the system under investigation (untargeted metabolomics), in such cases large number of metabolites is quantified and all the obtained variables are considered

simultaneously, making univariate statistical methods unfeasible for analysis. Univariate methods fail to discriminate between groups if there are only minor differences on single molecule level. Normally in large data set, analysis is usually performed using multivariate statistical methods such as principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA) (Trygg et al. 2007) (Fig. 4b). Dimensionality reduction techniques help data filtering into a few key features that contribute to maximal variance or discriminatory covariance between the study groups and thus can be used as chemical fingerprints to define the observed phenotype.

Principal component analysis (PCA) is arguably the most widely used multivariate analysis method for metabolic fingerprinting. Unsupervised PCA is a linear modeling technique that provides a means to achieve unbiased dimensionality reduction (Bartel et al. 2013). Its application only reveals group structure when within-group variation is sufficiently less than between-group variation. Supervised forms of discriminant analysis such as partial least squares (PLS-DA).

Partial least squares (PLS-DA) or alternatively partial least squares projections to latent structures that rely on the class membership of each observation are also commonly applied in metabolic fingerprinting experiments (Wold et al. 2001). PLS-DA is usually used for classification purposes either to infer the variables that maximize the discrimination between predefined sample groups or even to predict class affiliations of unclassified samples based on a calibration set of known class distributions (Bartel et al. 2013). PCA is often used as a starting point for data analysis, especially in untargeted metabolic profiling. Some applications among many in the field of metabolomics include the analysis of γ and heavy ion radiation exposure on mouse intestinal metabolomics (Cheema et al. 2014), urine metabolomics in the diagnosis of some inherited metabolic disorders (Janeckova et al. 2014), and serum metabolites in Parkinson's disease (Michell et al. 2008). PLS-DA was used for the identification of discriminant biomarkers of cocoa product consumption on free-living healthy subjects and also in biomarker discovery of different types of cancers (Kind et al. 2007; Kim et al. 2009).

Evaluation

The main focus of metabolomics research in the recent years has been toward the discovery of novel biomarkers for an array of clinical and translational studies. Despite a large number of publications and research in metabolomics, there has been little success in translating actual biomarker discovery into clinical study. Unfortunately, among the many published metabolomics studies focusing on biomarker discovery, there is very little consistency and little rigor in how researchers select, assess, or report their candidate biomarkers. Among several discrepancies is an example of ROC curve analysis. ROC curve is generally considered the standard method for performance assessment, but very few studies explicitly describe or release the biomarker model used to generate their ROC curves (Xia et al. 2013). The main limitation of most metabolomics studies results from the lack of reliable validation on large cohorts of heterogeneous human population. Discovery-based studies need to be followed up by large-scale validation studies in diverse clinical cohorts so as to enable potential clinical use of the biomarkers. Validation studies for candidate biomarkers selected at discovery stage are imperative for demonstrating reliable diagnostic or prognostic disease biomarkers. Since metabolomics experiments are highly susceptible to pre-analytical variables due to inconsistencies in sample collection, sample size, sample handling, and storage conditions, there is a need to standardize the way metabolomics studies are designed and data are reported. As a result of an increasing need to put standards in place, Metabolomics Society in 2005 started metabolomics standards initiative (MSI) program and formed an oversight committee to monitor, coordinate, and review the efforts of working groups (WG) in specialist areas that will examine standardization and make recommendations to propose the standardize protocol for reporting the metabolomics data (Sansone et al. 2007). The success of using this high-

through put molecular phenotyping technology will depend on cross-platform and inter-laboratory reproducibility studies aimed towards credentializing research discoveries and translating them into regular clinical practice.

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Quantitative Glycoproteomics

Radoslav Goldman

The term "proteome" was coined by Wilkins et al. (1995) to describe the protein complement of the genome. The term "proteomics" was first used to describe the complexity of proteins expressed in an organism using two-dimensional gel electrophoresis (2-DE) followed by quantitative analysis. 2-DE remains the highest-resolution protein separation method available, but the ability to concentrate and identify low-abundance proteins has always been an extremely difficult problem. Mass spectrometry (MS) has been an integral part of approaching this problem (de Hoog and Mann 2004; Lane 2005). Although improvements in 2-D gel technology have been made since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics (Patterson 2000). The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and the widespread introduction of mass spectrometers, capable of data-dependent ion selection for fragmentation (MS/MS) (i. e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying together the first technological advances. The mass spectrometer instruments are named for their type of ionization source and mass analyzer (Patterson and Aebersold 1995; Carr and Annan 1997; Patterson 1998). To measure the mass of molecules, the test material must be charged (ionized) and desolvated. The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI, the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer that measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region (Kowalski and Stoerker 2000). The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC column or a nanospray device (Wilm and Mann 1994), similar to a microinjection needle. The ions formed during this process are directed into the mass analyzer, which could be a triple-quadrupole, an ion trap, a

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Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qp-TOF) type (Morris et al. 1996). Although unambiguous identification of a protein cannot always be derived from the masses of a few of its peptides in the tandem mass spectrometer, peptide ions from the first mass spectrometer run are fragmented and identified in a second run to yield a more valuable commodity of a peptide sequence.

Annis et al. (2004) and Whitehurst et al. (2006) proposed affinity selection-mass spectroscopy (AS-MS) as a useful high-throughput assay system for the discovery and characterization of all classes of integral membrane protein ligands, including allosteric modulators.

The goal of proteomics is a comprehensive, quantitative description of protein expression and its change under the influence of biological perturbations, such as disease and drug treatment (Anderson and Anderson 1998, 2002; Müller et al. 1998; Blackstock and Weir 1999; Dove 1999; Hatzimanikatis et al. 1999; Jungblut et al. 1999; Williams 1999). A combination of mRNA and protein expression patterns has to be simultaneously considered to develop a conceptual understanding of the functional architecture of genomes and gene networks (Kreider 2000). New methods are created such as automated proteomics platforms (Quadroni and James 1999; Nielsen et al. 1999), combining two-dimensional electrophoresis, automated spot picking, and mass spectrometry (Binz et al. 1999; Dancik et al. 1999; Loo et al. 1999; Dutt and Lee 2000; Feng 2000; Patterson et al. 2000; Ryu and Nam 2000; Service 2000; Yates 2000; James 2001; Jain 2001; Rabilloud 2001).

Improvements in quality, ability, and utility of large-scale tertiary and quarternary protein structural information are enabling a revolution in rational design, having a particular impact on drug discovery and optimization (Maggio and Ramnarayan 2001).

Disease proteomics will give a better understanding of disease processes, develop new biomarkers for diagnosis and early detection of disease, and accelerate drug development (Hanash 2003).

One may expect that with the new approach of drug research, including combinatorial chemistry, genomics, pharmacogenomics, proteomics, and bioinformatics, unprecedented results will be generated (Browne 2000; Burley et al. 1999; Debouck and Metcalf 2000; Drews 2000; Haystead 2001).

The difficulties arising in proteomic experiments from data analysis are discussed by Patterson (2003).

Duncan and Hunsucker (2005) discussed the value of proteomics as a tool for clinically relevant biomarker discovery and validation. A critical review on the impact of "OMIC" technology in drug development is given by Bilello (2005).

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Personalized Medicine

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Contents

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Purpose and Rationale

Personalized, or precision medicine, capitalizes on knowledge of an individual's molecular, genomic, cellular, clinical, behavioral, physiological, and environmental parameters to inform guidance toward the prevention, diagnosis, and treatment of disease (Chan and Ginsburg 2011; Collins and Varmus 2015). The growing body of evidence attests that personalized medicine (PM) is supportable by increasingly powerful high throughput methodology with excellent economy of scale to acquire detailed, informative data including clinically relevant profiles of biomarkers such as DNA, RNA, and other macromolecules (Abul-Husn et al. 2014). The capacity to extract and intelligently interpret the data in a clinically relevant manner creates the opportunity to implement personalized, informed preventive lifestyle changes and real-time interventions promoting healthier outcomes for patients while incurring measurable health care cost savings (Altman and Klein 2002; Altman et al. 2013; Ginsburg and Willard 2009; O'Donnell et al. 2012).

Pharmacogenomics (PGx) forms the basis of PM through the use of genetic information to select the right drug at the right dose at the right time (Bielinski et al. 2014), thereby tailoring therapy to a patient to optimize drug efficacy, promote adherence, and minimize side effects while reducing costs (Dietel and Sers 2006; Dolan et al. 2013; March 2000; Murphy 2000; Owusu-Obeng et al. 2014; Sadee 1999; Spear 2001; Spear et al. 2001).

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Drugs are metabolized and interact with proteins encoded by an individual's genome. Specific genetic variants can dramatically affect the conversion of predrugs into their active forms as well as the breakdown of drugs in the body. Variability in response to therapy is an accepted feature of virtually all drug treatments. The adoption of contemporary molecular biological tools available since the 1980s has resulted in the definition of key genes mediating drug metabolism, transport, and targets. Specific variants in at least 84 genes are known to impact on metabolism of over 100 drugs and most individuals encode at least one of these variants (Frueh et al. 2008; Relling and Klein 2011). Association of severe adverse drug reactions (ADRs) has been demonstrated for some of these variants (Pirmohamed et al. 2004). Recently, the application of high throughput sequencing has resulted in identification of large numbers of rarer variants in these genes (Drogemoller et al. 2011; Feero et al. 2010; Forde and O'Toole 2013; Koboldt et al. 2013; Mardis and Dhillon 2014; Mardis 2013; Pareek et al. 2011; Shendure and Ji 2008). Collectively, these less common variants seem likely to contribute to variable drug responses in an individual but are not incorporated into current genotyping platforms.

Clinical use of genomic markers can be considered in two broad contexts. A **reactive** strategy involves genotyping specific variants in individual patients at point-of-care, with clinical action responsive to results as they become available. An alternative, **preemptive** strategy performs genotyping of specific variants in individual patients in advance with routine cache of dense genotypic information in electronic health record (EHR) systems, thereby allowing genotype-informed guidance to be delivered to health care providers at the point-of-care in real time. Further, due to the comprehensive nature of the available genetic data, incidental findings may also be identified (Haga and Moaddeb 2014). Each of the aforementioned strategies has benefits and shortcomings. Aligning ourselves with a PM approach that is preventive, predictive, personalized, and participatory (Hood et al. 2012), we advocate preemptive (or predictive) genomic testing (PGx). This approach includes proactive identification of patients with a significant likelihood of benefit from available, personal genetic data prior to disease onset, therefore actionable for preventive or therapeutic measures.

Clinical decision support (CDS) systems are an essential feature in most EHRs and assist clinicians with clinical decision-making at point-of-care. CDS alerts generally follow two forms: passive or active (Bell et al. 2014). **Passive CDS alerts** provide information to a clinician in a way that does not interrupt the clinical workflow and may include as examples: patient reports, order sets, or clinical practice guidelines. **Active CDS alerts** interrupt the clinician's work flow and require that some type of action be taken or acknowledgement given before the workflow can continue. Most PGx CDS implementations have opted to use active CDS in order to provide information on the right drug and the right dose at the right time to a clinician prescribing the medication (Bell et al. 2014; Shuldiner et al. 2014; Johnson et al. 2013; Pulley et al. 2012; Bielinski et al. 2014).

Given the role of Adverse Drug Reactions (ADRs) as a major cause of morbidity and mortality (Lazarou et al. 1998; Pirmohamed et al. 2004), the increasing number of recognized variants included in FDA labels as mediators of both efficacy and toxicity [(Pirmohamed et al. 2004) and the relative lack of stigma attached to being identified as a carrier of variants in "pharmacogenes"] (McLeod and Evans 2001; Wang et al. 2011) we posit a high level of benefit associated with implementing PGx alerts into the EHR. Recognizing this inherent potential, the National Institute of Health (NIH) has funded several research networks to identify and investigate obstacles surrounding the integration of genetics into healthcare. Table 1 provides a brief description of these networks and their focus of genomic medicine research. These networks have also developed tools that can assist others in understanding various aspects of genomic medicine.

Concurrently, a limited number of institutions engaged in developing PGx systems to alert physicians of possible adverse events due to variant-drug interactions. Table 2 provides an overview of

Table 1 National Institute of Health research initiatives focused around genomic medicine. Many of these networks have publicly available resources available

Network	Sponsor	Year created	Goal	Available tools	References
Pharmacogenomics Research Network (PGRN)	National Institute of General Medical Sciences	2000	Focused on understanding how individual genetic makeup influences response to medication. Network resources include: Next-Gen sequencing, ontology, statistical analysis, large EHR populations and global alliances along with several scientific research projects.	Pharmacogenomics KnowledgeBase (PharmGKB) (Thorn et al. 2010) – knowledge resource that encompasses clinical information regarding dosing guidelines and drug labels, potential clinically actionable gene-drug associations and genotype-phenotype relationships. https://www.pharmgkb.org/	Shuldiner et al. (2014)
Clinical Pharmacogenetics Implementation Consortium (CPIC)	Collaboration with PGRN.	2009	Formed to address the need to provide evidence-based guidelines to facilitate the translation of pharmacogenomics knowledge into clinical practice. CPIC created a framework that defines the types and levels of evidence needed to justify inclusion of pharmacogenomics into clinical practice	CPIC Dosing Guidelines https://www.pharmgkb.org/view/dosing-guidelines.do?source=CPIC	Relling and Klein (2011), Hewett et al. (2002)
Translational Pharmacogenomics Program (TPP)	National Institute of Health in collaboration with PGRN	2011	Program goal is implementation of CPIC gene-drug guidelines into patient care; identify barriers to implementation, followed by dissemination of real-world solutions	TPP lookup tables by gene which contain phenotype and clinical decision support information based on haplotypes and diplotypes. https://www.pharmgkb.org/page/tppTables	Shuldiner et al. (2013)
Clinical Sequencing Exploratory Research Program (CSER)	National Human Genome Research Institute and National Cancer Institute	2010	Program objective is to explore and share best practices in the integration and application of genomic sequencing into clinical care	CSER Resources – a variety of databases, tools and applications that provide annotated databases, tools and applications; https://cser-consortium.org/resources	Jarvik et al. (2014)
Electronic Medical Records and Genomics Network (eMERGE)	National Human Genome Research Institute	2007	Network is engaged in the development of best practices, using the EMR as a tool for genomic research and validating the concept that EMRs could successfully provide clinical genome-wide association studies (GWAS). eMERGE is advancing knowledge in EMR-based phenotyping, GWAS, genomic medicine implementations, ethical and regulatory issues and research on returning results to study participants	Phenotype KnowledgeBase (PheKB) – Phenotype knowledge base containing EHR developed phenotyping algorithms; https://phekb.org/ Sequence, Phenotype, and Pharmacogenomics Integration Exchange (SPHINX) – web-based tool for exploring data for hypothesis generation around drug response implications of genetic variation; https://www.emergesphinx.org/	Cronin et al. (2014), Gottesman et al. (2013a, b), Jarvik et al. (2014), McCarty et al. (2011), McGuire et al. (2011), Newton et al. (2013), Peissig et al. (2012), Rasmussen-Torvik et al. (2014), Clayton et al. (2010)

Table 2 Overview of institutions involved in pharmacogenomics clinical practice integration efforts

Institution/ Reference (s)	St Jude's[a–c]	Maryland[d]	University of Florida[e–g]	Vanderbilt PREDICT[h]	Mayo[i]	University. of Chicago[j–l]
Associated Network	PGRN	Not reported	UF Clinical Translational Science Institute	PGRN	eMERGE	Not reported
Genotyping approach	Preemptive	Preemptive	Preemptive	Preemptive	Preemptive	Preemptive
Drug-variant(s)	TPMT, CYP2D6, SLCO1B1, CYP2C19 Connected with 12 high-risk drugs and 55 clinical decision support rules	CYP2C19 Cardiac catheterization/ clopidogrel	CYP2C19 Percutaneous coronary intervention (PCI) clopidogrel	CYP2C19, SLCO1B1, VKORC1 & CYP2C9, CYP3A5	HLA-B*1502, HLA-B*5701, TPMT, IL28B, CYP2C19, CYP2D6, SLCO1B1	LTC4S, REN, ADD1, SLCO1B1, GNB3, KIF6, CYP3A4, CACNA1C, ADRB1, GRK4, ABCB1, CYP2C19, LDLR, GNB3, AGT, CYP2C9, VKORC1
Institution resources	CLIA/CAP lab offsite Robust Biomedical informatics/EHR Pharmacy support Institutional support Education strategy	CLIA/CAP lab onsite Biomedical informatics/EHR Pharmacy support Institutional support Education strategy	CLIA/CAP lab onsite Robust biomedical informatics/EHR Pharmacy support Institutional support Education strategy	CLIA/CAP lab onsite Robust biomedical informatics/EHR Pharmacy support Institutional support Education strategy Patient engagement QI vs. research	CLIA/CAP lab onsite Robust biomedical informatics/EHR Pharmacy support Institutional support Education strategy Prediction model CAB community engagement	CLIA/CAP l ab onsite Customized web based interface Genomic prescribing system (GPS) with virtual PGx support Institutional support Education strategy
Institution decision makers	PG4KDS team to pharmacogenetics Oversight Committee (varied specialty physicians, clinical pharmacists, pathology, clinical informatics, and an external advisor) Hospital Pharmacy and Therapeutic Committee Medical Exec Committee	Internal work group Physicians, cardiac catheterization lab, biomedical informaticians (CIO), directors and counselors of genetics, pathology, finance, etc.	Pharmacy and Therapeutics Committee PMP sub-committee	Leadership with clinicians, (cardiologists) geneticists, biomedical informaticians, user interface experts, pharmacogenomics/ pharmacists, ethicists, clinical pathologists, program manager, finance, etc. Pharmacy and Therapeutics Committee Post Therapeutics Sub-committee	Mayo Clinic Center for Individualized Medicine Pharmacogenomics Task Force Disease Oriented Task Force under Mayo Pharmacy Formulary Committee Mayo CDS Subcommittee and Pharmacy and Therapeutics Committee	

Clinical decision support method(s)	***Active CDS*** *and* ***Static CDS*** ***Active CDS*** intercepts provider at POC pre- and post-test when hi-risk drug prescribed. Pretest alerts issued if very hi-risk drug prescribed. Reviewed by pharmacogenetic pharmacist. ***Static CDS*** offers general gene-based Rx guidance Email used for numerous supplemental CDS alerts. Patient may opt for letter with genotype results and relevance to med use High risk genotypes are entered into EHR Problem List. Decision Support links high-risk genotypes to drug ordering, prescribing and administration	***Active CDS*** in 2 groups: (1) intercept provider at POC (some manual, call, fax, email) d/t EHR systems and external lab use (2) intercept provider at POC with CDS in EHR, call pharmacist	***Active CDS*** electronic to physician and cardiovascular clinical pharmacist Best Practice Advisory alerts are presented to ordering physician in the EHR when clopidogrel order is linked with an actionable CYP2C19 genotype	***Active CDS*** Pharmacogenomic information is entered into EHR and the POC decision support pops up when a physician enters a drug that might conflict with the patient's genotype. People who participate may access their results online	***Active CDS*** RIGHT protocol Synchronous real-time CDS integrated in EHR flags potential patient-specific drug-gene interactions inpatient and outpatient order entry and provides therapeutic guidance including link to additional related info in "AskMayoExpert." Results available to patients online and genetic counseling services available	***Static CDS*** Results not integrated into EHR; separately available in a genomic prescribing system (GPS) as traffic lights: green = favorable, yellow = caution, and red = high risk Additional information available as virtual PGx consult Physicians "reminded" of availability for enrolled patients
Reported results	Interruptive CDS appropriately guided RX in 95% for whom issued	31.9% enrollees IM/PMs, 27 of these = PCI of which 17 (63%) RX alternative	1,097 preemptively genotyped possible PCI patients in one year. 80 of 291 PCI patients had an actionable genotype with drug therapy changes implemented in 56 individuals	Not reported	Not reported	Results were delivered via GPS for 86% of enrolled patients seen in clinic. 57% of results accessed were green with 20% further info sought; 41% of results accessed were yellow with 72% further info sought; 1.4% results accessed were red; 100% further info sought

[a]Bell et al. (2014), [b]Hoffman et al. (2014), [c]Relling and Klein (2011), [d]Shuldiner et al. (2014), [e]Johnson et al. (2012), [f]Johnson et al. (2013), [g]Weitzel et al. (2014), [h]Pulley et al. (2012), [i]Bielinski et al. (2014), [J]Dolan et al. (2013),[k]O'Donnell et al. (2012), [l]O'Donnell et al. (2014)

Table 3 Challenges identified from PGx integration efforts

Interpretation of variants	Lab and quality control	Institution reluctance	EHR	Miscellaneous
Lack of consensus guidelines on what to recommend for high-priority/ high-risk diplotypes[a–c] Many diplotypes are ambiguous[a–c] Interpretation of diplotypes is complex and will change[a–c] Paucity of clear recommendations for pharmacogenetic testing by professional associations[d] Accuracy of genetic testing[a–c] PGx test availability and delay in result reporting [k]	Must be mechanism for QC, sample identity and genotype reproducibility[a–c] CLIA lab use logistics[d] Preemptive chip based genotyping feasibility [e–g] Establishment of a validated custom designed genotyping panel to generate accurate genotype calls in a CLIA environment was "not trivial"[j–l] Continuous assay performance and monitoring CLIA lab[e–h]	Commitment and collaboration across disciplines and leadership is critical[h] While evidence for genetic predictor to Clopidogrel response is strong, controversy remains over utility of genotyping[h] Prescriber uncertainty re benefits, both clinical and economic, for genome guided therapy[i,k] Lack of prospective genotype directed RCTs validating the advantage of using pharmacogenetic based dosing over soc treatment algorithms[d] Regulatory body to define clinically actionable pharmacogenetic cases [e–g]	Must be able to update information (e.g,. add drugs to genes as data accumulate)[a–c] Alert fatigue considerations[a–c] EHRs do not support formats to record genetic results[d] Lack of robust infrastructure to provide decision support for genomic medicine[d] Developing and refining decision support rules is time consuming[h] Lack of support for commercial EMRs to integrate large scale genomic data linked to automated CDS[i] Development of quality CDS[i] Suggested treatment algorithms[a–c] Methodologies to report results[a–c]	Education and experience of clinicians interpreting and acting on pharmacogenetic information is lacking [d] Cost and reimbursement issues [d,k] Ethical and medico-legal concerns[d] Education to phlebotomists critical d/t specific requirements of handling genotype samples[e–g] Ethical, legal, social and financial concerns re genomic medicine by patients and families.[i] Delay in treatment when traditional reactive ordering of PGx testing at POC used[i] Lack of physician knowledge[k]

[a]Bell et al. (2014), [b]Hoffman et al. (2014), [c]Relling and Klein (2011), [d]Shuldiner et al. (2014), [e]Johnson et al. (2012), [f]Johnson et al. (2013), [g]Weitzel et al. (2014), [h]Pulley et al. (2012), [i]Bielinski et al. (2014), [j]Dolan et al. (2013),[k]O'Donnell et al. (2012), [l]O'Donnell et al. (2014)

these efforts. Of the institutions highlighted, all used a preemptive genomic testing strategy making PGx information available and actionable at point-of-care. In most models, the electronic health record (EHR) was used to deliver a PGx alert when the physician prescribed a medication, although one institution developed a customized web-based interface to a separate genomic prescribing system which did not trigger as an alert but was available for providers choosing to access it. All institutions used a multidisciplinary committee (comprised of physicians, pharmacists, pathologists, genetic counselors, and informaticians) to guide decisions surrounding the implementation of PGx alerts.

Challenges encountered by institutions implementing PGx systems have been similar and focus on themes of variant-drug interpretations, laboratory and quality control requirements, institutional support and acceptance, physician education, and EHR integration (Table 3). Key activities that support the adoption of PGx systems include patient and healthcare provider engagement, identifying specific relevant genetic variants for clinical implementation, establishing assay reliability, developing point-of-care

decision support, and employing additional personnel and instrumentation (Bell et al. 2014; Bielinski et al. 2014; Dolan et al. 2013; Hoffman et al. 2014; Johnson et al. 2012; Johnson et al. 2013; March 2011; Pulley et al. 2012; Ramos et al. 2014; Relling and Klein 2011; Shuldiner et al. 2014; Weitzel et al. 2014).

Marshfield Clinic Experience

Marshfield Clinic developed a proof-of-concept project to begin to define processes and procedures required to: (1) determine which variants to test for clinical use, (2) establish Clinical Laboratory Improved Amendment- Certified (CLIA) testing and validation, (3) develop PGx variant translation and clinical decision support (CDS) architecture, and (4) implement and evaluate such architecture. A review of resources, outline of procedure, and an update on the progress of these initial and ongoing efforts follows.

Marshfield Clinic (MC) is a regional health care system that serves the population of northern, central, and western Wisconsin and the Upper Peninsula of Michigan. Primary and specialty care services are offered to patients at regional outpatient clinics, and care is often integrated with local hospitals in order to offer high quality health care service access to patients residing in these rural and underserved areas.

MC has over 3.5 million patient encounters reported annually and relies on the EHR to coordinate care efforts, document diagnoses, observations and treatment, and provide CDS to its physicians. Cattails, the internally developed EHR at MC, has been in use since the mid-1980s, having available coded diagnoses dating back to 1963. Cattails is used by over 17,000+ users at 55+ MC rural outpatient clinics, three hospitals, a diagnostic treatment center, and a variety of non-Marshfield owned outpatient clinics and hospitals. This full feature EHR is deployed into clinical practice on laptop, tablet, and PDA technology and supports a variety of clinical decision support (CDS) applications facilitating care management, prevention, radiology test ordering, and medication prescribing.

Marshfield Clinic has a long history of genetics research. In 2002, the Personalized Medicine Research Cohort was established and included approximately 20,000 consented individuals that provided DNA, plasma, and serum biological samples. The participants in the project have on average 30 years of EHR data and over 10,000 participants have dense phenotyping by Illumina's HumanCoreExome BeadChip.

MC has developed an environment to expand genomic discovery research and implement best practices in genomic medicine into the EHR while engaging patients and providers. MC's proof-of-concept has focused on three known variant-drug applications as determined by the FDA and Clinical Pharmacogenetics Implementation Consortium (CPIC) and includes the following: (1) SLCO1B1 and Simvastatin, a cholesterol lowering medication; (2) CYP2C19 and Clopidogrel, a medication given to patients with a recent placement of drug-eluting coronary artery stents; and (3) CYP2C9, VKORC1, and Warfarin, a medication given to thin the blood. Herein lays a description of the approach used and resources developed at Marshfield Clinic to support the genomic medicine initiative.

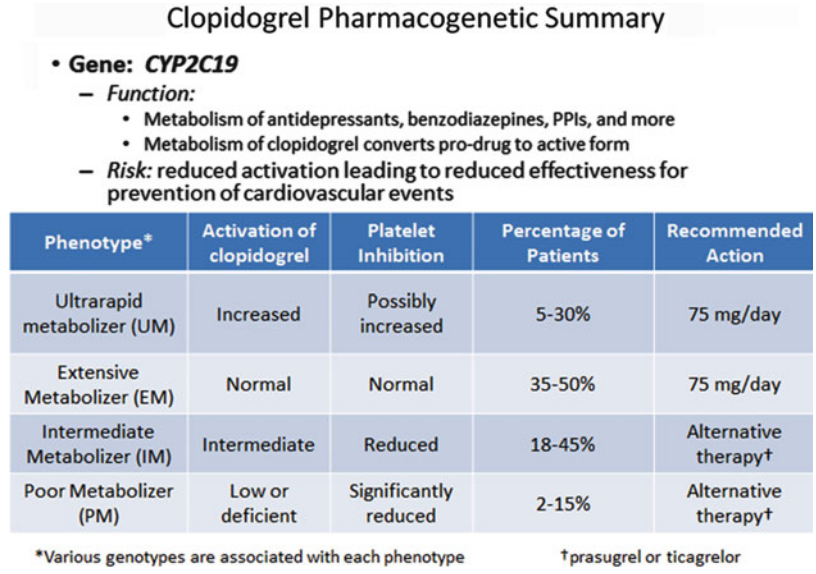
Procedure

The primary goal of this work was to integrate validated genetic sequence data into the clinical EHR environment at MC and assess uptake, acceptance, and clinical value with the ultimate goal of improving patient care. Marshfield Clinic Institutional Review Board approved this research prior to protocol execution. To advance this proof-of-concept, 750 subjects greater than 50 years of age with a high likelihood of being prescribed simvastatin, clopidogrel, and/or warfarin were recruited for the study.

Determination of Variants to Test for Clinical Use

In collaboration with other eMERGE and PGRN institutions, a PGRN-Seq assay was defined

Fig. 1 Example of the clopidogrel-CYP2C19 translation



Clopidogrel Pharmacogenetic Summary

- **Gene:** ***CYP2C19***
  - *Function:*
    - Metabolism of antidepressants, benzodiazepines, PPIs, and more
    - Metabolism of clopidogrel converts pro-drug to active form
  - *Risk:* reduced activation leading to reduced effectiveness for prevention of cardiovascular events

Phenotype*	Activation of clopidogrel	Platelet Inhibition	Percentage of Patients	Recommended Action
Ultrarapid metabolizer (UM)	Increased	Possibly increased	5-30%	75 mg/day
Extensive Metabolizer (EM)	Normal	Normal	35-50%	75 mg/day
Intermediate Metabolizer (IM)	Intermediate	Reduced	18-45%	Alternative therapy†
Poor Metabolizer (PM)	Low or deficient	Significantly reduced	2-15%	Alternative therapy†

*Various genotypes are associated with each phenotype †prasugrel or ticagrelor

consisting of 84 genes with high impact in PGx. The panel included gene encoded systems for drug metabolism and transport, as well as drug targets selected by PGRN investigators and other modulators of drug action representing the spectrum of scientific interests across the PGRN.

DNA samples were collected from consented individuals and then made available for CLIA-certified testing per an approved protocol. The Center for Inherited Disease Research (CIDR), a CLIA certified genotyping facility, genotyped half of the samples using the PGRN-Seq assay. The remaining samples were genotyped at MC laboratories, using the same assay with a Sequenom Mass Array genotyping platform. Genotyping lab procedures included validation of assay results by comparison to reference samples to ensure continued reproducibility.

Clinical Oversight

As in most clinical environments, there are oversight committees established to examine the safety and effectiveness of various clinical interventions prior to use in clinical practice. A PGx Committee was formed to evaluate the significance, merit, and proposed efficacy of PGx variant-drug alert candidates and proposed uncommon variant interventions. Organizational stakeholders including laboratory and pharmacy operations, prescribing physicians, clinical genetics, genetic research, and informatics were members of the PGx Committee.

A subgroup of PGx Committee members (pharmacists, laboratory administration, researchers, and medical geneticists) reviewed existing evidence and made recommendations on variant-drug phenotype interpretations, alert actions, and clinical practice guidelines for each PGx variant-drug pair under review (Fig. 1). The PGx Committee approved the translation and presentation of PGx verbiage for the alerts prior to EHR integration. Recommendations were presented to the Safety Committee for final review pending approval.

Establish CLIA Certified Testing and Validation

A Sequenom Mass Array genotyping platform was used for assay processing. Genotyping laboratory procedures included the validation of assay results (e.g., >99% correlation with a specific genotyping platform(s) such as the ADME and DMET panels) by comparing the results to reference samples, and a panel of multiethnic HapMap

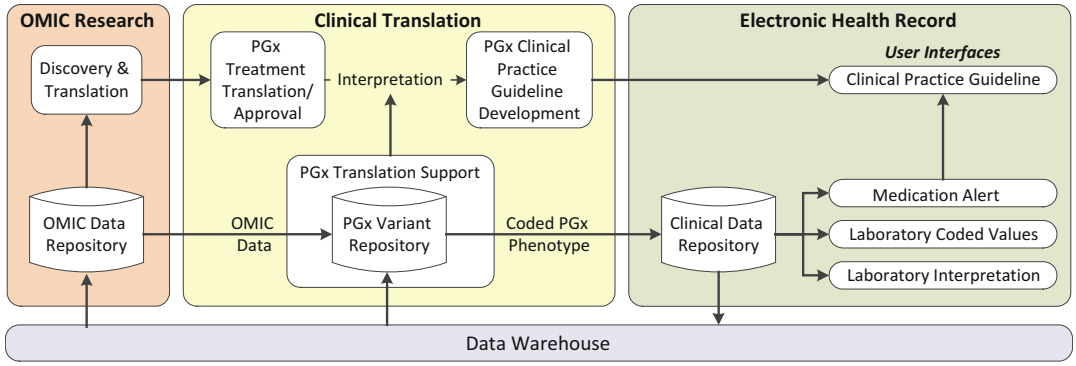


Fig. 2 Overview of computing environment required to support PGx variant-drug translation and integration into clinical practice

trios, prior to use in the CLIA-certified laboratory. Periodic reassays of samples with known results were also undertaken to ensure continued reproducibility.

CLIA system testing ensures the integrity of the data as it passes from one system to another and is presented to a clinician. We utilized workflow diagrams to identify points in the system in which data was either transformed or transferred between information systems. These diagrams guided the development of use cases that tested program logic and assignment of interpretations for PGx alerts. A dataset representative of the various use cases was prepared and used during CLIA-certification testing after software development was complete. Laboratory compliance staff documented the results of the CLIA testing prior to clinical use of the system.

Development of PGx Variant-Drug Translation and CDS Architecture

Following commonly used information system development principles, PGx system requirements were defined by a committee of stakeholders. An analysis of existing resources (laboratory, EHR, data storage, and computing environments) and clinical workflow identified areas for EHR integration and gaps in the areas of data storage, translation capability, and PGx alerting. From this analysis, a four-tier computing architecture was designed (Fig. 2) to support new genomic discovery, translation of variant-drug pairs, and EHR integration.

A well-accepted axiom in informatics is that it is difficult, if not impossible, for a single database to perform optimally for single patient queries (retrieving all the data for one patient) and cross-patient queries (such as finding all patients with specific condition, e.g., diabetes). Marshfield Clinic has utilized a model of maintaining similar copies of clinical data: a Clinical Data Repository (CDR) supporting the EHR that is optimized for single patient queries and a Data Warehouse (DW) supporting research and population evaluation activities that is optimized for cross-patient queries. Due to the needs of personalized medicine two additional tiers were added: an OMIC Research area featuring an OMIC Data Repository for OMIC research and data storage and a Clinical Translation area highlighting a PGx Variant Repository and PGx Translation Support to translate variant-drug pairs for patient care.

The OMIC data repository requires data storage to hold large amounts of "raw" or unprocessed OMIC data and computing resources to support computationally intense OMIC translations. OMIC data is transferred into the Clinical Translation area when a variant-drug pair is deemed to have clinical relevance. Software residing in the Clinical Translation tier evaluates patient-specific variant-drug pairs to determine the PGx phenotype for a drug. Software (using approved PGx

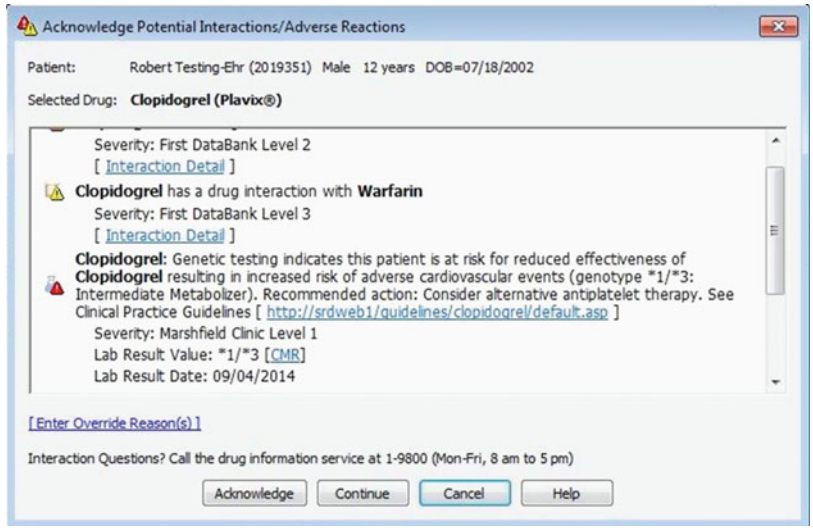


Fig. 3 Example of a CYP2C19 variant PGx alert for Clopidogrel

variant-drug guidelines) classifies the PGx phenotype and stores it as a PGx laboratory result in the CDR. The coded PGx laboratory result is made available to several clinical user interfaces for interpretation. A software application, developed to extend the functionality of the existing Medication Alert CDS system, checks the CDR for a clinically relevant PGx-laboratory result when a physician prescribes a PGx-related medication to the patient. If a PGx phenotype exists the resultant action provides an alert to the physician suggesting an alternative course of treatment.

It is a well-known fact that clinicians can suffer from alert fatigue due to the presentation of nonaction oriented alerts (Kesselheim et al. 2011; Slight et al. 2013; van der Sijs et al. 2006; Yeh et al. 2013). We limited alerts to those that required some type of action or change to the current prescribing standards. The PGx alerts are context sensitive and can be viewed in the medication prescribing application when the PGx medication is prescribed and the patient has a variant that requires either switching to another medication or change in dosage. Figure 3 shows an example of a Clopidogrel PGx alert. Embedded in the PGx alert is a link to supporting reference materials to facilitate ease in finding additional information. Implementation of the PGx alerts was preceded with communications to physicians via an internal news publication, EHR change announcement, and a Grand Rounds presentation. The Marshfield Clinic EHR is also used by non-Marshfield physicians so a communication was distributed outlining the changes made to the system.

Implementation of PGx Computing Architecture

A three-step process involving organizational approval, variant-drug translation, and EHR implementation tasks was developed to accommodate new PGx integration efforts or to modify existing PGx interpretations. Communication and education efforts are required throughout the process to ensure organizational support. Figure 4 provides an overview of the implementation process.

Members of the PGx Committee discuss and approve proposals for new PGx alerts. The pharmacy and medical genetic staff prepare PGx translation materials used to create the PGx Clinical Practice Guideline and interpretation materials. Informatics and laboratory staff develop computerized-PGx variant-drug translations that reflect the interpretations approved by the PGx Committee and assign appropriate PGx phenotype codes that reflect each unique variant-drug phenotype. Databases are modified to reflect the

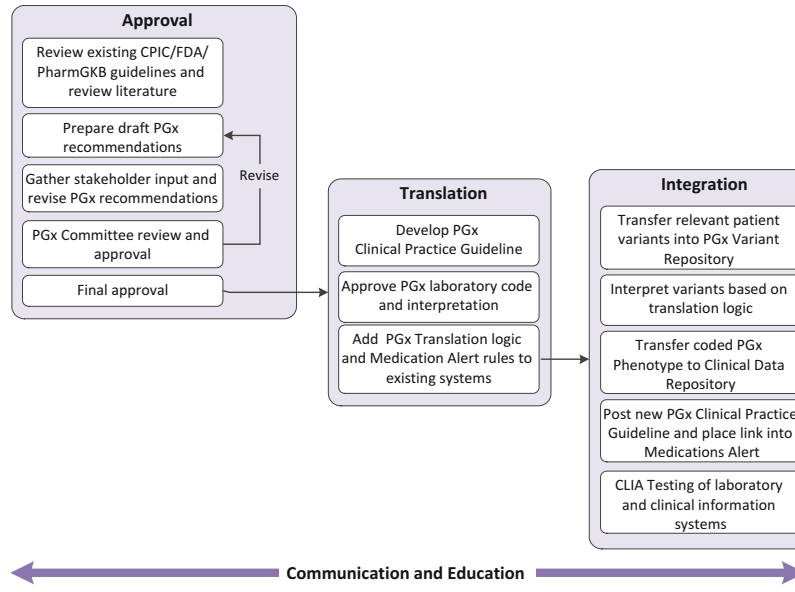


Fig. 4 PGx Alert Implementation Process

Table 4 Evaluation metrics and progress

Evaluation type	Metric type	Metric description	Progress
Assay results	Process	Establish and maintain quality control in a CLIA environment Report genotype and diplotype results for actionable variants	Complete
		Report the number, in silicon predictions of function(GERP scores, polyphen, etc.) for variants of uncertain significance	Not evaluated
Clinical Operations	Process	How frequently was a decision support module deployed? For which drug-gene pairs What action was taken by the physician? Were other consultations requested/deployed?	Not evaluated
Patient perceptions and concerns	Attitudes	Surveys prior to implementation and at 2 years	In progress

changes and CLIA testing ensues. Upon successful completion of testing, the systems are released into the production environment and made available for use by laboratory and clinical staff.

Evaluation

This proof-of-concept study recruited 750 participants for preemptive genomic testing. Variant-drug combinations for SLCO1B1-simvastatin, CYP2C19-clopidogrel, and CYPC9 and VKORC1-warfarin were evaluated resulting in 1114 active PGx alerts being placed in the EHR for the study participants. As part of eMERGE, we defined process and outcome metrics to assess the effectiveness of the PGx integration effort. Table 4 describes some of the evaluation metrics relevant to this effort and progress to date. Due to recent implementation many of the measurements are pending and not currently available.

Modifications of the Method

An alternative approach is *reactive* genomic testing which genotypes specific variant(s) of an individual at point-of-care with clinical action responsive to the results when they become available. This reactive approach has shown to be cost

effective by reducing hospitalizations and adverse events (Epstein et al. 2010; Nutescu et al. 2013; Roberts et al. 2012) but can become costly if multiple genetic tests are needed for an individual. Preemptive testing has limited evidence on cost effectiveness to date (Grosse 2014). Some speculate that preemptive testing may be more cost effective in individuals that require multiple genetic tests. More research is needed to assess the cost effectiveness of both approaches and develop best practices for the delivery of genomic medicine.

Conclusion

Much has been accomplished toward implementing personalized medicine, yet progress is pedantic and ongoing challenges remain requiring continuous collaboration among researchers, health care professionals, and patients. This report describes a personalized medicine demonstration project focused on using preemptive genetic testing to guide the development of PGx alerts in the EHR. A four-tier computing environment was established to support the variant-drug translation and integration into clinical practice. Involvement of stakeholders and continuous communication and education is pivotal to the success of the PGx implementation. Although not addressed in this chapter, ethical, legal, social, and financial implications should be considered when designing any personalized medicine intervention.

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Herbal Medicine

Jufeng Wang, Fanghua Huang, and Wei Li

Contents

Herbal medicine played an important role throughout human history as a way to cure and prevent diseases and has a longer history than chemical drugs and biological products. Herbal medicine has different definitions and different scopes in different countries. It is usually called botanical drug product in the USA and herbal medicinal product in the EU. In China, it is usually called traditional Chinese medicine (TCM).

In the modern medicine system, herbal medicine plays different roles in the health-care system in different countries. Herbal medicines usually are marketed as food or dietary supplements rather than as drugs. However, they play a similar role as chemical drugs in China due to their special application background in China. Because of the different roles of herbal medicines in different cultures, there are different regulations for herbal medicines in the USA, the EU, and China. In this chapter, the different regulatory requirements in the EU, the USA, and China are described, especially for preclinical safety evaluation.

Regulatory Requirement for the Safety of Herbal Medicine in the USA

A botanical product may be a food (including a dietary supplement), a drug (including a biological drug), a medical device (e.g., gutta-percha), or a cosmetic under the law in the USA. Which way a botanical product is marketed depends on its intended use, and most of botanical products are

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marketed as dietary supplements. The Food and Drug Administration (FDA) is cautious in its regulation of herbal medicine (called botanical drug product) and had no specific regulatory requirements for them in the twentieth century, resulting in the relatively slow R&D of new herbal medicines int he USA before 2000.

A drug is defined as articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease or articles intended to affect the anatomy or any function of the body under the Federal Food, Drug, and Cosmetic Act (FD&C Act). Dietary supplements are considered as medicine only if they are stated to prevent and treat diseases under the Dietary Supplement Health and Education Act, USA (DSHEA), which was issued in 1994. As we all know, many botanical products were used as drugs, although they are marketed as dietary supplements and lack sufficient safety and efficacy evidence. However, the use of traditional herbal medicine (especially TCM) is increasing over the years in health-care practices, and their therapeutic value is being acknowledged; In the early 2000s, the FDA began to reexamine its regulatory policies for herbal medicines. In July 2004, the Center for Drug Evaluation and Research (CDER), FDA, issued Guidance for Industry Botanical Drug Products. It is the first guidance for the regulation of botanical drug products, and it is significant for its acknowledgement of herbal medicine's role and value.

On October 31, 2006, Veregen was approved by FDA as the first botanical drug 2 years later after the guidance was issued. Veregen is indicated for the topical treatment of external genital and perianal warts (condyloma acuminata) in immunocompetent patients 18 years and older. The second botanical drug that was approved on November 31, 2012, was Fulyzaq (crofelemer). Fulyzaq, which comes from *Croton lechleri*. It is the first drug approved for HIV-related diarrhea for HIV/AIDS patients and the first oral botanical drug.

Guidance for Industry Botanical Drug Products interprets FDA regulation of botanical products. If a botanical product is intended for use in diagnosing, mitigating, treating, or curing disease, it is a drug under the Act. The term botanicals includes plant materials, algae, macroscopic fungi, and combinations thereof. It does not include materials derived from genetically modified botanical species, fermentation products (including when plants are used as a substrate), highly purified substances (e.g., paclitaxel), or chemically modified substances (e.g., estrogens synthesized from yam extracts) derived from botanical sources. It does not include materials derived from animals and minerals.

In general, the FDA looks at botanical drug products the same as chemical drugs, which should meet the requirements for CMC control and clinical data. The FDA recognizes that botanical drugs have certain unique characteristics and difficulties in R&D that should be taken into account when regulating botanical drug products. FDA considers it appropriate to apply regulatory policies that differ from those applied to synthetic, semisynthetic, or otherwise highly purified or chemically modified drugs (including antibiotics derived from microorganisms). But these adjustments only apply to the IND for the initial clinical trial. When conducting expanded (i.e., phase 3) clinical studies or submitting for marketing approval as a drug for a botanical drug product, it will be treated like any other new drug under development.

A botanical drug product may be marketed in the USA under (1) an OTC drug monograph or (2) an approved NDA or ANDA. A botanical product that has been marketed in the USA for a certain time and extent for a specific OTC drug indication may be eligible for inclusion in an OTC drug monograph, and it may be marketed under FDA's OTC drug monograph system. However, most of botanical drug products cannot be applied as an OTC drug because there are very strict limitations, and a sponsor must submit an NDA to obtain FDA approval to market the product. The requirements of pharmacology and toxicology for IND will be described as follows.

For IND of botanical products, FDA recognizes that preclinical pharmacological and toxicological studies are useful in guiding early clinical studies and in predicting the potential toxicity of a

new drug, but because of special characteristics of botanical products, less nonclinical information are necessary to support the initial clinical trials (including phase I and phase II). For a botanical product that is not currently lawfully marketed in the USA, but is administered orally and prepared, processed, and used according to methodologies for which there is prior human experience, sufficient information may be available to support initial clinical studies without standard nonclinical testing. However, for a botanical drug with a route of administration other than oral, additional pharmacology/toxicology information may be necessary before initial clinical studies. The demand for preclinical pharmacology/toxicology studies in USA are varied and depend on whether the products have been marketed in the USA or abroad, whether they are traditional preparations, or whether they have safety issues.

Initial studies for botanical products with no known safety concerns and that have been marketed in the USA as dietary supplements may generally be conducted without further pharmacological/toxicological studies. Nevertheless, all available information should be provided. A database search should be conducted, when feasible, to identify information relevant to the safety and effectiveness. Using the information gathered from literature, the sponsor should address, as appropriate for the proposed study, the following safety issues concerning the botanical drug products: general toxicity; target organs or systems of toxicity; teratogenic, carcinogenic, or mutagenic potential of any botanical ingredient in the product; relationship of dosage and duration to toxic responses; and pharmacological activity.

Additional information must be provided for a botanical product that has been previously marketed but not in the USA with no known safety concerns. The amount of preclinical pharmacology/toxicology information needed before a sponsor conducts an initial clinical study will be determined on a case-by-case basis, depending on the indications, proposed dose, duration and size of the study, and available data supporting safe human experience.

For the botanical drug product that is not currently lawfully marketed in the USA or elsewhere but is prepared, processed, and used by humans according to an established methodology, sufficient information might be enough to support clinical studies without standard nonclinical testing. However, for a botanical product that is not prepared according to a traditional methodology, information in addition to that described above is required. The nature of preclinical pharmacology/toxicology information needed before conducting an initial clinical study will be determined on a case-by-case basis, depending on the indications, extent of safe human experience, and safety concerns about the new formulation, preparation, or processing methodology used.

For the initiation of clinical studies on botanic drugs with known safety issues, the nature of the preclinical pharmacology/toxicology information needed will be determined on a case-by-case basis to address those issues.

For INDs for expanded (i.e., phase 3) clinical studies or NDAs of all botanical drug products, toxicity data from standard toxicological studies in animals may be needed to demonstrate safety for expanded clinical studies or to support marketing approval of a botanical drug product, which is treated like any other new drug under development. Previous human experience may be insufficient to demonstrate the safety of a botanical drug product, especially when it is indicated for chronic therapy, so systematic toxicological evaluations may be needed to supplement available knowledge on the general toxicity, teratogenicity, mutagenicity, and carcinogenicity of the final botanical drug product. Depending on the indication (e.g., target patient population, disease to be treated), route of administration, and duration of recommended drug exposure, the timing of these animal studies in relation to concurrent clinical trials and other requirements for nonclinical animal studies can vary. In general, animal studies should, as much as possible, be conducted using the same drug substance prepared and processed in the same manner as the drug substance used in clinical trials. Registration of Pharmaceuticals for Human Use (ICH) guidances should be referenced when conducting toxicological studies.

Regulatory Requirement for Safety of Herbal Medicine in the EU

Botanical drugs are usually called herbal medicinal products or herbal medicine in Europe. Herbal medicinal products have been used widely in EU countries. However, different countries have their own regulations for herbal medicine. For uniform regulations and requirements for herbal medicinal products, the Herbal Directive (Directive 2004/24/EC) was adopted to facilitate the introduction of traditional herbal medicinal products to the market by the European Parliament and the Council on March 31, 2004. A Committee on Herbal Medicinal Products (HMPC) was set up at the European Medicines Agency (EMA) in September 2004 in view of the establishment of an EU list of herbal preparations or herbal substances by the European Commission. HMPC is responsible for compiling and assessing scientific data on herbal substances, preparations, and combinations with a focus on safety and efficacy.

Directive 2004/24/EC aims to regulate herbal medicinal products. It is amendment to Directive 2001/83/EC, which is a directive that regulates medicinal products for human use. Directive 2004/24/EC gives the definition of herbal medicinal product: any medicinal product exclusively containing active ingredients of one or more herbal substances or one or more herbal preparations or one or more such herbal substances in combination with one or more such herbal preparations. The Directive classifies the scope of traditional herbal medicinal product. A simplified registration procedure is established for herbal medicinal products which fulfill all of the criteria. For a simplified registration procedure, the Directive notes that the long tradition of the medicinal product makes it possible to reduce the need for clinical trials, insofar as the efficacy of the medicinal product is plausible on the basis of long-standing use and experience, and preclinical tests do not seem necessary, where the medicinal product on the basis of the information on its traditional use proves not to be harmful in specified conditions of use. However, even a long tradition does not exclude the possibility that there may be concerns with regard to the product's safety, and therefore the appropriate authorities should be entitled to ask for all data necessary for assessing the safety. In summary, for a traditional herbal medicinal product, if an applicant can demonstrate by detailed references to published scientific literature that the product has acceptable level of safety within the meaning of Directive 2001/83/EC, preclinical toxicity data is not necessary.

However, if a herbal medicinal product cannot fulfill the criteria of traditional herbal medicinal product, the product must meet the requirements of a full marketing authorization similar to other medicinal products rather than the simplified procedure. The sponsor needs to systematize toxicological and pharmacological evaluations which meet CTD format under Directive 2001/83/EC. For this kind of herbal medicinal product, the demands are strict, the same as chemical drugs. Toxicological data includes single-dose toxicity; repeated-dose toxicity; genotoxicity, carcinogenicity, reproductive, and development toxicity; local tolerance; and other toxicity studies.

Regulatory Requirement for the Safety of Herbal Medicine in China

In China, herbal medicine is usually called TCM. Because TCM has a long history for thousands of years and plays an important role in Chinese health care, the regulations for TCM are different from other countries, especially the USA and EU countries. TCM, chemical drug, and biological products constitute the drug system of China, and they have the same statutes. With the implementation of the Drug Administration Law of the People's Republic of China and the foundation of Drug Evaluation Agency in 1985, China has built a fairly comprehensive regulatory system for

TCM through 30 years of continuous development and improvement.

The Scope, Regulation System, and Registration Procedure of TCMs

The scope of herbal medicine is different from other countries. Broad definition of herbal medicine includes TCM and natural drugs. Under Provisions for Drug Registration (2007), TCM refers to the medical substance and its preparation used under the guidance of Chinese traditional medical theory. Natural drugs refer to the medical natural substance and its preparation used under the guidance of modern medical theory. Most of the applications are TCMs, and few are natural drugs in the recent 10 years of botanical drug application.

TCM review and approval process are consistent with the chemical drugs and biological products, and it can be divided into IND and NDA, namely, the application for clinical trials and application for marketing. China Food and Drug Administration (CFDA) is responsible for drug regulation. The Center for Drug Evaluation (CDE), CFDA, is responsible for the technical review of a drug including TCM.

Regulation and Guidance on TCMs

Provisions for Drug Registration which promulgated in 2007 is the most important directive on drug regulations. It includes the demands for all kinds of drug application, including application procedure and data requirements. As a registration for drug, both the Chinese domestic and foreign applicants should comply with.

For specific requirements for all kinds of toxicity studies, China has issued a series of guidances to direct the development of R&D of new TCMs. Up to date, preclinical safety evaluation system has been established, and the frame is similar to ICH safety guidances, with the general principle of evaluation, keeping up with international requirements. When conducting a study, you should refer to the relevant guidance.

Registration Categories of TCMs

TCM is divided into different categories to facilitate registration management. Categories are divided mainly based on different sources (including medicinal source, components, nature of substances, etc.). Specific categories are as follows (see Table 1).

Pharmacology/Toxicology Data Requirements of TCMs

Pharmacology/toxicology data should be submitted for new drug application (IND and NDA) of new TCM as follows:

1. Summary of pharmacological and toxicological studies
2. Primary pharmacodynamics study and literature
3. Safety pharmacological study and literature
4. Acute/single-dose toxicity study and literature
5. Repeated-dose toxicity study and literature
6. Special safety study and literature of hypersensitive (topical, systemic, and phototoxicity), hemolytic, and topical irritative (the blood vessel, skin, mucous membrane, and muscle) reaction related to topical and systemic use of the drugs
7. Study and literature of genotoxicity
8. Study and literature of reproductive and development toxicity
9. Study and literature of carcinogenicity
10. Study and literature of nonclinical pharmacokinetics

As drug registration varies, preclinical safety evaluation documents required are different. This is mainly determined by the properties of material basis and clinical history and accordingly different risk management requirements. Specific requirements are as follows (see Table 2).

Table 1 Registration categories of TCMs

Registration category	Definition
1. Active ingredients and its preparation extracted from plant, animal, or minerals, which have not been marketed in China	Refer to the single component or its preparation, which are extracted from plant, animal, or minerals and not yet collected in the National Drug Standards, where the content of this single component should be more than 90 % of the extraction
2. Newly found drug material and its preparations	Refer to the drug material and preparations not yet collected in the National Drug Standards or provincial drug formulary (statutory standards)
3. New Chinese medicinal material substitute	Refer to a new Chinese medicinal material used to substitute the toxic drug material of the formula in the National Drug Standards or the endangered Chinese medicinal material, which is not yet collected by statutory standards
4. New part of Chinese medicinal material and its preparations	Refer to the new part of existing Chinese medicinal material from plants or animals, which is already in the statutory standards
5. New active part of Chinese medicinal material and its preparation, which are extracted from plant, animal, or minerals and have not been marketed in China	Refer to active parts of similar or multiple components and its preparation, which are extracted from plant, animal, or minerals and not yet collected in the National Drug Standards, where the active part should be more than 50 % of the extraction
6. Combined preparation of TCM or natural drugs, which are not yet marketed in China, include: 6.1 Combined preparation of TCM 6.2 Combined preparation of natural drugs 6.3 Combined preparation of TCM, natural drugs, and chemical drugs	Combined preparation of TCM should be formulated under traditional Chinese medical theory, including combined preparation of TCM from ancient classic formula, combined preparation with indication to syndrome, or combined preparation with indication to combine with disease and syndrome Combined preparation of natural drugs should be formulated under modern medical theory, where indication should be described in modern medical term Combined preparation of TCM, natural drugs, and chemical drugs includes combined preparation of TCM and chemical drugs, combined preparation of natural drugs and chemical drugs, and combined preparation of TCM, natural drugs, and chemical drugs
7. Preparations with change in route of administration of the TCM or natural drugs already marketed in China	Refer to the preparation which transfer route of administration or absorption location
8. Preparations with change in dosage form of the TCM or natural drugs already marketed in China	Refer to the preparation of change in dosage form but no change in route of administration
9. Generic drugs	Refer to the registration application of TCM or natural drug already approved to be marketed in China

Principle and Consideration for TCM Safety Evaluation

Nonclinical safety evaluation should comply with GLP under Provisions for Drug Registration. There are over 50 GLP Laboratories approved by CFDA which have abilities to conduct safety evaluation of TCMs.

Case-by-case is the principle of preclinical safety evaluation for TCM. Guidelines are the minimum requirements. Each TCM has its own characteristics, such as prescription form, different safety information, and so on. The sponsor should determine the appropriate studies and design of the program based on a case-by-case basis. When some problems are found or safety concerns arise, additional and/or follow-up studies may be required.

The test article for preclinical safety evaluation of TCM should be a full representative of the proposed clinical trial samples and/or the proposed marketing sample in CMC qualities. If the manufacturing procedure of herbal extracts and products changes during the clinical trial, nonclinical toxicological bridging studies should be considered and all nonclinical toxicological bridging studies may need to be repeated.

Table 2 Pharmacology/toxicology information required for TCM and natural medicine

Type	Item	Registry classification and application material requirements										
		1	2	3	4	5	6			7	8	9
							6.1	6.2	6.3			
Pharmacology/toxicology documents	1	+	+	*	+	+	+	+	+	+	±	–
	2	+	+	*	+	+	±	+	+	+	±	–
	3	+	+	*	+	+	±	+	+	–	–	–
	4	+	+	*	+	+	+	+	+	+	±	–
	5	+	+	±	+	+	+	+	+	+	±	–
	6	*	*	*	*	*	*	*	*	*	*	*
	7	+	+	▲	+	*	*	*	*	*	–	–
	8	+	+	*	*	*	*	*	*	*	–	–
	9	*	*	*	*	*	*	*	*	*	–	–
	10	+	–	*	–	–	–	–	–	–	–	–

Description
"+" mandatory to submit
"–" may exempt from submission
"±" research data may be replaced by literatures or exempted
"▲" information must be provided, except those who meet statutory standards
"*" in accordance with the application instructions and specific descriptions

Safety Evaluation Requirements for TCM Combined Preparation

TCM combined preparation is the most representative of TCM. Because many of them have some clinical data and have a certain understanding on safety and efficacy, it does not need to undergo standard nonclinical safety evaluation. In general, single species testing will be satisfactory, and clinical administration route is used for single-dose and repeated-dose toxicity studies. Rodent (usually rats) is preferred. When obvious toxicity was found in rodent repeated-dose toxicity study, a second kind (non-rodent) of animal will be used for further study. For most of TCM combined preparation, there is no requirement on genotoxicity, reproductive, and development toxicity studies, unless there are special safety concerns. Some drug targets reproductive population that may have effects on the reproductive system (e.g., contraceptives, hormone, sexual dysfunction treatment, promoting spermatogenesis, tocolytic drug, or drugs with other cytotoxic effects). In these cases, genotoxicity data should be provided, and reproductive toxicity data should be submitted according to the particular case. For instance, a TCM combined preparation to be used in postpartum women, as the indications include postpartum hemorrhage and postpartum prolactin, the reproductive and development toxicity study should be considered. If cytotoxicity or abnormal growth-promoting effects for certain organs is observed during repeated-dose toxicity study, in addition to positive results in genotoxicity tests, carcinogenic research data and literature review should be provided. Detailed requirements are described in the related guidelines.

Safety Evaluation Requirements for Active-Ingredient Preparation (Category 1) or New Active Part of Chinese Medicinal Material and Its Preparation (Category 5)

The two TCM categories are developed as modern TCM. Although they derive from Chinese medicinal material, big changes have occurred, and resulted in research and development ideas that is different from conventional TCM. Because of big changes and less human application experiences, toxicology demands are relatively higher for TCM combined preparation.

For an adequate understanding of the toxicity of test articles, single-dose toxicity and

repeated-dose toxicity should be carried out in two animals (usually rodent and non-rodent). Safety pharmacological studies should be included to test their effects in fundamental physiological systems (the central nervous system, cardiovascular system, and respiratory system). For active-ingredient preparation (Registration 1) (single component, purity reaches more than 90 %), the requirements are similar to chemical medicine, and genetic toxicity, reproductive and development toxicity, and carcinogenicity (when needed) studies should be performed. For detailed study requirements, refer to the respective guidelines. In short, comprehensive toxicological information should be available through the above studies in order to support the subsequent clinical trials or marketing. Below gives an example for safety evaluation of a new TCM that belongs to Category 1.

Example

A new TCM belongs to TCM Category 1, which contains active ingredients extracted from *Rheum tanguticum Maxim et. Balf.* The content of this component is more than 90.0 %. Dosage form is capsule, administration is oral, and indication is diabetic nephropathy. Nonclinical toxicity studies of it include the following:

Study title	Project
Safety pharmacological study	In vivo studies included the assessment on CNS (mice, ig), respiratory system, and cardiovascular system functions (dogs, through duodenum)
Acute toxicity study	Mice acute toxicity study (ig) Rat acute toxicity study (ig) Beagle dog acute toxicity study (ig)
Repeated-dose toxicity study	26-week study in rats (with 8-week recovery period) (ig) 39-week study in beagle dogs (with 8-week recovery period) (ig)
Genotoxicity study	Ames test In vitro chromosome aberration assay (Chinese hamster lung cells) In vivo mouse bone marrow micronucleus test (ig)
Reproductive and development toxicity study	Fertility and early embryonic development study in rats (ig) Embryo-fetal development study in rats (ig)[a]
Carcinogenicity study	Is not yet done[b]

[a]Embryo-fetal development study in rabbits and pre- and postnatal development study in rats should be conducted during clinical trial and submitted in NDA

[b]The need for carcinogenicity study depends on ICH guidance on the need of carcinogenicity test for pharmaceuticals, and if needed, carcinogenicity data should be submitted in NDA

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Part XX

Guidelines for the Care and Use of Laboratory Animals

Regulations for the Care and Use of Laboratory Animals in Various Countries

Andreas W. Herling

In nearly all countries in the world, the use of laboratory animals for biomedical research in academia as well as in industry is regulated by national legislations. Regulations for the care and use of laboratory animals in various countries are subject of permanent legislative changes, adaptations, improvements, etc. Therefore, any information on the legislation in various countries can be only a snapshot for the time being.

In many countries, legislation of research is regulated in a specific animal experimentation act, while in other countries, this is included in the animal welfare/protection act. In nearly all countries, animal experimentation needs an approval by the local authorities before the start of the experimentation, as well as regular inspections by the local veterinary authorities (bureau) with respect to animal housing, handling, and experimentation.

In the European Union (EU) in November 2008, it was decided that the European directive concerning the use of animals in biomedical research should be revised, with the aim of more standardization of the legislation in the European Member States. The final text of the directive was finalized and signed on 22 September 2010 and named 2010/63/EU. When the directive was in force, the deadline for the Member States to transpose it into their own words into national laws until end of 2012 was also put. The directive is formally applied across Europe as of 1 January 2013. There are 28 Member States of the EU as of the end of 2014: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and the United Kingdom.

Institutional Animal Care and Use Committees (IACUC) are of central importance to the application of laws to animal research in many counties, e.g., the United States, in all European countries according to the EU directive 210/63/EU, etc. In some countries, IACUC are mandatory for all biomedical research using laboratory animals (e.g., EU countries, China), while in other countries, this committee is only necessary for every institution that uses animals for federally funded laboratory research (e.g., the Unites States). Each local IACUC reviews research protocols and conducts evaluations of the institution's animal care and use, which includes the results of inspections of facilities that are required by law. The IACUC must consist of several members appointed by the institution. The appointed members must be qualified to regulate animal care at that institution. The IACUC must include a veterinarian with expertise in the species used at the institution, practicing scientist(s) experienced in animal research, as well as a lay member with the knowledge of critical concerns that are in nonscientific areas.

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Techniques of Blood Collection in Laboratory Animals

Andreas W. Herling

Contents

Introduction

Blood is collected from laboratory animals for various scientific purposes, for example, to study the effects of a test drug on various constituents, such as hormones, substrates, or blood cells. In the field of pharmacokinetics and drug metabolism, blood samples are necessary for analytical determination of the drug and its metabolites. Blood is also needed for some in vitro assays using blood cells or defined plasma protein fractions.

The techniques for blood collection depend on specific factors which differ from one experiment to the other. There is a difference between terminal and nonterminal blood collection techniques. The conditions of blood collection at the end of an experiment which includes death of the animal (terminal experiment) are completely different (anesthesia, volume of blood) from those of single or repeated blood collections from a conscious animal. Terminal blood collection under anesthesia allows the use of techniques which are not acceptable for nonterminal blood collections.

Aspects of Animal Welfare

Minimizing any pain and distress in laboratory animals during the procedure have to be as important as achieving the desired experimental results. This is important not only for humanitarian reasons but also as part of good scientific practice. Blood collection may be stressful to the animal

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due to the handling and the discomfort associated with a particular technique. Many biochemical and physiological changes are associated with stress which affects the results, e.g., increases in the blood levels of catecholamines, prolactin, and glucocorticosteroids can influence certain metabolic parameters, such as glucose, as well as the counts of erythrocytes, white cells, and packed cell volume. Therefore, stress should be reduced to an absolute minimum if it is not possible to avoid it at all; this is not only in the interest of animal welfare but also in the interest of good science to obtain representative data. To minimize stress during blood collection, e.g., from dogs or cats, it may be helpful for the animal as well as for the operator first to do some dummy runs and provide rewards to the animal.

During nonterminal blood collection, it is important not to withdraw too much blood which could reduce total blood volume and lead to false results. A reduced total blood volume is accompanied by a reduced hemoglobin content and oxygen transport capacity (Gainer et al. 1995) as well as by a fall in blood pressure and an increase in the concentrations of stress-related hormones. It may be further accompanied by other factors such as necrosis of the gastric mucosa.

The welfare of the individual animal should not be endangered by removal of too large a volume of blood or by too frequent collections. This may be the case more often when small laboratory animals, e.g., mice, gerbils, rats, or hamsters, are used. In these cases the study protocol should be adapted to use more animals to minimize distress for the individual animal.

Total Blood Volume

The total blood volume is very difficult to determine (McGuill and Rowan 1989) and depends on species, sex, age, and health as well as nutritional condition.

The calculation of limit volumes for blood sampling relies on accurate data on circulating blood volumes. A review of the literature indicates that there is considerable variation in these values, probably relating to the techniques used, the strain and gender of animal, etc. Table 1 gives the circulating blood volumes of the species commonly used in safety evaluation studies (Diehl et al. 2001). The values shown have been adapted from different sources assuming that the animal is mature, healthy, and on an adequate plane of nutrition. Total blood volume is smaller in larger animals than in smaller animals of the same species in relation to body weight. It is also smaller in older and obese animals compared to normal weight and young animals. Total circulating blood volume is in the range of 55–70 ml/kg body weight.

Table 1 Circulating blood volume in laboratory animals

Species	Blood volume (ml kg^{-1})	
	Mean	Range
Mouse	72	63–80
Rat	64	58–70
Rabbit	56	44–70
Dog (beagle)	85	79–90
Macaque (*Macaca mulatta*)	56	44–67
Macaque (*Macaca fascicularis*)	65	55–75
Marmoset	70	58–82
Minipig	65	61–68

Terminal Blood Collection

Terminal blood collection represents (i) exsanguination as a single process of blood removal to collect as much blood as possible and (ii) multiple blood sampling during a terminal experiment under general anesthesia. Basically, exsanguination should only be performed after the animal has been rendered unconscious by another method, e.g., physical stunning or general anesthesia. This is due to the fact that stress occurs with extreme hypovolemia and accessing deeper blood vessels causes pain. Due to the anesthetized condition of the animal and the terminal nature of the experiment, methods can be used for exsanguination which can never be recommended for nonterminal blood collections with recovery of the animal. These include:

- Blood withdrawal from the V. cava caudalis or the aorta after laparotomy when as much blood as possible should be removed in a sterile manner

Table 2 Blood vessels for venous blood withdrawal

Species	*V. coccygica* + tt	*V. auricularis*	Orbital venous	*V. jugularis*	*V. cephalica*, *V. saphena*	*V. femoralis*	*V. mammarica*
Mouse	cc + cc	–	a	–	–	–	–
Gerbil	cc + cc	–	aa	a	–	–	–
Hamster	–	–	aa	a	–	–	–
Rat	ccc + cc	–	a	aa	–	–	–
Guinea pig	–	c	–	a	–	–	–
Rabbit	–	ccc	–	c	–	–	–
Cat	–	–	–	aa/c	ccc	cc	–
Dog	–	–	–	ccc	ccc	a	–
Rhesus monkey	–	–	–	aa/c	ccc	cc	–
Pig	–	iii	–	iii/cc(cvc)	–	–	–
Sheep	–	–	–	ccc	c	– c	–
Goat	–	–	–	ccc	c	–	– c
Cattle	cc	c	–	ccc	c	–	– cc
Horse	–	–	–	ccc	–	–	–

c/cc/ccc conscious animal, *c/i/a* possible alternative, *i/ii/iii* immobilized animal, – not recommended or impossible, *a/aa/aaa* anesthetized animal, *cvc* cranial vena cava, *ccc/iii/aaa* recommended route, *tt* amputation tail tip, *cc/ii/aa* acceptable route repetition of letters indicates the preferred condition

- Exsanguination after decapitation, incision of the jugular vein or carotid artery, or techniques in the slaughterhouse, when a non-sterile collection is possible
- Retro-orbital bleeding of smaller laboratory animals like mice, gerbils, hamsters, and rats which can also be a method of exsanguination

Nonterminal Blood Collection

Nonterminal blood collections can be differentiated into single and multiple blood withdrawals. Possible peripheral veins for blood withdrawal are listed in Table 2.

Single Blood Removal

A single withdrawal of up to 15 % of total blood volume does not influence the well-being of the animal. However, the removal of 15–20 % might be accompanied by side effects such as fall in cardiac output or blood pressure. Hemorrhagic shock can be induced by the withdrawal of 30–40 % of total blood volume, and the loss of 40 % causes mortality in up to 50 % of pigs and rats (McGuill and Rowan 1989).

A single removal of up to 15 % of total blood volume may be repeated after 3–4 weeks from normal and healthy animals with no detectable adverse effects. This does not mean that the animal does not experience any adverse effects, but it does not show any.

Symptoms of hypovolemic shock are fast pulse, pale mucous membranes, hyperventilation, and a subnormal body temperature including cold skin and extremities. In these animals therapeutic intervention consists of volume substitution with warm isotonic intravenous infusion.

Multiple Blood Removal

Multiple withdrawals of blood samples should not exceed 1 % of total blood volume every 24 h (0.6 ml/kg/d). More frequent withdrawals and/or removal of larger volumes of blood causes anemia.

Symptoms of anemia are pale mucous membranes of the conjunctiva or inside the mouth,

intolerance to exercise, and an increased respiratory rate in cases of severe anemia. Anemia can be easily detected by determination of erythrocyte cell count and packed cell volume (hematocrit), hemoglobin level, as well as reticulocyte count in a blood sample. In case of anemia the animal should be treated with iron and vitamin B_{12} and should be monitored for the abovementioned blood parameters during therapy until normal values are reached again.

Technical Aspects of Blood Removal

A common method in mice and rats for collecting up to 0.1 ml capillary blood is to remove the tip of the tail. For repeated blood sampling the blood clot on the tail has to be removed to get fresh capillary blood. This method is sufficient for multiple blood collections to determine, e.g., blood glucose or total radioactivity after the administration of radiolabeled drugs. In tailless animals such as guinea pigs and hamsters, cardiac puncture under general anesthesia may be the preferred technique.

Blood collections from larger animals will preferably be performed from a superficial vein. The person holding the animal and raising the vein plays a key role in collecting blood without undue stress to the animal by talking to and stroking the animal. Some animals, e.g., dogs and some primates, may be trained to present a limb for blood removal without the use of any physical restraint.

It is important to locate the vessel accurately before insertion of the needle or the catheter. In most cases obstruction of the venous return is necessary for distension of the vessel and to successfully insert the needle. The bore of the needle should be as large as possible to ensure rapid blood withdrawal with minimal risk of blood clotting within the needle. When the sample is taken too quickly by a syringe, the vein will collapse. After the needle has been withdrawn, continuous pressure should be applied immediately to the puncture site and maintained for at least 30 s. The animal should be monitored 15 min later to check for after-bleeding.

Permanent Venous Cannulation

For multiple blood collections a permanent venous access by chronic cannulation is often recommended. In most cases, particularly in rats, it is necessary to restrain the animal in harnesses or jackets to prevent it from damaging or removing the cannula. In these cases the signs of stress are often apparent by an increase in serum levels of stress hormones. However, a few days after implantation of catheters, hormone levels are normal in restrained rats (Tsukamoto et al. 1984; Wiersma and Kastelijn 1985). Such animals are usually housed alone, and the tethering restricts normal movements such as lying on the back and rolling over. Such restrictions may be considered as potential sources of stress. This can be prevented by having the catheter exit the back of the animal for only 2 cm and capping it with a steel needle. At the time of the experiment, a longer catheter is attached for blood collections.

A simple device for serial blood collection has been described by Sir-Petermann et al. (1995).

One has to balance very carefully the distress and discomfort of the individual animal with a permanent cannulation under restraint conditions for a longer period with multiple blood samplings without permanent cannulation. In the first case, multiple values from the same animal can be obtained showing perhaps individual differences among a group of animals. In the latter case it may be necessary to use a larger number of animals, but there is less discomfort for the individual animal.

Short-term cannulation (less than a day) of a peripheral blood vessel in larger animals is easy to perform. A butterfly needle can be inserted under aseptic conditions, and multiple blood samples can readily be collected. Long-term cannulation (longer than 2 days) in larger and smaller animals often presents complications such as blockage of the cannula by thrombi. The infusion and administration of substances via the permanent cannula are much easier than the removal of blood. Thrombi attached to the end of the cannula function as a one-way valve. Clotting can be prevented by repeatedly filling the catheter with saline containing heparin.

Retro-orbital Bleeding

Blood sampling by orbital puncture is a controversial technique. The puncture of the orbital venous plexus is often performed in tailless animals, e.g., hamsters. This technique is also used in rats and mice, when larger volumes are required which cannot be obtained from the tail vein. Basically, retro-orbital bleeding should always be performed under anesthesia. Pasteur pipettes, micropipettes, or microcapillary tubes are used and pushed with a rotating movement through the conjunctiva laterally, dorsally, or medially of the eye to the back wall of the orbit. In general, inflammatory reactions can be seen histologically in the puncture track 4 days after punction. After 4 weeks the lesions have healed without detectable scars (van Herck et al. 1992). However severe side effects such as retro-orbital hematoma with subsequent pressure on the eye cannot be completely excluded. This pressure can damage the optical nerve. The animal may be unable to close its eye. Bleeding from the orbital venous plexus should only be performed with recovery of the animal in exceptional circumstances when there is no other method available. The technique should be performed only by a well-trained staff and only one eye should be used.

Cardiac Puncture

The collection of blood by cardiac puncture has been performed in guinea pigs, gerbils, and hamsters. In these species it is difficult to collect blood by alternative methods except retro-orbital bleeding. In general, cardiac puncture should be performed under general anesthesia with atropine as premedication to prevent cardiac arrhythmia. If cardiac puncture is used for a nonterminal blood withdrawal with recovery, the animal has to be separated from other animals until it is fully conscious. It should be carefully watched for adverse effects and sacrificed if found in distress due to complications like bleeding into the pericardium or into the thorax.

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Anesthesia of Experimental Animals

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Contents

Introduction

In biomedical research, experiments should only be done with a conscious animal if it is not possible to do the study in an anesthetized one. Anesthetic conditions should always be chosen to exclude stress, discomfort, and pain for the animal which could have negative influences on the pharmacological results and reproducibility of the data. Therefore, an experimental design causing minimal discomfort to the animal is always preferable. This is important not only for humanitarian reasons but also for good scientific practice.

Many pharmacological experiments are performed under anesthesia:

- Terminal experiments under anesthesia followed by euthanasia
- Experiments under anesthesia with recovery at the end of the study
- Experiments in which an animal is surgically prepared under anesthesia and continuation of the experiment occurs with the conscious animal after recovery

Generally, two possibilities exist for immobilization of aggressive animals and to prevent escape: (i) physical restraint (e.g., immobilization cages or immobilization tubes) and (ii) chemical restraint with anesthetic compounds. As a rule of thumb, it is recommended to use physical restraint for animal studies in which no anesthesia would be used in comparable studies in man. Physical

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restraint can be used for short and painless interventions like administration of substances or blood sampling from a vein.

In general, physical restraint produces fear, distress, and anxiety in experimental animals with the result of stress symptoms which could affect the results of the study. To minimize pain for the animals, to obtain correct and reproducible results, and to protect the handlers from aggression by the animals, it is often necessary to use chemical restraint. However, the chemical used can affect the biochemistry or physiology of the animal.

It is possible to anesthetize special areas of the animal (local anesthesia) or the whole animal (general anesthesia). Local anesthesia plays only a minor role for experimental animals as compared to general anesthesia.

Local Anesthesia

Local anesthesia is the regional and reversible elimination of pain with chemical compounds. Circulatory, pulmonary, and renal functions are not disturbed and the animals are conscious. Surface anesthesia has to be distinguished from the anesthesia produced after a local injection. The most common compounds for surface anesthesia are tetracaine and proparacaine. Procaine, butanilicaine, lidocaine, mepivacaine, and etidocaine are commonly used injectable local anesthetics. Local anesthesia is only recommended for gentle and calm animals (cattle, sheep). For most laboratory animals, general anesthesia is the method of choice.

General Anesthesia

Preparation

It is very important to check the general condition of the animal prior to anesthesia. This check should include a clinical examination (inspection, auscultation, palpation) of the animal concerned. Sometimes it could be useful to perform a few laboratory tests, e.g., hematocrit, hemoglobin, pH value of blood, and acid/base parameters.

Those animals with a vomiting reflex should be fasted prior to anesthesia. Most animal species should be fasted for a period of at least 12 h but pigs and cattle for at least 24 h. Water should be offered during the fasting period ad libitum.

Premedication

Premedication is recommended prior to anesthesia for easier administration of the anesthetic and for elimination of side effects of the anesthetic used, such as disturbing autonomic reflexes.

Hydration and Base Excess

Based on hematocrit, hemoglobin, and erythrocyte values, the hydration of the animal should be normalized prior to anesthesia. Infusions of glucose or Ringer solution can be used for this purpose. To check the success of the treatment, repeated determinations of the abovementioned values are necessary.

In cases of acidosis (pH of blood <7.36), treatment of the animals with $NaHCO_3$ is recommended. If measurement of base excess is possible, the amount of $NaHCO_3$ can be determined from the following formula:

$$\text{Dose } NaHCO_3\ (\text{ml}) = \text{g body weight} \times \frac{\text{base excess}}{0.6}$$

Atropine

To avoid cardiopulmonary problems and to decrease saliva production, atropine should be administered intramuscularly prior to general anesthesia. The recommended dose varies considerably and is usually between 0.05 and 0.1 mg/kg body weight. Cats and rodents have a higher activity of atropine-esterase in the liver, and these species need higher amounts of atropine (up to 0.25 mg/kg).

Sedation and Pain Elimination

Indications for sedation and elimination of pain are to calm the animals and to stabilize the autonomic nervous system.

For sedation the following compounds are used:

- Minor tranquilizers without autonomic effects:
- Meprobamate and diazepam
- Major tranquilizers with autonomic side effects:
- Propionylpromazine, acetylpromazine, azaperone, dehydrobenzperidol, xylazine, and detomidine

Anesthesia does not necessarily lead to analgesia (elimination of pain). Although general anesthesia produces loss of consciousness and pain is not perceived, the noxious stimuli will be transmitted to the CNS and will develop central hypersensitivity, leading to a postoperative heightened perception. To reduce the degree of central hypersensitivity, analgesics have to be administered before noxious stimulation begins (preemptive analgesia). Analgesia should also reduce or eliminate peripheral inflammation, which aggravates central hypersensitivity.

For analgesia, the opioids used are mainly:

- Methadone, meperidine, and fentanyl

In most species (dog, rabbit, guinea pig), a sedative effect is to be observed after administration of these compounds. In other species (pig, cat), an excitatory effect can occur.

Minor tranquilizers, major tranquilizers, and analgesic compounds are often used in common with anesthetics. A compilation of such combinations is summarized in Table 1.

Course of Anesthesia

The animal has always to be observed very carefully during anesthesia. Various systems can be checked with technical equipment, e.g., circulatory system (heart rate, pulse, blood pressure, ECG, peripheral perfusion, temperature) or pulmonary system (respiratory rate).

A very important procedure during anesthesia is the determination of the depth of anesthesia. There are four stages of anesthesia:

I. Stage of analgesia (from the first effect to unconsciousness):
   – Heart and respiratory rate increase, normal dilation of pupils
II. Stage of excitation (from the beginning of unconsciousness to the start of regular respiration): respiration irregular, dilated pupils, increased motor reflexes, nystagmus, and opisthotonus
III. Stage of tolerance (from the beginning of regular respiration to the termination of spontaneous respiration):
   This stage is divided into four steps:
   (A) Regular respiration, narrow pupils, and most reflexes present
   (B) Skeletal muscles relaxed, narrow pupils, no eyelid reflex, corneal reflex present, flat respiration, and good analgesia
      This is the optimal stage of anesthesia for surgery.
   (C) Only corneal reflex present, respiration very flat, and pupils dilated
   (D) No reflexes, respiration very flat, and pupils very dilated.
IV. Stage of asphyxia (after termination of the spontaneous diaphragmatic respiration). No reflexes, no respiration: danger of death, immediate use of antidotes is necessary to prevent death.

By using combinations of different anesthetics – mainly by using combinations with muscle-relaxing agents – the reactions of animals will differ from this scheme.

Routes of General Anesthesia

In general, there are two different routes to induce general anesthesia: (i) injection and (ii) inhalation anesthesia. Sometimes combinations of both routes are used. The decision for one or the other route depends on the animal species, the purpose of the study, and the necessity of control during anesthesia.

Table 1 Anesthesia of experimental animals (values are in mg/kg)

Species	Premedication	Sedation	Short anesthesia	Medium anesthesia	Long anesthesia
Rat	Atropine (0.2 s.c.)	Diazepam (2.5 i.m.)	Alfentanil + etomidate (0.03 + 2 i.m.) or inhalation (isoflurane)	Xylazine + ketamine (5 + 100 i.m.) or pentobarbitone (50 i.p.)	Xylazine + ketamine (16 + 100 i.m.) or urethane (1,500 i.m.)
Mouse	Atropine (0.1–0.25 s.c.)	Diazepam (5 i.p.)	Alfentanil + etomidate (0.03 + 2 i.m.) or inhalation (isoflurane)	Xylazine + ketamine (5 + 100 i.m.) or pentobarbitone (50 i.p.)	Xylazine + ketamine (16 + 100 i.m.)
Hamster	Atropine (0.1–0.2 s.c.)	Diazepam (5 i.p.)	Inhalation (isoflurane)	Xylazine + ketamine (5 + 50 i.m.) or pentobarbitone (35 i.p.)	Xylazine + ketamine (10 + 200 i.m.)
Guinea pig	Atropine (0.1–0.2 s.c.)	Diazepam (2.5–5 i.m.)	Inhalation (isoflurane)	Xylazine + ketamine (2 + 80 i.m.)	Xylazine + ketamine (4 + 100 i.m.) or pentobarbitone + chloral hydrate (30 i.p. + 300 i.v.)
Rabbit	Atropine (0.1–0.2 s.c.)	Diazepam (1–5 i.m.)	Inhalation (isoflurane)	Xylazine + ketamine (5 + 25–80 i.m.)	Xylazine + ketamine (5 + 100 i.m.) or pentobarbitone + chloral hydrate (30 i.p. + 300 i.v.)
Cat	Atropine (0.05–0.2 s.c.)	Diazepam (0.2–1 i.m.)	Acetylpromazine (0.5–1 i.v./i.m.) or propionylpromazine (0.5–1 i.v.) or xylazine (2 i.m.)	Xylazine + ketamine (2 + 10 i.m.) or ketamine (5 i.v.) or inhalation (isoflurane)	Pentobarbitone (35 i.v./i.p.)
Dog	Atropine (0.05 s.c.)	Xylazine (3 i.m.) or acetylpromazine (0.5 i.m.) or propionylpromazine (0.5 i.m.) or droperidol (1 i.m.) or diazepam (1 i.m.)	Thiopental (17 i.v.) or metomidate + fentanyl (4 + 0.005 i.m.) or alfentanil + etomidate (0.03 + 1 i.m.) or inhalation/intubation (isoflurane)	Xylazine + methadone (2 + 1 i.m.) or xylazine + ketamine (2 + 10 i.m.) or comb. with diazepam (0.6 i.m.) or propionylpromazine + methadone (0.5 + 1 i.v.) or acetylpromazine + methadone (0.5 + 0.5–1 i.v.)	Pentobarbitone (30 i.v.) or xylazine + ketamine (2 + 15 i.m.) or intubation (isoflurane)
Pig		Azaperone (1–2 i.m.) or chlorpromazine (1–2 i.m.)	Thiopental (10 i.v; 5 % solution)	Azaperone + metomidate (0.05–5 + 2.5–5 i.m. + i.v./i.p.) or tiletamine + zolazepam + xylazine (2 + 2 + 0.5–1 i.m.)	Pentobarbitone (10–25 i.v.)
Sheep and goat		Xylazine (0.05–0.1 i.m.) or diazepam (2 i.m.)	Xylazine + ketamine (1–2 + 5–10 i.m.) or thiopental (7.5–10 i.v.)	Pentobarbitone (20–30 i.m.)	Ketamine after pretreatment with xylazine/ketamine 3–4 h or intubation (isoflurane)
Monkey[a]	Atropine (0.05–0.1 s.c.)	Diazepam (1.0 i.m.) or xylazine (1–2 i.m.) or ketamine (10–30 i.m.)	Inhalation/intubation (isoflurane)	Xylazine + ketamine (2 + 10 i.m.)	Pentobarbitone (20–30 i.v./i.p.) or intubation (isoflurane)

[a]Anesthesia of monkeys depends very much on the monkey species

Injection

By using this route of anesthesia, the narcotic compound is dissolved in a liquid. The route of administration can be intravenous, intramuscular, subcutaneous, or intraperitoneal. The mostly frequently used compounds are mentioned below:

Barbiturates

There are three groups of barbiturates: long-acting, short-acting, and very short-acting barbiturates. For laboratory animals, short- and very short-acting barbiturates are used predominantly (sodium pentobarbitone, thiopental, hexobarbital).

Barbiturates are metabolized in the liver and mainly excreted via the bile. They are very fat soluble. Their short duration of action is caused by a distribution into adipose tissue. Fat represents a large compartment for these compounds with a relatively slow excretion. This can lead to prolongation of the narcotic effects after repeated dosing. The dosing of barbiturates should be adjusted according to the observed reactions of the individual animal as there are individual differences due to age, body weight, size, fat content, and general condition of the animal. Barbiturates are not analgesic and should not be given without analgesics.

Chloral Hydrate

Chloral hydrate is a relatively old soporific compound. By using it for anesthesia, cardiovascular side effects are often observed. The range of dosing is very narrow. Its use for laboratory animals is therefore limited. Intraperitoneal injections in rats can lead to paralysis of the ileus.

Combinations of Analgesic with Neuroleptic Compounds

This method is often used for dogs and rodents. Strong analgesics (morphine, methadone, meperidine, fentanyl) are combined with neuroleptics like phenothiazine, acetylpromazine, or butyrophenone. The anesthesia can be rapidly terminated by available antagonists.

Ketamine

Ketamine is an anesthetic compound with a very fast onset of action following intramuscular administration. It can be used for nearly all species. Its therapeutic window is huge. A side effect of this compound is an increased tonus of skeletal muscles, but this can be prevented by the simultaneous administration of xylazine or diazepam.

Hypnotic Agents

Hypnotics are compounds which produce a very deep sleep without analgesia (metomidate). Therefore, combination with neuroleptic compounds is recommended (e.g., combination of azaperone and metomidate for pigs). As a single compound, metomidate can only cause anesthesia in birds.

Xylazine

Xylazine is frequently used for anesthesia in combination with other substances (Table 1). As a single compound, it is only used to produce anesthesia in cattle.

Urethane

Urethane was formerly used as a hypnotic agent. It can, at the appropriate dose, produce a long-acting (about 10 h) anesthesia in rats. Urethane is liver toxic and therefore its use is limited to some pharmacological models in which liver metabolism is of no importance. Due to its carcinogenic properties, it should not be used anymore.

The important criteria of anesthesia are sedation, unconsciousness, analgesia, and relaxation. These cannot be achieved with a single compound. Therefore, a combination of different compounds is necessary. The most common combinations for different species are listed in the table with respect to the duration of anesthesia: short (up to 30 min), medium (up to 120 min), and long anesthesia (longer than 120 min).

Inhalation

Inhalation anesthesia is more common for the large laboratory animals such as dogs, cats, sheep, goats, and monkeys. However, it has gained an increased importance in small laboratory animals like rodents. The advantages of this form of anesthesia are the possibilities of

controlling exactly the depth of anesthesia and of fast management of complications.

The parts of an inhalation system include:

- Bottle with oxygen (blue bottles)
- Valve to regulate pressure (reduces the pressure of the oxygen bottle)
- Flowmeter (monitors the gas flow to the animal)
- Evaporator (evaporation of liquid anesthetic compounds)
- Oxygen bypass (fast supply of oxygen to the animal in case of need)
- Tube to the system

Different techniques are used for laboratory animals:

Technique of Insufflation

Administration of anesthetic compounds is performed via a mask. Expiration occurs into the air of the room. Advantages are the simple procedure without valves and CO_2 absorber and the very small dead volume of the system. Disadvantages are the waste of compounds, drying of the trachea of the animals, the impossibility of checking the respiration volume, and the expiration of narcotic compounds into the room air (jeopardy to the staff).

Open System

Inspired and expired gases are separated by a valve. The inspired air consists of the fresh mixture of gases. The expiration reaches completely the atmosphere. The "Stephen slater" is the most used system of this group. It is recommended for smaller animals.

Half-Closed and Closed Systems

In closed systems all of the expired air passes to a CO_2 absorber. The CO_2 is removed chemically and the air is inspired again with newly evaporated anesthetic compounds mixed with oxygen. In a half-closed system, part of the expired air reaches the atmosphere. Advantages of closed systems include the economic benefit, the decrease of fluid and body heat loss from the animal, and no risk to the laboratory staff. Disadvantages are the necessity to change the absorber every 8–10 h during anesthesia, the production of heat, and the increase of resistance to breathing.

Summary

If it is possible, inhalation should be done by intubating the animal. The risk of aspiration of stomach contents with the danger of an aspiration pneumonia can then be minimized. It is very important to use a tube with the correct diameter and length. An animal should be unconscious for intubation (see Table 1). In order to avoid gulp or cough reflexes, it is recommended to administer succinylcholine, a muscle relaxant. Atropine can also be administered to decrease saliva production. Generally, all methods of injection anesthesia mentioned (Table 1) can be combined with an inhalation method. Such a "balanced anesthesia" is recommended for long and highly sophisticated operations.

Inhalation Compounds

The inhalation mixture has to include 21 % oxygen. Sometimes it is better to administer 33 % oxygen. Isoflurane, methoxyflurane, and enflurane are widely used compounds for inhalation anesthesia. Ether is no longer recommended for anesthesia, due to the hazard of explosion and fire and because it is highly irritating to the respiratory tract. By using a mixture of N_2O and O_2, the amount of the evaporated compounds can be reduced drastically (Table 2).

Table 2 Inhalation compounds and their characteristics

Compound	Conc. with N_2O/O_2 (%)	Conc. without N_2O/O_2 (%)	MAC
Methoxyflurane	0.4–1	3.5	0.23
Isoflurane	1.0–2.5	1.5–3.5	1.4
Enflurane	0.5–1.5	2	2.2

MAC minimal alveolar concentration for an anesthetic effect

Termination of Anesthesia

Inhalation anesthesia can be stopped by removing the supply of evaporated compounds. To hasten the elimination of anesthetic compounds, the concentration of oxygen in the system can be increased for a period of 5 min.

The elimination of injected compounds is difficult to influence. It may be possible to accelerate metabolism of the anesthetic by using agents which stimulate metabolism in the liver and excretion by the kidney.

It is very important to check the body temperature of the animal during and after anesthesia. In cases of low body temperature, the use of heating lamps or pads is necessary. After termination of anesthesia, the animals go through the same phases as mentioned above but in the reverse order (tolerance, excitation, analgesia).

During anesthesia it might be necessary to stimulate respiration or circulation. Stimulatory agents for respiration are doxapram, pentamethylentetrazole, nikethamide, lobeline, or micoren. Stimulatory agents for circulation are adrenaline, etilefrin, dopamine, and ephedrine. The application of pure oxygen via a mask is also recommended during an injection anesthesia. Antidotes to morphine and its derivatives are morphine antagonists like naloxone and levallorphan. The α_2 antagonists yohimbine and atipamezole are antagonists of xylazine. The antidote for diazepam is flumazenil. There are no direct antagonists for ketamine and barbiturates.

Postoperative Analgesia

To effectively reduce pain in animals, a pain assessment has to be made using behavior, stress response, etc. Pain assessment will be facilitated by:

- A good knowledge of the species-specific behaviors of the animal being assessed
- A knowledge and comparison of the individual animal's behavior before and after the onset of pain (e.g., pre- and postoperatively)
- The use of palpation or manipulation of the affected area and assessment of the responses obtained
- Examination of the level of function of the affected area: e.g., leg use following injury or limb surgery, together with a knowledge of any mechanical interference with function
- The use of analgesic regimens or dose rates that have been shown to be effective in controlled clinical studies and evaluation of the change in behavior this brings about
- A knowledge of the nonspecific effects of any analgesic, anesthetic, or other drugs that have been administered

Analgesics can be broadly divided into two groups: the opioids or narcotic analgesics and the nonsteroidal anti-inflammatory drugs (NSAID). Clinical pain involves several pathways, mechanisms, and transmitter systems. To provide the most effective pain relief, drugs of different classes should be applied, acting on different parts of the pain system, for example, combining opioids and NSAIDs.

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Euthanasia of Experimental Animals

Andreas W. Herling

Contents

Euthanasia means a gentle death and should be regarded as an act of a human method of sacrificing an animal with a minimum of physical and mental suffering. The method of euthanasia should be appropriate for the species and the age of the animals. The method should be painless, avoid excitement, and achieve rapid unconsciousness and death. Additionally, the method should be reliable, reproducible, and irreversible.

Prior to euthanasia, it is important to recognize symptoms of fear, distress, and anxiety; these symptoms are species specific. Depending on the species, these symptoms may include distress vocalization, attempts to escape, aggression, freezing, salivation, urination, and defecation. Distress vocalization and release of certain odors or pheromones by a frightened animal may cause anxiety in other animals housed nearby. In this context, it has to be stressed that many vocalizations of animals are in a range of frequencies which are out of the human hearing range. Therefore, animals should not be present during euthanasia of other animals, especially of their own species. If possible, an animal should not be killed in a room where other animals are housed, in particular in case of a bloody method of euthanasia, e.g., decapitation.

Euthanasia usually requires some physical control over the animals. Suitable control minimizes pain, distress, fear, and anxiety in the animal and depends on animal species, size, state of domestication, and method of euthanasia. Gentle handling, stroking, and talking to the animal

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during euthanasia often have a calming effect on many animals. The use of sedating and immobilizing drugs may be necessary in those cases, where capture or restraint may cause pain, injury, or anxiety to the animal.

The person performing euthanasia is the most relevant factor during sacrificing an animal in order to cause a minimum of pain, fear, and distress. A suitable method of euthanasia can be extremely harmful to the animal if it is badly performed. All persons performing euthanasia should be well trained, demonstrate professionalism, and be sensitive to the value of animal life.

After euthanasia it is essential to confirm death. Signs of death are cessation of heartbeat and respiration and absence of reflexes. Death must be guaranteed by exsanguination or removal of the heart, destruction of the brain, decapitation, evisceration, or the presence of rigor mortis.

Methods for euthanasia of laboratory animals can be separated into physical and chemical methods.

Physical Methods Recommended for Euthanasia of Laboratory Animals

Physical methods are stunning (concussion, electrical stunning, and stunning with a captive bolt), cervical dislocation, decapitation, and microwave irradiation. The different methods of stunning as well as cervical dislocation cause a rapid loss of consciousness which must be followed immediately by a method to force and guarantee death of the animal.

Concussion may be sufficient in smaller animals, e.g., rodents, to achieve unconsciousness and is performed by a blow to the head. Electrical stunning is a common method in the slaughterhouse predominantly for pigs. Only specific equipment must be used for this method either in the slaughterhouse or in laboratory. Stunning with a captive bolt is also a common and an effective method for larger animals in the slaughterhouse to achieve unconsciousness. Adapted equipment can also be used for larger rabbits in biomedical laboratories. The correct localization of the captive bolt is important in order to achieve immediate destruction of the brain. Cervical dislocation destroys the brainstem but the large vessels to the brain are often intact. All these methods have to be followed immediately by an act to force and guarantee death, e.g., exsanguination, removal of the heart, or destruction of the brain.

During the decapitation process, the head is separated from the neck which causes an immediate interruption of the blood circulation to the brain and a fall in blood pressure in the brain with subsequent loss of consciousness. This is valid only for warm-blooded animals. In cold-blooded vertebrates, it is recommended to stun the animals prior to decapitation due to their higher resistance against anoxia. For decapitation of smaller laboratory animals, specific guillotines have been developed.

Euthanasia by microwave irradiation is used by neurobiologists for fixation of brain metabolites without destruction of brain anatomy. Only specific equipment developed for this purpose must be used (no domestic microwave ovens). It is essential to localize correctly the microwave beam onto the brain of the animal.

Chemical Agents Recommended for Euthanasia of Laboratory Animals

Many chemicals can cause death due to their toxicity, but only a few are recommended for euthanasia. The most suitable chemicals for euthanasia are certain anesthetics in overdose. In this case, the anesthetic agent causes unconsciousness, followed by death.

Volatile anesthetics such as halothane, enflurane, isoflurane, and methoxyflurane should only be used in a gas-scavenging apparatus. Carbon dioxide at high concentrations of 80–100 % causes unconsciousness within a few seconds.

Injectable anesthetics, predominantly barbiturates such as sodium pentobarbitone, are the most widely used and the most appropriate agents for euthanasia for most animals. Three times the anesthetic dose causes generally rapid unconsciousness and death. Intravenous injection is the most reliable and rapid route. Intraperitoneal injection may also be used in smaller rodents but it needs more time for death to occur. The intracardial and

intrapulmonal administration can only be recommended in unconscious animals, because it is painful and, in the case of intracardial injection, difficult to perform successfully on the first attempt.

The agent T61 is a mixture of a local anesthetic, a hypnotic and a curariform component. It is used only intravenously. Due to the curariform component, it is not allowed in some countries, but it has been demonstrated that unconsciousness and neuromuscular blockade occur simultaneously in dogs and rabbits. Nevertheless, prior sedation should be performed if possible.

Methods and Agents Not to Be Used for Euthanasia of Laboratory Animals

Physical methods not to be used for euthanasia are exsanguination, rapid freezing, pithing, decompression, hyperthermia, hypothermia, asphyxia, drowning, and strangulation. Chemicals not to be used are carbon monoxide, nitrogen, nitrous oxide, cyclopropane, chloroform, trichloroethylene, hydrogen cyanide, magnesium sulfate, potassium chloride, nicotine, strychnine, chloral hydrate, and ethanol. Some of the abovementioned chemicals are not recommended for euthanasia because they are extremely noxious and dangerous to the experimenter.

Neuromuscular blocking agents such as curare, succinylcholine, or suxamethonium which do not cause rapid unconsciousness prior to death should also not be used. Ketamine is a very good anesthetic with a wide therapeutic safety margin for most animal species. Therefore, it is unsuitable for euthanasia.

However, non-acceptable methods of euthanasia can be used if animals are anesthetized or rendered insensible and unconscious by a recommended method. This is used for, e.g., exsanguination, rapid freezing, and pithing. Exsanguination must not be performed within the sight or smell of other animals. Rapid freezing is important to minimize enzymatic processes prior to subsequent biochemical determinations in tissues and organs. Pithing is a quick method of brain destruction achieved by insertion of a needle through the foramen magnum.

Recommended Methods for Euthanasia for Specific Animal Species

Mouse

- Decapitation
- Cervical dislocation with subsequent exsanguination
- Euthanasia within an 80 % carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rat

- Concussion, cervical dislocation (both with subsequent exsanguination), and decapitation, conducted only by well-trained persons
- Euthanasia within an 80 % carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 150 mg/kg i.p.
- Microwave irradiation

Hamster

- Decapitation, conducted only by well-trained persons
- Euthanasia within an 80 % carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 300 mg/kg i.p.

Guinea Pig

- Concussion (with subsequent exsanguination) and decapitation, conducted only by well-trained persons

- Euthanasia within an 80 % carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rabbit

- Stunning with captive bolt
- Concussion (with subsequent exsanguination), conducted only by well-trained persons
- Sodium pentobarbitone at a dose of 120 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter

Cat

- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 200 mg/kg i.p.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter; it is recommended to anesthetize the animal beforehand with 20–30 mg/kg ketamine i.m. or 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Dog

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v.; it is recommended to anesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Ferret

- Sodium pentobarbitone at a dose of 120 mg/kg i.p.

Cattle, Sheep, Goat, Horse, and Pig

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; for larger animals, prior sedation is recommended (xylazine).
- All other acceptable methods which are used for slaughtering.

Primate

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; it is recommended to anesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.
- T61 at a dose of 0.3 ml/kg strictly i.v.; it is recommended to anesthetize the animal beforehand with xylazine/ketamine i.m.

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